Biofuel Technologies

Vijai Kumar Gupta · Maria G. Tuohy
Editors

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Recent Developments

Springer

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Foreword by M. A. Mazutti

Biomass was the world's dominant source of energy and its consumption grew from approximately 50 million tons of oil equivalent in the beginning of the Christian era to 1,000 tons of oil equivalent today (a 20-fold increase). Presently, biomass accounts for about 10 % of the world's primary energy consumption. The other 90 % is made up of nonrenewable fossil fuels (80 %), hydroelectricity (2 %), nuclear energy (6 %), and renewable solar energies (2 %). Worldwide, there is a great interest from researchers and industries to increase the percent of biofuel use on the total energy consumption. The production of bioethanol from biomass is well-reported, but, more recently, the production of biobutanol and biohydrogen, which are more energetic than bioethanol, have aroused interest. However, to obtain biofuel from biomass requires a proper pretreatment to enable efficient saccharification of cellulose and hemicellulose components to their constituent sugars due to the complex structure of biomass. Breakthrough technologies are needed to overcome barriers to develop cost-effective processes for converting biomass into biofuel, and the knowledge of the molecular mechanisms of the enzymatic hydrolysis of lignocellulosic feedstocks is one of the important parts of this process. Although the production and use of biofuels is an eminent technology, there are little references compiling the recent developments and trends on the field. By this reason, the book "Biofuel Technologies: Recent Developments" will bring the readers with the tendency and developments concerning the use of biomass to obtain traditional biofuel as ethanol and biodiesel as well as modern and more efficient biofuels namely butanol and hydrogen.

Brazil, 14 September 2012

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Foreword by R. C. Kuhad

Because of the limitations that first generation biofuels produced from food crops have caused, greater emphasis is to be placed on second generation biofuels produced from secondary agriculture feedstocks. Large-scale production of crop-based (first generation) biofuels may not be feasible without adversely affecting global food supply or encroaching on other important land uses. Because alternatives to liquid fossil fuels are important to develop in order to address greenhouse gas mitigation and other energy policy objectives, the potential for increased use of advanced (noncrop, second generation) biofuel production technologies has significant policy relevance.

Biofuel technology is a lignocellulose-based technology that converts wood and nonwood wastes into biofuels. For a longer term, one should look to expand the biofuel feedstock base through the use of new second generation technologies along with refining technology to biomass to extract energy, high value biochemicals, and fibers. Biofuels can be produced using various feedstocks and bioconversion technologies. Bioethanol can be produced from lignocellulosic materials and biodiesel from animal fats and microorganisms, such as microalgae. Emerging biofuel technologies include cellulosic ethanol and microorganism-based biodiesel as advanced biofuel technologies.

Research is necessary to improve the efficiencies in these areas and explore developing new technologies to convert lignocelluloses into ethanol. Similarly, the major challenge for microalgal biodiesel production is the high cost of producing microalgal biomass. The major issues to be solved are cost-effective algal harvesting and protection of the high-oil microalgae from contamination with wild algae. Another important issue for both lignocellulosic ethanol and microalgal biodiesel processes is by-products development. Both processes utilize only a portion of the raw materials for biofuel generation; only cellulose and hemicelluloses are used in ethanol production, while lipids are the only materials used for biodiesel production. There are sufficient residual by-products generated and the residues need to be processed for by-products through refinery to improve the economics of the whole process. The logistics to providing a competitive, all-year-round, supply of biomass feedstock to a commercial-scale plant is challenging, as

is improving the performance of the conversion process to reduce costs. The biochemical route, being less mature, probably has a greater cost reduction potential than the thermochemical route. The rapid expansion of biofuels in many countries poses significant challenges for policymaking. The issues surrounding the expansion of biofuels production and utilization are complex and highly dependent on crop type, local circumstances, and production management systems.

Therefore, advanced biofuel production technologies including lignocellulosic ethanol and microalgae have a good technical potential to substantially replace fossil fuels in the near future. Lignocellulosic materials such as agricultural residues, woods, and grasses are abundant in most land areas of the world and their utilization does not necessarily compete for arable land against food and feed production. Microalgae can produce a huge amount of oil on a small footprint, hundreds or thousands of times higher yield than most oil plants, if managed to be produced in larger quantity. It is technically possible to produce a high volume of biodiesel that is equivalent to or higher than the current level of diesel consumption using microalgae as feedstock that are grown on a small portion of land areas. Full commercialization of either biochemical or thermochemical conversion routes for producing second generation biofuels are still under progress. So, there is no doubt that good progress with bioethanol production has been made during the past decades following increasing investments in R&D. Successful outcomes include the development of improved microorganisms and the evaluation of innovative conversion technologies with improved performance and efficiencies. There is also a better understanding by the industry of the overall feedstock supply chain (whether from crop and forest residues or from purpose-grown crops), necessary to provide consistent quality feedstock delivered all-year-round to the conversion plant gate. There have also been successful developments relating to the construction of pilot-scale biorefineries to produce a range of coproducts.

It is considered that second generation technologies to produce liquid transport biofuels will be a long-term view for the potential of biofuels, but still more efforts are required to bring these technologies closer to the market. International cooperation is paramount and collaboration through international organizations should be enhanced with various sectors playing active roles to develop and sustain the second generation biofuels technologies for the long term. This edited book entitled "Biofuel Technologies: Recent Developments" reviews the current status of several advanced biofuel technologies. The book will be of interest to teachers, scientists, and researchers, whether in academia or industry.

14 September 2012

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Preface

Bioresources represent an important part of the available renewable resources. One of the key challenges facing sustainable industrial development is the transition from fossil-based feedstocks to renewable alternatives to meet the growing demand for energy, fuel, and chemicals. The sustainable use of biofeedstocks in energy/biofuels and chemical/material production is currently receiving much international attention, with significant efforts being made to translate scientific advances into commercial reality. The term biofuel is used here to mean any liquid fuels made from plant materials, residues, and wastes that can be used as a substitute for petroleum-derived fuels. Biofuels are increasingly attracting renewed attention worldwide as substitutes for petroleum-derived transportation fuels to help address resource limitations, security of supply and global warming concerns associated with liquid fossil fuels. Second generation biofuels include those made by biological processing and those made by thermochemical processing, which represent two fundamentally different approaches. Success in the commercial development of second generation biofuel technologies requires significant progress in feedstock selection and optimization through genetic and crop breeding strategies, as well as in crop husbandry and production practices. Technology improvements are essential for biofuel production in biomass conversion processes, either by the biochemical route (e.g. feedstock pretreatment, low-cost biocatalysts, or enzymes with improved efficiency, better microbial strains for biofuel production) and thermochemical route. Thermochemical processing has the important advantage of greater feedstock flexibility than biological processing, but the scale required to achieve an economically process may be larger than for biological processing. Many efforts are ongoing worldwide to commercialize second generation biofuels derived from both routes.

The technologies described in this publication reflect a number of the issues and challenges relevant to the development of the biofuels industry. Some research and development breakthroughs, followed by commercial-scale demonstrations, are needed to prove the viability of unsubsidized cellulosic ethanol. In contrast, because thermochemical biofuels are identical to some fuels that are already being made from fossil fuels, little or no fundamental research and development

breakthroughs are needed, but commercial-scale demonstrations are still required. Recovery and utilization of valuable coproducts generated during the production of second generation biofuels offers the potential to increase the overall revenue from biomass to biofuel processes. Optimization of the conversion process to maximize the value and yield of coproducts (heat, electricity, various chemicals, etc.) needs to be pursued for different feedstocks and conversion pathways. The development of improved microorganisms and the evaluation of innovative, more efficient conversion technologies are required. To support sustainable development, greater understanding of the overall feedstock supply chain, whether from crop and forest residues or from purpose-grown crops, is of paramount importance to provide consistent high-quality feedstock that can be delivered all-year-round to conversion plants. Sustainability is critical to the successful development of biofuels. Therefore, in the context of global trade, sustainability certification may be pivotal to ensure that global biofuel production is accompanied by the achievement of social and environmental goals. The overall chain of biomass production, conversion to biofuels, and end use is complex and requires integrated collaboration of many diverse stakeholder groups; farmers, crop producers and managers, engineers, scientists, chemical companies, fuel distributors, engine designers, and vehicle manufacturers. In order to address and reflect this complexity and understand the flow of activities involved, contributions to this publication have been collated under three main technical areas/heading: biomass production, conversion processes, and product end-use.

This publication provides valuable information to help understand technology-related implications of biofuels development. The chapters presented in the book cater for the needs of postgraduate researchers and scientists across diverse disciplines and industrial sectors where biofuel technologies and related research and experimentation are undertaken. Moreover, this book describes recent updates on biofuel feedstocks, biofuel types, and associated co-/by products and their applications. Therefore, this publication will be very useful not only to experienced researchers, but also to those new to the area.

Galway, Ireland, 14 September 2012

Vijai Kumar Gupta
Maria G. Tuohy

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Contents

Part I
Sustainability and Technical Challenges

Chapter 1
Fermentable Sugars from Lignocellulosic Biomass: Technical Challenges

Ravichandra Potumarthi, Rama Raju Baadhe and Sankar Bhattacharya

Abstract Lignocelluloses, the most abundant renewable biomass on earth, are composed mainly of cellulose, hemicellulose, and lignin. Both the cellulose and hemicellulose fractions are polymers of sugars and thereby a potential source of fermentable sugars. Lignin can be used for the production of chemicals, combining heat and power, or for other purposes. Energy crisis and environmental pollution drive the scientific community toward the potential exploitation of lignocellulosic biomass. To crack their complex structures various pretreatment technologies including biological, mechanical, chemical methods, and various other combinational methods are available. We cannot relate the best and common pretreatment method to all types of the lignocellulosic biomass. It mostly depends on the type of lignocellulosic biomass and the desired products. The final aim of pretreatments must be improvement in the hydrolysis rate of lignocellulosic biomass. Currently, there is a large scope to investigate and restore the challenges in the pretreatment processes which finally leads to develop the tailor-made effective pretreatment methods for diverse types of lignocellulosic biomass.

1.1 Introduction

The world is facing two environmental challenges at present, energy crises and environmental pollution. Energy is a key ingredient for all sectors of a modern

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economy and plays a fundamental role in improving the quality of life. Approximately 80 % of the total world energy supply depends upon fossil fuels, which are depleting rapidly. It is estimated that the reserves of crude oil, natural gas, and coal are only expected to last around 50, 60, and 120 years, respectively, at the current rate of consumption (British Petroleum Statistical Review 2011). Another challenge with fossil fuels is emission of greenhouse gases (GHG). In 2009, 37 % of CO_2 (one of the major GHG) was emitted from oil combustion (International Energy Agency statistics 2011). Future energy needs, environmental hazards that are concerned with the consumption of fossil fuels, and national security, have heightened attention toward clean liquid fuel (Bioethanol) as a suitable alternative source of energy. Bioethanol not only reduces the dependence on oil trade and reduces the uncertainties caused by the fluctuations in oil price, but also secures reductions in environmental pollution problems due to its high oxygen content (Huang et al. 2008).

Bioethanol is produced from simple sugars that are derived from various sources such as corn, sugar cane, or switchgrass. Though these resources are connected with the forest diversity (GE Trees 2008), food security (Pimentel 2003), and also affect the available agriculture land for food grains cultivation. Large amounts of renewable carbon is produced in the ecosystem and about 77×10^9 tons are fixed via photosynthesis, leading to production of 100 billion tons of biomass annually (lignocellulosic materials) (Bozell 2001). These lignocellulosic waste materials are available as agricultural, industrial, and forest residues around the world (Prasad et al. 2007). This biomass is a potential source for bioethanol production. Usage of agricultural waste materials in an effective way minimizes environmental pollution, food security problems, and also proves to be a good agricultural waste management process.

Production of bioethanol comprises three important processes: pretreatment, saccharification, and fermentation. These copious and inexpensive lignocellulosic biomass are better sources of diverse polymeric sugars: glucose (cellulose) and xylose (hemicellulose), which can be converted biotechnologically into valuable products like sugars, ethanol, chemicals, and a wide variety of enzymes (Bozell 2001; Torrea et al. 2008; Miura et al. 2004; Kahar et al. 2010).

Because of their high complex structure with lignin, efficient enzymatic hydrolysis is difficult to produce fermentable sugars. This is possible with the breakdown of complex polysaccharide chains attached with lignin using pretreatment and subsequent enzymatic hydrolysis which are commonly known as saccharification in order to release the monomeric glucose units from cellulose. Finally, these glucose units are converted into ethanol by using yeast. Various pretreatment methods have been developed to facilitate the enzymatic hydrolysis of a variety of lignocellulosic materials (Kirk-Othmer 2001; Chen et al. 2009; Ken-Lin et al. 2011; Erdei et al. 2010; Pedersen et al. 2011; Michael et al. 2009)

There is a continuing need for research to find suitable and economical pretreatment techniques. Major challenges for the lignocellulosic bioethanol production include the pretreatment step, which is generally considered the most costly step in the whole process. Development of low cost enzymes or microbes

for production of sugars at industrial level is another challenge. An additional challenge is optimizing the ethanol fermenting microorganisms to tolerate all adverse conditions (Bothwell et al. 2012). In this chapter, some of the technical challenges with respect to bioprocesses for pretreatment are discussed.

1.2 Lignocellulosic Materials

1.2.1 Physical Properties

"Lignocellulosic biomass" refers to higher plants, softwood, or hardwood. The major components of lignocellulosic materials are cellulose, hemicellulose, and lignin. They also include water and a small amount of proteins and other compounds, which do not participate significantly in forming the structure of the material (Raven et al. 1992). Inside the lignocellulose complex, cellulose retains the crystalline fibrous structure which appears to be the core of the complex. Hemicellulose is positioned both between the micro- and the macrofibrils of cellulose. Lignin provides a structural role to the matrix in which cellulose and hemicellulose are embedded (Faulon et al.1994).

The composition of lignocellulosic material strongly depends on its source. There is a significant variation in the lignin and (hemi) cellulose content of lignocellulosic depending on whether it is derived from hardwood, softwood, or grasses. The contents of cellulose, hemicellulose, and lignin in common agricultural residues are listed in Table 1.1. The contents of the structural components are from various materials.

1.2.1.1 Cellulose

Cellulose is the most abundant polysaccharide on earth, approximately 75 billion tons of cellulose are produced and consumed annually (Kirk-Othmer 2001). It is a highly ordered polymer of cellobiose (D-glucopyranosyl-β-1, 4-D-glucopyranose) (Fig. 1.1), representing over 50 % of the wood mass. Cellulose is commonly considered as a polymer of glucose, since cellobiose consists of two molecules of glucose. The chemical formula of cellulose is $(C_6H_{10}O_5)_{n.}$

The properties of cellulose depend on its degree of polymerization (DP), i.e., the number of glucose units that make up one polymer molecule. The DP of cellulose is more commonly a number of 800–10,000 units (Kirk-Othmer 2001). It can also extend up to 17,000 units (wood pulp). Cellulose contains β-1, 4 glucosidic bonds (between glucose molecules), and leads to formation of long straight chains (Fig. 1.2). Later hydroxides are evenly distributed on both sides of the monomers. This leads to numerous strong intermolecular hydrogen bonds between hydroxyl groups of adjacent molecules in the parallel chains (Faulon et al. 1994).

Table 1.1 Composition of lignocellulose in several sources on dry basis (Sun and Cheng 2002)

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–302	25–30	30–40
Corncobs	45	35	15
Paper	85–99	0	0–15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Sorted refuse	60	20	20
Leaves	15–32	80–85	0
Cotton seeds hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste paper from chemical pulps	60–70	10–20	5–10
Primary waste water solids	15–20	NA	24–29
Fresh bagasse	33.4	30	18.9
Swine waste	6	28	NA
Solid cattle manure	1.6-4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switchgrass	45	31.4	12

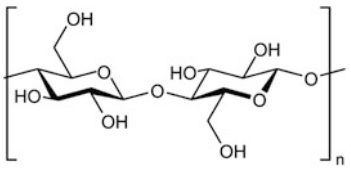

Fig. 1.1 Structural unit of cellulose (cellobiose)

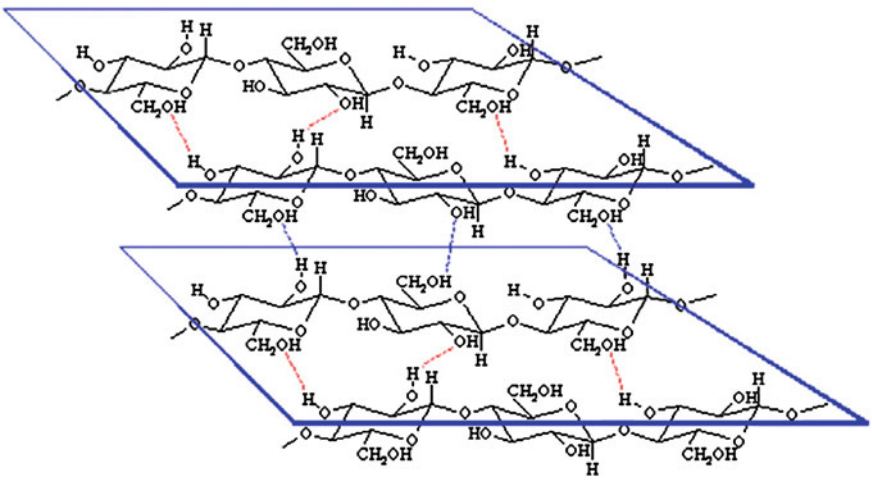

Fig. 1.2 Representation of the hydrogen bonding which allows the *parallel arrangement* of the cellulose polymer chains (Harmsen et al. 2010)

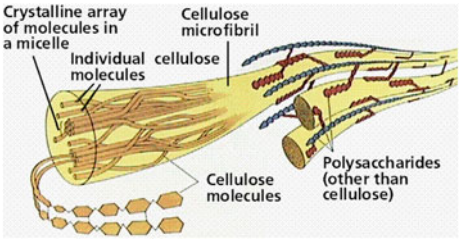

Fig. 1.3 Formation of microfibrils (fibers) of cellulose (Purves et al. 1995)

Cellulose is found in both crystalline and noncrystalline structures. The fusion of several polymer chains leads to the formation of microfibrils, which in turn are united to form fibers. In this way cellulose can obtain a crystalline structure (Fig. 1.3).

1.2.1.2 Hemicelluloses

Polyoses are the linking material between cellulose and lignin. Unlike cellulose, hemicelluloses consist of different monosaccharide units. In addition, the polymer chains of hemicelluloses have short branches and are amorphous. Because of the amorphous morphology, hemicelluloses are partially soluble or swell in water. The backbone of the chains of hemicelluloses can be a homopolymer (generally consisting of single sugar repeat unit) or a heteropolymer (mixture of different sugars). Formulas of the sugar component of hemicelluloses are listed in Fig. 1.5. Among the most important sugars of hemicelluloses component is xylose. In hardwood xylan, the backbone chain consists of xylose units which are linked by β-(1, 4)-glycosidic bonds and branched by β-(1, 2)-glycosidic bonds with 4-O-methyl glucuronic acid groups. In addition, O-acetyl groups sometimes replace the OH groups in positions C2 and C3 (Fig. 1.4). This leads to the lack of crystalline structure (Kirk-Othmer 2001). For softwood xylan, the acetyl groups are fewer in the backbone chain. However, softwood xylan has additional branches consisting of arabinofuranose units linked by β-(1, 3)-glycosidic bonds to the backbone (Fig. 1.4).

1.2.1.3 Lignin

By contrast, Lignin is a three-dimensional polyphenolic network built of dimethoxylated (syringyl), monomethoxylated (guaiacyl), and nonmethoxylated (p-hydroxyphenil) phenylpropanoid units, derived from the corresponding p-hydroxycinnamyl alcohols, which give rise to a variety of subunits including different ether and C–C bonds. Lignin is hydrophobic and highly resistant toward chemical and biological degradation. It is located in the middle lamella, acting as cement between the plant cells, and in the layers of the cell wall, forming, together with hemicellulose, an amorphous matrix in which the

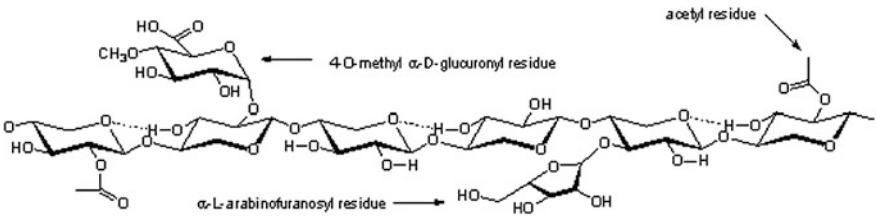

Fig. 1.4 Schematic representation of the hemicellulose backbone (Harmsen et al. 2010)

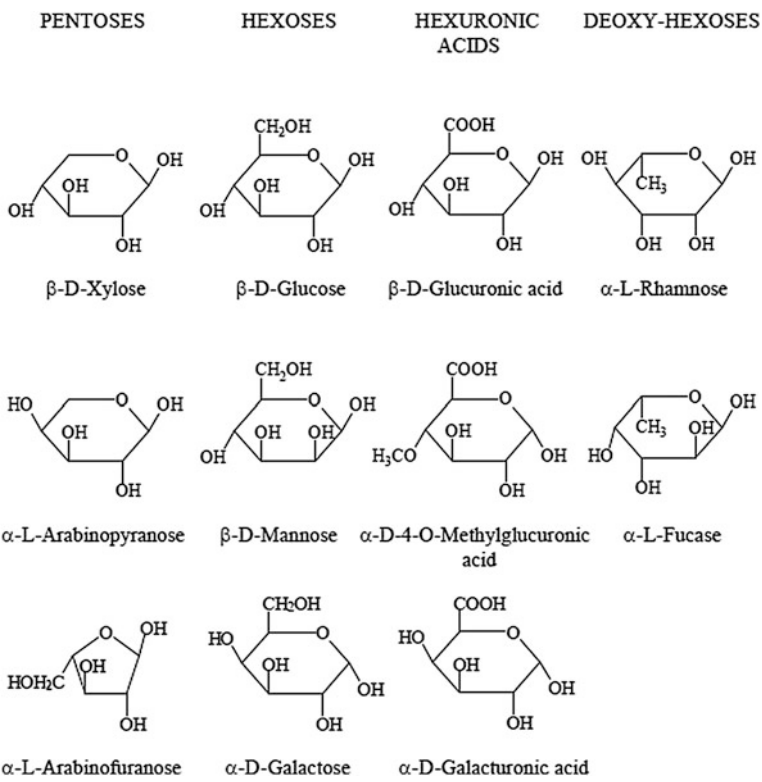

Fig. 1.5 Formulas of the sugar component of hemicelluloses (Mustafa et al. 2009)

cellulose fibrils are embedded and protected against biodegradation. Lignin content and composition vary among different plant groups. Moreover, the lignin composition varies between the different wood tissues and cell wall layers. The monomeric building units of lignin are shown in Fig. 1.6. The guaiacyl unit is dominant in the softwoods. In contrast, syringyl units are dominant in hardwoods (Kirk-Othmer 2001) (Fig. 1.7)

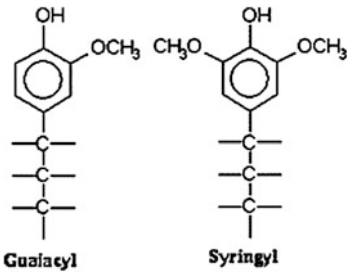

Fig. 1.6 The monomeric building units of lignin (Mustafa et al. 2009)

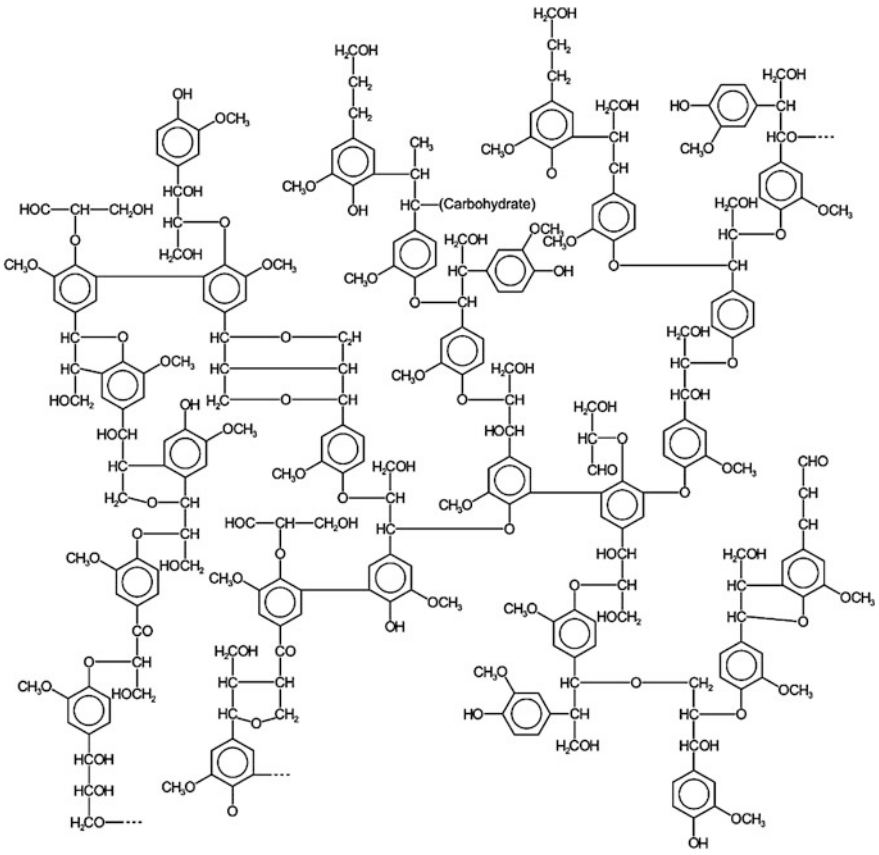

Fig. 1.7 Structure of lignin (Glazer et al. 1995)

Table 1.2 Overview of linkages between the monomer units and polymers to form lignocellulose (Harmsen et al. 2010)

Bonds within different components (*intrapolymer linkages*)	
Ether bond	Lignin, (hemi)cellulose
Carbon to carbon	Lignin
Hydrogen bond	Cellulose
Ester bond	Hemicellulose
Bonds connecting different components (*interpolymer linkages*)	
Ether bond	Cellulose-lignin
	Hemicellulose lignin
Ester bond	Hemicellulose-lignin
Hydrogen bond	Cellulose-hemicellulose
	Hemicellulose-lignin
	Cellulose-lignin

1.2.1.4 Chemical Characteristics

There are four main types of bonds recognized in the lignocellulosic complex. They are (1) Ether, (2) Ester, (3) Carbon-to-carbon, and (4) Hydrogen bonds. These four bonds are the major types of bonds that provide linkages within (intrapolymer linkages), and between the different components of lignocellulosic material to form complex (interpolymer) linkages (Table 1.2) (Faulon et al. 1994).

1.2.1.5 Intrapolymer Linkages

The main types of bonds that connect the building molecules within the lignin polymer are ether bonds and carbon-to-carbon bonds. Ether bonds may appear between allylic and aryl carbon atoms, or between aryl and aryl carbon atoms, or even between two allylic carbon atoms. The total fraction of ether-type bonds in the lignin molecule is around 70 % of the total bonds between the monomer units. The carbon-to-carbon linkages form the remaining 30 % of the total bonds between the units. They can also appear between two aryl carbon atoms or two allylic carbon atoms, or between one aryl and one allylic carbon atom (Kirk-Othmer 2001).

The polymer of cellulose is formed on the basis of two main linkages, the glucosidic linkage and the hydrogen bond.

The glucosidic linkage is one that forms the initial polymer chain. More specifically, it is a 1–4 β D-glucosidic bond that connects the glucose units together. The glucosidic bond can also be considered as an ether bond, since it is in fact the connection between two carbon atoms with an elementary oxygen interfering (Solomon 1988).

The hydrogen bond is considered to be responsible for the crystalline fibrous structure of cellulose. The arrangement of the polymer in long straight parallel

chains together with the fact that the hydroxyl groups are evenly distributed on both sides of the glucose monomer, allow the formation of hydrogen bond between two hydroxyl groups of different polymer chains (Faulon et al. 1994).

1.2.1.6 Interpolymer Linkages

Lignocellulosic complex break down by using different methods (Franklin 1988); they are broken down and the individual components are separated in order to know their interactions. However, their separation modifies their original structure. As a result, there is no clear conclusion on the interpolymer linkages. Faulon et al. (1994) projected a three-dimensional model using molecular mechanics and dynamics in order to understand the organization of these polymers. According to this model, It has been identified that hydrogen bonds not only present between cellulose and hemicelluloses, but also connect lignin with cellulose and hemicellulose. Furthermore, the existence of covalent bonds between lignin and polysaccharides has been identified by examining the ester bonds between lignin and hemicelluloses. There is no clear evidence whether the ether bonds are formed between lignin and cellulose, or hemicelluloses. Hemicellulose lacks primary alcohol functional group external to the pyranoside ring due to this hydrogen linkage being weak between cellulose and hemicelluloses.

1.3 Pretreatment Methods and Associated Challenges

Pretreatment is an important first step for practical cellulose conversion processes, and is required to alter the structure of lignocellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars as represented in the schematic diagram in Fig. 1.8. The goal is to break the lignin seal and disrupt the crystalline structure of cellulose. Pretreatment is considered as one of the most expensive processing steps in cellulosic biomass-to-fermentable sugars conversion with costs as high as 30$/gallon ethanol produced. Pretreatment also has great potential for improvement of efficiency and

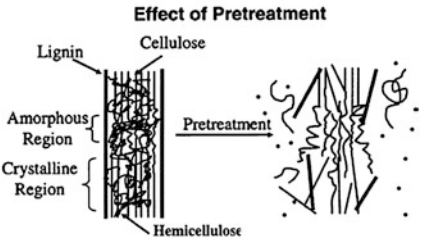

Fig. 1.8 Schematic representation of the effect of pretreatment (Hsu et al. 1980; Mosier et al. 2005)

lowering of cost through research and development (Lynd et al. 1996; Lee et al. 1994; Kohlmann et al. 1995; Mosier et al. 2003a, b).

The following consequences during pretreatment process lead to an improvement in (enzymatic) hydrolysis of lignocellulosic material in order to produce bioethanol:

1. Increase in the surface area and porosity.
2. Modification of lignin structure.
3. Removal of lignin.
4. (Partial) depolymerization of hemicellulose.
5. Removal of hemicellulose.
6. Reduction of the crystallinity of cellulose.

The most Important and current challenges of current pretreatment technologies are choosing the economic pretreatment technique with low enzymatic hydrolysis inhibitors formation. Pretreatment technologies are diverse and numerous, but can be categorized into four main groups (Sun and Cheng 2002).

- Physical/Mechanical pretreatment:
  - Milling
  - Ultrasonic
- Physicochemical pretreatment:
  - Steam explosion
  - Ammonia fiber explosion (AFEX)
  - CO_2 explosion
- Chemical pretreatment:
  - Ozonolysis
  - Acid hydrolysis (dilute acid)
  - Alkaline hydrolysis
  - Oxidative delignification
  - Organosolv process
- Biological pretreatment.

1.3.1 Physical/Mechanical Pretreatment

1.3.1.1 Milling

Reduction in size increases the surface/volume ratio of a spherical particle. This can be done by milling or grinding. Mechanical pretreatment is usually carried out before a following processing step, and the desired particle size is reliant on the subsequent steps. For mechanical pretreatment, factors such as operating costs, capital costs, scale-up possibilities, and depreciation of equipment, are very important.

1.3.1.2 Ultrasonic Pretreatment

This process is widely used for the treatment of sludge from wastewater treatment plants. An experiment on carboxyl methyl cellulose (CMC), with energy by irradiation, increased the rate of the succeeding enzymatic hydrolysis to approximately 200 % (Imai et al. 2004). The mechanism of action, however, remains unknown. One approximation is that the hydrogen bonds of the cellulose crystalline structure were broken due to irradiation energy which is higher than the hydrogen bond energy (Bochek 2003).

1.3.2 Chemical Pretreatment

Pure chemicals or combination of chemicals used as the catalyst in order to mediate the pretreatment.

1.3.2.1 Liquid Hot Water

Hydrolysis using liquid hot water (LHW) is also known as hydrothermolysis, hydrothermal pretreatment, aqueous fractionation, solvolysis, or aquasolv (Mosier et al. 2005). In this process, biomass is pretreated with water at high temperature and pressure. In solvolysis hot compressed water contacts with biomass for up to 15 min at temperatures of 200–230 °C. Between 40 and 60 % of the total biomass is dissolved in the process, with 4–22 % of the cellulose, 35–60 % of the lignin, and all of the hemicellulose being removed. The resulting liquid yields over 90 % of the hemicellulose recovered as monomeric sugars when hydrolyzed with acid. In addition, formation of acetic acid during the treatment catalyzes the polysaccharide hydrolysis leading to the formation of monomeric sugars that may further decompose to furfural which is an inhibitor to fermentation. The amount of sugars released from this method will depend upon the type of biomass and lignin ratio in the overall biomass composition (Mok and Antal 1992).

1.3.2.2 Weak Acid Hydrolysis

Dilute acid treatment is one of the most efficient pretreatment methods for lignocellulosic biomass. Generally there are two types of weak acid hydrolysis:

1. High temperature and continuous flow process for low-solids loading ($T > 160$ °C, 5–10 wt % substrate concentration).
2. Low temperature and batch process for high-solids loading ($T \leq 160$ °C, 10–40 % substrate concentration).

Dilute (mostly sulphuric) acid is mixed with the raw material and the mixture is held at 160–220 °C for short periods for a few minutes. Hydrolysis of hemicellulose occurs, releasing monomeric sugars and soluble oligomers into the hydrolysates. Removal of hemicellulose increases porosity and improves enzymatic hydrolysis and almost completely removes the hemicellulose (Chen et al. 2007). The hemicellulose monomeric sugars might be further degraded to furfural and hydroxymethyl furfural (HMF), strong inhibitors to microbial fermentation. As an alternative to inorganic acids, organic acids (e.g., maleic acid, fumaric acid, and oxalic acid) can be used for dilute acid pretreatment, which neither promotes the degradation of free sugars nor to furfural and HMF (Kootstra et al. 2009; Lee et al. 2010). The treatment offers good performance in terms of recovering hemicellulose sugars; however, there are also some drawbacks that acids can be corrosive and neutralization results in the formation of solid waste. The method is especially suitable for biomass with low lignin content, as almost no lignin is removed from the biomass.

1.3.2.3 Strong Acid Hydrolysis

Hydrolysis using concentrated acid for producing sugars and ethanol from lignocellulosic biomass has been reported since 1883 (Harris 1949). The concentrated acid disrupts the hydrogen bonding among the cellulose chains, converting it into a completely amorphous state. Once the cellulose has been decrystallized, it forms a homogeneous gelatin with the acid. The cellulose is extremely susceptible to hydrolysis at this point. Thus, dilution with water at modest temperatures provides complete and rapid hydrolysis to glucose, with little degradation. Apart from hydrolysis, use of concentrated sulfuric acid is widely accepted as a test method for quantifying the potential glucose content of cellulose (Fan et al. 1987) and for quantifying the lignin content. Concentrated strong acids such as H_2SO_4 and HCl have been widely used for treating lignocellulosic materials (Sun and Cheng 2002), and no enzymes are needed subsequent to the acid hydrolysis. The advantages of concentrated acid hydrolysis are the suppleness in terms of feedstock choice, high monomeric sugar yield, as well as mild temperature conditions that are needed. But concentrated acids are corrosive in nature and recycling of acids is required in order to lower cost. There have been significant efforts for commercializing strong acid hydrolysis processes of lignocellulosic biomass for microbial fermentation purposes (BlueFire Ethanol 2010; Biosulfurol 2010).

1.3.2.4 Alkaline Hydrolysis

The major consequence after alkaline pretreatment is the removal of lignin from the biomass based on saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components such as lignin (Sun and Cheng 2002), thereby improving the digestibility of the residual polysaccharides. In addition,

acetyl and various uronic acid substitutions on hemicellulose were removed by alkali treatment that lowers the accessibility of the enzyme to the hemicellulose and cellulose surfaces (Chang and Holtzapple 2000).

a. **Calcium or sodium hydroxide**

Usually lime (calcium hydroxide) or sodium hydroxide is used. Salts are formed during the treatment which can be included in the biomass and need to be removed or recycled (González et al. 1986). Process conditions are relatively soft but reaction times can be longer about 3–13 h (Kim and Holtzapple 2006). These mild conditions prevent condensation of lignin, improve lignin solubility, and particularly biomass with low lignin content. Due to the mild conditions, degradation of sugars to furfural, HMF, and organic acids is limited. The addition of air or oxygen to the reaction mixture greatly improves the delignification, with highly lignified materials (Chang and Holtzapple 2000).

b. **Ammonia**

Pretreatment of biomass with aqueous ammonia at elevated temperatures reduces lignin content and removes some hemicellulose while decrystallizing cellulose. Ammonia pretreatment techniques include the AFEX-method, ammonia recycle percolation (ARP), and soaking in aqueous ammonia (SAA).

AFEX is discussed under physicochemical pretreatment (Sect. 1.3.2.5). In ARP, the biomass is packed in a column reactor and pretreated with aqueous ammonia in a flow-through reactor at high temperature. To avoid flash evaporation the reactor system must be slightly pressurized (e.g. 2.3 MPa) (Kim et al. 2003; Kim and Lee 2005). After reaction, the solid fraction is separated from liquid, which is rich in cellulose and hemicellulose. For recovery of ammonia liquid fraction is sent into a steam-heated evaporator. After that lignin and other sugars get separated. Ammonia is then recycled to the reactor inlet, whereas the separated fraction is sent into a crystallizer. After crystallization, a washing step is carried out in order to extract the sugars that have been retained in the solid residuals.

SAA at low temperature removes efficiently the lignin by minimizing the interaction with hemicellulose. This results in increase in surface area and pore size. Consequently, retained hemicellulose and cellulose can be hydrolyzed to fermentable sugars by microbial cellulase and hemicellulases. SAA was used (Kim et al. 2008) to destarch the barley hull which was treated with 15–30 % aqueous ammonia (solid-to-liquid ratio 1:12) at 30–75 °C for 12 h to 77 days with no agitation. After soaking, the solids were recovered by filtrating, washed, and analyzed. As a result, they obtained 66 % of lignin solubilization and observed saccharification yields of 83 % for glucan and 63 % for xylan when treating biomass with 15 % aqueous ammonia at 75 °C during 48 h.

c. **Organosolv**

The organosolv process was originally developed as an alternative pulping process for paper making (Kleinert and Tayenthal 1931; Pye and Lora 1991). Since then it has been demonstrated as a potential and promising pretreatment

strategy for lignocellulosic materials and successfully applied for biomass fractionation/pretreatment (Papatheofanous et al. 1995). First, the lignocellulosic biomass is separated into its three main constituents (cellulose, hemicellulose, and lignin). Second, by fractionation and the treatment during organosolv, the cellulose fraction can be made more susceptible for enzymatic hydrolysis to fermentable sugars. Organosolv processes use an organic solvent or mixtures of organic solvents with water for removal of lignin before enzymatic hydrolysis of the cellulose fraction. In addition to lignin removal, hemicellulose hydrolysis occurs leading to improved enzymatic digestibility of the cellulose fraction. High yield of xylose can usually be obtained with the addition of acids such as HCl, H_2SO_4, oxalic, or salicylic acids. Common solvents for the process include ethanol, methanol, acetone, and ethylene glycol, tetrahydrofurfuryl alcohol, and so on. (Zhao et al. 2009) (Temperatures used for the process can be >200 °C, but lower temperatures (120–200 °C) can be sufficient depending on the type of biomass and the use of a catalyst (Ghose et al. 1983)). The solvent must be removed prior to fermentation, because the solvent itself can be an inhibitor for the enzymatic hydrolysis and the fermentation step. Generally, it is removed by evaporation and condensation, removal and recovery of the solvent is required for reducing its cost and environmental impact as well. Normally, low-molecular weight alcohols with lower boiling points such as ethanol and methanol are economically favored (Alvira et al. 2010).

Benefits of organosolv pretreatment include:

- Lowering the enzyme costs improved production of cellulose.
- More absorption of cellulase enzymes.
- Recovery of high-quality lignin, which make it possible to produce higher value derivate chemicals of lignin.
- Minimum cellulose loss.

d. **Oxidative delignification**

Delignification of lignocellulose can also be achieved by treatment with an oxidizing agent such as hydrogen peroxide, ozone, oxygen, or air. Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2 (Azzam 1989). The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis. About 50 % of the lignin and most of the hemicellulose were solubilized by 2 % H_2O_2 at 30 °C within 8 h, and 95 % efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45 °C for 24 h (Azzam 1989).

Wet oxidation was successfully applied for the treatment of lignocellulosic material (Schmidt and Thomsen 1998; Bjerre et al. 1996). In recent studies on alkaline wet oxidation of wheat straw, the main degradation products found from hemicellulose and lignin were carboxylic acids, CO_2, and H_2O. Compared to other pretreatment processes, wet oxidation proved to be efficient for treating lignocellulosic materials, because the crystalline structure of cellulose is open during the process (Panagiotou and Olsson 2007).

e. **Ionic liquids (ILs)**

The use of Ionic liquids (ILs) as solvents for pretreatment of cellulosic biomass has recently received much attention. ILs are salts that are in liquid phase typically at temperatures below 100 °C. Different kinds of ILs with common characteristics are available; which is that they usually comprise an inorganic anion and an organic cation of very heterogeneous molecular structure. The difference in the molecular structure renders the bonding of the ions weak enough for the salt to appear as liquid at room temperature (Van Rantwijk 2003). They do not produce any toxic or explosive gases so they are known as "green" solvents. ILs form hydrogen bonds between the nonhydrated chloride ions of the IL and the sugar hydroxyl protons, thus leading to disruption of the complex network of noncovalent interactions among the polymers cellulose, hemicelluloses, and lignin. Recently, a few studies demonstrated the IL pretreatment on wheat straw (Li et al. 2009) and wood (Lee et al. 2009). There is no toxicity effect observed on the growth of *Saccharomyces cerevisiae* grown on the IL (1-ethyl-3-methyl imidazolium diethyl phosphate) treated wheat straw (Li et al. 2009). Only limited information is available on the different ILs and their effects and further research is needed to improve the economics of ILs pretreatment and their recycle.

1.3.2.5 Physicochemical pretreatment

These methods combine mechanical and chemical actions. To this group belong the following pretreatments:

a. **Steam explosion**

Steam explosion (uncatalyzed or catalyzed) is one of the most applied pretreatment processes owing to its low use of chemicals and limited energy consumption. In this method, high-pressure saturated steam is injected into a batch or continuous reactor filled with biomass. During the steam injection, the temperature rises to 160–260 °C. Subsequently, pressure is suddenly reduced and the biomass undergoes an explosive decompression with hemicellulose degradation and lignin matrix disruption as result. Results of steam explosion pretreatment depend on residence time, temperature, particle size, and moisture content (Sun and Cheng 2002).

Studies have been carried out to try to improve the results of steam explosion by addition of chemicals such as acid or alkali (Cara et al. 2008; Stenberg et al. 1998; Zimbardi et al. 2007). In a recent study, steam explosion treatment was optimized at the batch scale on the basis of carbohydrate recovery for wheat, barley, and oat straws. The yields of fodder, lignin, and hemicellulose were found to be dependent on the nature of the starting straw. Delignified fodder (insoluble fraction) was produced with yields of 0.64, 0.59, and 0.55 from wheat, barley, and oat straw, respectively. Steam explosion improved the digestibility of the straw by 25 % (Viola et al. 2008). Limitations of steam explosion include the formation of degradation products that may inhibit downstream processes (Garcia-Aparicio et al. 2006).

b. **AFEX**

AFEX is a physicochemical pretreatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is suddenly reduced. The AFEX process is similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1–2 kg of ammonia/kg of dry biomass, the temperature is 90 °C, and the residence time is 30 min. (Teymouri et al. 2005). It reduces the lignin content and removes some hemicellulose while decrystallizing cellulose. The cost of ammonia and especially of ammonia recovery drives the cost of the pretreatment (Holtzapple et al. 1994), although ammonia is easily recovered due to its volatility.

c. **CO2 explosion**

This method is similar to steam and ammonia fiber explosion; high pressure CO_2 (supercritical) explosion is injected into the batch reactor, and then liberated by an explosive decompression. It is believed that CO_2 reacts to carbonic acid (carbon dioxide in water). Carbon dioxide molecules are comparable in size to water and ammonia, and are usually able to penetrate small pores accessible to water and ammonia molecules, thereby improving the hydrolysis rate. Yields of CO_2 explosion are lower than those obtained with steam or ammonia explosion, but they are higher than those reached with enzymatic hydrolysis without pretreatment (Sun and Cheng, 2002). Due to low temperature, utilization cost of this process is potentially lower compared to ammonia explosion. Low temperature also prevents decomposition of monosaccharides by the acid.

Explosive release of the carbon dioxide pressure and the disruption of the cellulosic structure increases the accessible surface area of the substrate to hydrolysis (Sahle et al. 1995; Kim and Hong 2001).

1.3.3 Biological Pretreatment

Most pretreatment technologies require exclusive and expensive instruments or equipment that have high energy requirements, depending on the process. In particular, physical and thermochemical processes require abundant energy for biomass conversion. Biological treatment using various types of rot fungi, a safe and environmentally friendly method, is increasingly being advocated as a process that does not require high energy for lignin removal from a lignocellulosic biomass, despite extensive lignin degradation (Okano et al. 2005).

In biological pretreatment processes, microorganisms such as brown-, white-, and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials (Galbe and Zacchi 2007). Brown rots mainly attack cellulose, whereas white and soft rots attack both cellulose and lignin. Lignin degradation by white-rot fungi occurs through the action of lignin-degrading peroxidases and laccase (Lee et al. 2007). These enzymes are regulated by carbon and nitrogen sources. White-rot

fungi are the most effective for biological pretreatment of lignocellulosic materials (Fan et al. 1987). An extensive range of studies has been carried out on the pretreatment of wheat straw by 19 white-rot fungi (Hatakka 1983).

The white-rot fungus *P. chrysosporium* produces lignin degrading enzymes, lignin peroxidases, and manganese-dependent peroxidases, during secondary metabolism, in response to carbon or nitrogen limitation (Boominathan and Reddy 1992). Both enzymes have been found in the extracellular filtrates of many white-rot fungi for the degradation of wood cell walls (Kirk and Farrell 1987; Waldner et al. 1988). Singh et al. (2008) evaluated eight bioagents, including fungi and bacteria, for their pretreatment effects on sugarcane trash. They narrowed down the C/N ratio of trash from 108:1 to a varying range from approximately 42:1–60:1. The maximum drop in C/N ratio of 61 % was observed using *Aspergillus terreus*, followed by those obtained using *Cellulomonas uda* (52 %) and *Trichoderma reesei* and *Zymomonas mobiliz* (49 %). The C/N ratio is important for biomass pretreatment, because degradation of lignocellulosic material depends on the material's C/N ratio. To degrade each molecule of carbon, a definite proportion of nitrogen is required by the microorganisms, and this varies with different kinds of micro flora. Fungi have a higher C/N ratio (30:1) as compared to bacteria (10:1); hence, fungi are more capable of degrading any lignocellulosic material, as their dependency on nitrogen is comparatively lower (Wichern et al. 2004). Report by Ravichandra et al. (2012) on the microbial and cell free hydrolysis of corncobs using *P. chrysosporium* has shown the efficient release of sugars (424.50 mg/2 g for 120 h and 395.15 mg/2 g for 48 h).

1.3.3.1 Cellulase Producing Microorganisms

Cellulase producing microorganisms primarily depend upon simple carbohydrates and are unable to use complex components like lipids and proteins for energy generation (Lynd et al. 2002). Some of the cellulolytic bacteria, *cellulomonas* and *cytophyga*, depends upon a variety of carbohydrates in addition to the cellulose (Poulsen and Petersen 1988; Rajoka and Malik 1997). In contrast to anaerobic, cellulolytic species have narrow carbohydrate range and limited cellulose and its hydrolytic products (Sukumaran et al. 2005). Large-scale production of cellulase was achieved by certain fungi which secrete large amounts of extracellular protein as cellulase. The most extensively studied cellulolytic microorganisms are *Trichoderma, Pencelluim, Humicola, and Aspergillus*; Bacteria: *Bacilli, Pseudomonas, Celluluomonas* and Actinomycetes, *Actinomucor; Streptomyces sp.* Commercial production of cellulase is limited to some species like *T. ressi* and few other *species.* Some cellulase producing microorganisms are listed in Table 1.3.

In addition to cellulose degrading, some white fungi (*P. chrysosporium*) can also produce lignin peroxidases to degrade lignin. *P. chrysosporium* produces complex arrays of cellulases, hemicellulases, and lignin-degrading enzymes for the efficient degradation of all three major components of plant cell walls: cellulose, hemicellulose, and lignin (Broda et al. 1994; Copa-Patino et al. 1993; Covert et al.

Table 1.3 Major cellulase producing microorganisms

Group	Microorganism	Reference
Fungi	*Acremonium cellulolyticus*	Tatsuya et al. (2009)
	Aspergillus acculeatus	Sawao et al. (1988)
	Aspergillus fumigatus	Dongyang et al. (2011)
	Aspergillus niger	Acharya et al. (2008)
	Fusarium solani	Wood (1971)
	Irpex lacteus	Eiichi et al. (1993)
	Penicillium funmiculosum	Van Wyk (1999)
	Phanerochaete chrysosporium	István et al. (1996); Henriksson et al. (1999); Potumarthi et al. 2013)
	Schizophyllum commune	Steiner et al. (1987)
	Sclerotium rolfsii	Lachke and Deshpande (1988)
	Sporotrichum cellulophilum	Henri (1984); Shinichi et al. (1986)
	Talaromyces emersonii	Oso (1978)
	Thielavia terrestris	Henri (1984)
	Trichoderma koningii	Wood and McCrae (1978)
	Trichoderma reesei	Henri (1984); Van Wyk (1999); Tatsuya et al. (2009)
	Trichoderma viride	Beldman et al. (1985); Griffin et al. (1974)
Bacteria	*Clostridium thermocellum*	Thomas and Zeikus (1981)
	Ruminococcus albus	Leatherwood (1965)
Actinomycetes	*Streptomyces sp.*	Jang and Chang (2005)
	Thermoactinomyces sp.	Bärbel et al. (1978)
	Thermomonospora curvata	Fred (1971, 1972)

1992; Vanden et al. 1993). Cellulose and hemicellulose degradation occurs during primary metabolism, whereas lignin degradation is a secondary metabolic event triggered by limitation of carbon, nitrogen, or sulfur (Broda et al. 1996). During the growth on carbon or nitrogen limiting media ligninolytic activity is observed between 4 and 5 days. Whereas the nitrogen and carbohydrates were present in excess and sulfur was limited (20 μM), cultures became ligninolytic between 7 and 8 days. Sulfur concentration below this level (17 μM) did not trigger the lignin degradation due to poor growth (Thomas et al. 1981).

1.3.3.2 Bio Process for Cellulases: Fermentation, Production of Cellulolytic Enzymes

A majority of the reported work utilized submerged fermentation (SMF) in order to produce the cellulose; widely studied organisms are fungi *Trichoderma sp.* but

natural growth and cellulose utilization of anaerobic microorganism resembles solid-state fermentation (SSF), which led most of the investigators toward SSF. However, SMF has the advantage in terms of better handling and monitoring of microorganisms over SSF. Carbon source for the production of cellulase is primarily cellulosic biomass such as rice straw, rice husk, wheat bran, corncobs, bagasse, waste from paper industry, and other lignocellulosic materials (Hafedh et al. 2001; Reczey et al. 1996; Sukumaran et al. 2005). The cellulase producing process is primarily a batch process. There have been successful attempts to produce cellulase from batch (Hafedh et al. 2001; Ghose and Vikram 1979) and continuous mode (Bailey and Tähtiharju 2003) operation of reactors. This is helpful to overcome the repression mediated by reducing sugars. SSF for production of cellulase is gaining importance as it decreases the production cost of enzyme and also utilizes and converts the abundant, inexpensive, and renewable lignocellulosic biomass to valuable components. There is a 10-fold reduction in the production cost in SSF compared to SMF. There are also some reports with mixed cultures of *Trichoderma* and *Aspegillus* on SSF for production of cellulase (Maria et al. 1994; Jayant et al. 2011). Mixed culturing is found to be beneficial compared to single-culture for economic production of cellulase on nutritionally poor agricultural residues, without the need for expensive organic supplements (Marcel et al. 1999). The biomass productivity and enzyme productivity were also found to have improved to double that in the mixed-culture compared to the single-culture (Dueñas et al. 1995) operation.

1.4 Conclusions

Energy crisis and environmental pollution drive the scientific community toward the potential exploitation of lignocellulosic biomass which is widely available and relatively inexpensive. To crack their complex structures, various pretreatment technologies including biological, mechanical, chemical methods, and various other combinational methods are available. Biological pretreatments make use of lignocellulolytic microorganisms that degrade lignin and hemicellulose. Due to slow hydrolysis rate they are not fruitful. Mechanical methods (physical methods) reduce crystallinity and the particle size, and increase the surface/volume ratio, and thus it makes material handling simpler. However, these processes often require high energy and overheads. It shares more than 50 % of the production cost of bioethanol.

In chemical pretreatments chemicals such as alkalis, ozone, peroxide, or organic solvents effectively remove the lignin, consequently improving the enzymatic digestibility of cellulose. Pretreatments with acids affect the hydrolysis of hemicellulose and celluloses to sugars. A combination of mechanical and chemical treatment would give better results.

We cannot relate the best and common pretreatment method to all types of the lignocellulosic biomass. Mostly, it depends on the type of lignocellulosic biomass

under use and the desired final products. The final aim of pretreatments must be improvement in the hydrolysis rate of lignocellulosic biomass. Every pretreatment has its own effects on the cellulose, hemicellulose, and lignin fractions: Acid pretreatment acts on the hemicelluloses, whereas alkali and organosolv act on the lignin and organosolv treatment completely, removing the lignin and improving the cellulose accessibility. In contrast to these methods, biological pretreatment can act effectively on pretreated lignocellulosic materials or moderately on non-treated materials. For the production of ethanol at the industrial level, acid-based pretreatment methods are widely used. However, there is an unmet need to research and improve these pretreatment processes. The challenges to overcome include minimization of sugar loss; limit the production of inhibitors during pretreatment, increase the solids concentration, and decrease the water utilization. Last but not the least, there are needs to develop tailor-made effective pretreatment methods for different types of lignocellulosic biomass.

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Chapter 2
Sustainability Assessment of Palm Biodiesel Production in Thailand

Thapat Silalertruksa and Shabbir H. Gheewala

Abstract Palm oil is the world's largest source of edible oil and is recently being promoted to produce biodiesel in South–East Asian countries including Thailand. The impacts of palm biodiesel production and use to the environment and socioeconomic development in Thailand are presented. Substituting palm biodiesel for diesel can result in various positive externalities to the Thai society and economy such as GHG emissions reduction, employment generation, GDP development, and trade balance improvement. However, some potential environmental drawbacks of increased palm biodiesel production in the future such as the increase in eutrophication impact potential and land-use change from forest land to oil palm need to be considered and regulated. Improving the sustainability of palm oil and palm biodiesel industry in the future entails the investigation on the potential exploitation of palm-based biorefinery systems along with the concept of "cascade utilization" to fully utilizing biomass residues and wastes generated from palm oil and palm biodiesel industry.

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2.1 Introduction: Situation and Challenges of Global Biodiesel Production and Use for Transport

To alleviate the threat of fluctuating oil price and climate change, substituting agrofuels for petroleum fuels especially for the transportation sector has been used as a measure by nations around the world (WGBU 2010). Biodiesel can be used to substitute diesel in either pure or blended form and is considered as an attractive alternative fuel because of its environmental friendly characteristics while giving almost the same functional properties as conventional diesel. Global biodiesel production in 2010 was around 53.4 ml/day or sharing about 18 % of the total global biofuels production (EIA 2010). The annual figure has grown exponentially over the past decade i.e. more than tenfold since 2000. Germany is the largest biodiesel producing country contributing about 15 % of the global biodiesel production, followed by Brazil, France, Argentina, and the USA sharing about 12, 11, and 6 %, respectively (EIA 2010). The increased demand for biodiesel is anticipated to continue in the foreseeable years due to the established goals of biodiesel utilization set by many nations such as the United States, the European Commission (EU), Brazil, China, India, and countries in South–East Asia such as Indonesia, Malaysia, and Thailand (Smyth et al. 2010; USAID 2009).

Commercial biodiesel today is mainly produced by transesterification of vegetable oil and animal fats or so-called "first generation biodiesel". Rapeseed oil and sunflower oil are the principal feedstocks used for biodiesel in Europe. Soya oil is the outstanding feedstock in the US. Palm oil which so far has been mainly used and exported for food and oleo-chemical industries is being promoted to produce biodiesel in tropical America and South–East Asia. Palm oil nowadays has gained attention as the most attractive feedstock for commercial biodiesel production compared to other vegetable oils due to its high yield of oil, its lower production cost and its availability of supply (Vanichseni et al. 2002; Tan et al. 2009; Sumathi et al. 2008; Mekhilef et al. 2011). The importance of palm biodiesel is not only for domestic consumption in the producing countries but also international trade especially for the countries for which imported biodiesel is more cost-efficient than domestic production (Zah and Ruddy 2009).

Substituting palm biodiesel for diesel is expected to bring about several environmental and social benefits such as reduction of fossil resource depletion and mitigation of GHG emissions. Besides, for developing countries which generally are agriculture based, the development of biodiesel produced from their own indigenous feedstocks is expected to provide new social and economic opportunities for rural development. Several studies have addressed the environmental performance such as GHG emissions (Pleanjai et al. 2009; Wicke et al. 2008; Reijnders and Huijbregt 2008; Germer and Sauerborn 2008; Yusoff and Hansen 2007), energy efficiency (Pleanjai and Gheewala 2009; Kamahara et al. 2010), and cost performance (Silalertruksa et al. 2012) of palm oil and palm biodiesel production (Pleanjai and Gheewala 2009; Kamahara et al. 2010). These have shown large variations in the life-cycle greenhouse gas (GHG) emissions and net energy balance. This is due to

a number of factors such as different palm oil milling waste management; geographical conditions of oil palm plantation, allocation methods used to treatment byproducts in biodiesel system, different system boundaries e.g., including and excluding land-use change (Schmidt et al. 2009; Silalertruksa and Gheewala 2012).

Furthermore, the rapid growth of palm biodiesel production has raised many concerns amongst experts worldwide, in particular with regard to sustainability issues and the threat posed to ecosystems and society. For example, some studies have reported that increased palm oil demand for biodiesel can entail substantial ecological damage e.g., loss of biodiversity and increase in GHG emissions due to deforestation and land-use change (Stone 2007; Hooijer et al. 2006; PEACE 2007; Fargione et al. 2008). Therefore, case-specific analyses are necessary to evaluate environmental sustainability of biofuels production (Larson 2006). This is especially so for the life-cycle GHG emissions which nowadays is set as one of the key environmental sustainability indicators of bioenergy in various standards/initiatives (Ismail and Rossi 2010).

2.2 Biodiesel Development in Thailand and the Necessity for Sustainability Assessment

Thailand is also an agro-industrial country located in tropical region with a variety of suitable plant oils for biodiesel production such as palm, coconut, jatropha, sunflower, and so on. However, among those, oil palm is the most outstanding feedstock used today in Thailand and most of biodiesel in commerce today is produced from crude palm oil (CPO) and palm oil derivatives. In 2010, Thailand was ranked as the 8th world's largest producer with the production rate about 1.75 ml/day (EIA 2010). The Royal Thai Government (RTG) has promoted biodiesel since 2005 as one of the measures to counter the energy crisis caused by the increased oil price in the world market. At the initial stage, the production of biodiesel was insignificant until B2 biodiesel, a mix of 2 % B100 with 98 % of diesel oil was launched as a mandatory blend for the diesel on the market in 2008. This B2 blending mandate has been raised to B3 biodiesel since 2011 and B5 biodiesel has also been launched on the market as a voluntary program. In addition, the latest goals of biodiesel production have been set at 3.6 and 4.5 ml/day by 2016 and 2022, respectively, and B10 biodiesel is expected to be available for nationwide use by 2012 (DEDE 2011).

As the figures above indicate, the question for Thailand today is not if biodiesel plays a significant role in providing energy for transport, rather what the consequences and sustainability of its production and use will be—for the environment and society. To address the sustainability of palm biodiesel production and use in Thailand and to provide the appropriate recommendations for policy makers for directing biodiesel production in the future toward sustainability, the environmental and social/socioeconomic impacts of palm biodiesel from various systems

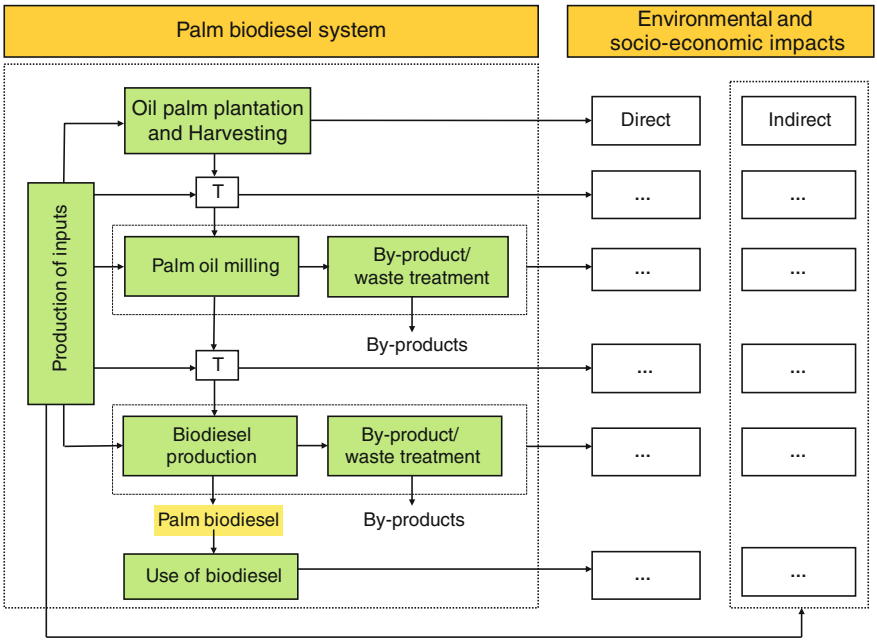

Fig. 2.1 Simplified palm biodiesel system and implication of direct and indirect impacts

need to be assessed throughout the entire life cycle and compared to the conventional diesel that being substituted. Figure 2.1 shows the simplified palm biodiesel production system and scope of impacts to be assessed. Direct impacts mean the environmental and socioeconomic impacts generated from the main stages of biodiesel production and indirect impacts mean those induced from the production of input materials and energy for the main stages.

Life-cycle assessment (LCA) and Input–Output (IO) analysis are two decision supporting tools for evaluating the environmental and socioeconomic impacts of palm biodiesel production in Thailand as both direct and indirect impacts will be accounted. LCA is an assessment tool to compile and evaluate the environmental impacts of a product or service system throughout its life cycle (ISO 2006; Dreyer et al. 2003; Guinée et al. 2002). According to this approach, all burdens including resources used, land occupied, airborne emissions i.e., CO_2, NO_x, SO_x, CH_4, N_2O and waterborne emissions i.e., COD, BOD, Total P, Total N, and suspended solids since oil palm plantation through palm oil and palm biodiesel production, to use will be considered in the environmental impacts assessment. This can help the policy makers draw a complete picture of environmental performance of palm biodiesel as the major environmental impacts of palm biodiesel will be revealed. IO analysis is an economic assessment tool to study the interrelationships within and between economic sectors of a country and it can be used to determine the impacts of an economic activity on the whole economy (Wicke et al. 2009; Suh 2009). As the direct and indirect impacts of an economic activity on the whole

economy can be calculated through the IO analysis; therefore, it can be applied to investigate the employment and other socioeconomic impacts i.e., GDP and national trade balance of palm biodiesel production in Thailand which will be discussed later.

2.3 Palm Biodiesel Systems in Thailand

In general, palm-based biodiesel system based on life-cycle approach can be simplified as Fig. 2.2. The system consists of five major stages i.e., oil palm plantation, palm oil milling, biodiesel conversion, transportation of raw materials, and use phase of biodiesel. Nevertheless, due to the complexity of palm milling stage where a variety of products, byproducts, and wastes are generated, palm biodiesel production systems in reality therefore will vary by approaches used to manage those byproducts and wastes. Different approaches of wastes and byproducts management at the mills, therefore, are evaluated in the study as scenarios to determine their influence on the environmental performance of palm biodiesel. Descriptions of the palm-based biodiesel systems in Thailand and assumptions used for the assessments are as follows:

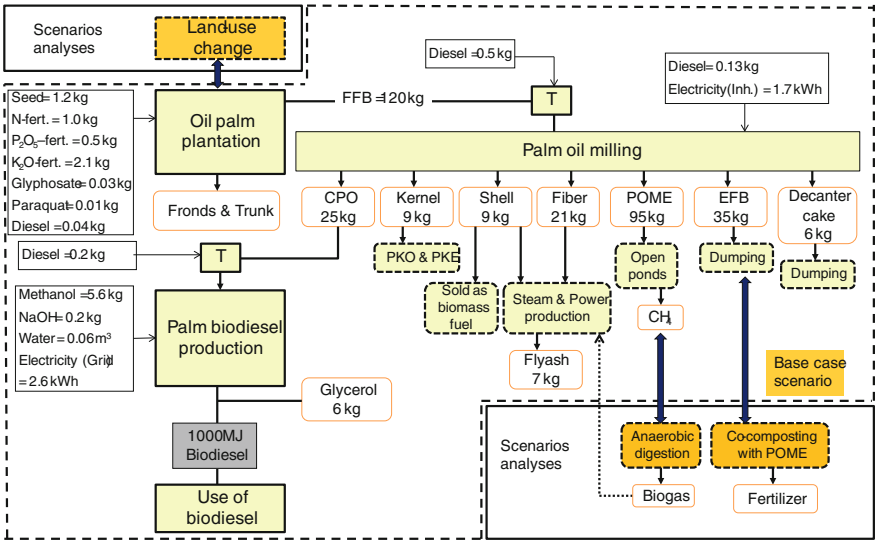

Fig. 2.2 Palm biodiesel system

2.3.1 Oil Palm Plantation

Palm oil is now the world's largest source of edible oil with total global production of about 38.5 Mt/year contributing around 25 % of the worldwide total edible oil and fats production (Shuit et al. 2009). Indonesia and Malaysia are the two leading producers and exporters of palm oil sharing between them around 87 % of the 48 Mt of global palm oil produced in 2010 (USDA 2011). Even though the scale of oil palm plantations and palm oil productions in Thailand is far smaller than Indonesia and Malaysia, there is a high capability to expand oil palm plantations in the future due to suitable climatic conditions particularly in the southern region. Over the past decade, the oil palm planted areas in Thailand have increased by 9 % annually. The total planted areas have reached 0.65 M.ha in 2010 and around 0.57 M.ha was recorded as the harvested area (OAE 2011).

The stage of oil palm plantation consists of growing oil palm at the nursery and planting it in the field. The whole nursery step takes approximately 12–13 months since sowing of seed in a small poly bags until seedling ready for planting. Oil palm starts bearing bunches 2.5–3 years after field planting and the first harvest is possible after 5 years. The oil palm's productive life actually can last as long as 50 years; however, after 25 or 30 years its stalk reaches a height that makes it difficult to harvest. Therefore, the average lifetime of oil palm used in the study is 25 years before replanting. The average fresh fruit bunches (FFB) yield in Thailand is 17.5 t/(ha.yr) (OAE 2010). In addition, biomass residues such as fronds and trunks are also generated in the plantation. The fronds, cut down regularly from the palm trees over the service life of the plantation, are estimated to be around 12 t of dry matter per hectare and year (APC 2007). However, the trunks will be available only one time (when cutting down for re-planting) over the 25 years; the total dry mass of these trunks was estimated to be around 57 t/hec (APC 2007). Fertilizers and agrochemicals such as glyphosate and paraquat are the major input material during this stage; as well as a few diesel is also required for farm equipment and truck for FFB transport, although most of the harvest is done manually.

2.3.2 Palm Oil Milling

Palm oil milling consists of several processing steps including: (1) Loading of FFB in the mill; (2) Sterilization of FFB; (3) Bunch stripping—the empty fruit bunches (EFB) will be separated; (4) Digestion of separated fruits; (5) CPO extraction from oil mash and separation of decanter cake; (6) Nut/fiber separation—fiber is used a fuel for boiler house to produce steam and electricity in the palm oil processing mill itself; (7) Nut cracking—kernels and shell are separated at this stage. Palm kernel oil (PKO) and palm kernel extract (PKE) are produced from the mechanical processing of kernels while the separated shells are partially be used as fuel in

boiler. The remaining shells can be sold as solid fuel or sold as material for producing activated carbon.

Many options can be used for treating the wastes generated at the mills nowadays. For example, the EFB which are conventionally treated by dumping or mulching in plantation can be managed in the other ways such as using it as biomass fuel for in-house steam and power generation, or together with palm oil mill effluent (POME) for co-composting. POME itself can also be treated in different ways depending on the mills. Direct discharge of POME to the environment is not allowed due to high organic loading; therefore, some mills collect it in open ponds and leave it there for self-purification. Others use anaerobic digestion systems for treatment and the treated POME is applied for irrigation. However, thanks to the Clean Development Mechanism (CDM) opportunities, recovery of biogas from POME has nowadays gained attention in Thailand. Biogas contains approximately 60 % CH_4 and 40 % CO_2 and can be generated from anaerobic treatment (Poh and Chong 2009). This CO_2 from biogas can be considered carbon neutral as the amount of carbon released is the same as carbon that had been sequestered during the growth of FFB. However, CH_4 emission cannot be considered neutral in terms of GHG emission as it has a higher GWP than the CO_2 that was taken up by FFB. In the study, a base case and three scenarios of EFB and POME management at the mills as defined in Table 2.1 are assessed to determine their influence to the environmental performance of biodiesel.

CPO is processed further for biodiesel production and palm kernels are used for crude palm kernel oil (CPKO) production. To produce a kilogram of CPO, 5.95 kg of FFB are required for the milling process and this will generate by-products and waste materials including fiber, shell, kernel, decanter cake, and EFB at about 0.83, 0.36, 0.45 and 1.43 kg, respectively (Chavalparit et al. 2006). Economic-based allocation technique was applied to share the environmental burdens from oil palm cultivation and palm oil mill between the main products i.e., CPO and the co-products that are sold as raw material or fuel i.e., palm kernel and shell. Referring to the average prices of CPO, palm kernel and shell as interviewed from palm oil mills in year 2010 i.e., 0.91, 0.56, and 0.06 US$/kg, respectively (based on the exchange rate in 2010 about 31.87 THB/US$), an allocation factor for CPO of about 0.81 is obtained. This is used to evaluate the environmental burdens from CPO production. Allocation is not required for the fiber and a part of shells that are used as fuel for in-house steam and power generation; this can be considered as an internal recycling. EFB and decanter cake which currently have no use but are actually dumped by some mills at the oil palm plantations, are primarily

Table 2.1 Scenarios for treatment of EFB and POME at the palm oil mills

	EFB	POME
Base case	EFB is dumped in the plantation	Open ponds with CH_4 leakage
Scenario 1	EFB is dumped in the plantation	Biogas recovery
Scenario 2	Co-composting with POME	Open ponds
Scenario 3	Co-composting with POME	Biogas recovery

considered in the base case as waste, and hence no environmental burdens are allocated to them. The inputs and outputs relevant to production of 1 MJ palm biodiesel can be summarized in Fig. 2.2.

2.3.3 Biodiesel Production

At the biodiesel plant, CPO is transesterified with the catalyst, sodium hydroxide (NaOH), and methanol. At this stage, the inputs are CPO, water, electricity, methanol, and NaoH. Outputs from the process include biodiesel, glycerol, and wastewater. Economic allocation is also used to share the environmental burdens between biodiesel and glycerol. The market values of biodiesel and glycerol are 1 US$/L (DEDE 2009) and 0.56 US$/kg (ICISpricing 2010), respectively. The allocation factors obtained are 0.92 for biodiesel and 0.08 for glycerol.

2.3.4 Transport

The small farmers generally sell their product (FFB) to middlemen, who operates the ramp and deal with different mills in terms of delivered volume and prices. Delivery of FFB by truck is a conventional transport method in Thailand. Transport capacity and distance traveled from field to mills and mills to biodiesel plant are retrieved from (Pleanjai and Gheewala 2009).

2.3.5 Use of Biodiesel

To evaluate the use phase emissions of diesel and biodiesel, the chassis dynamometer studies for a pick-up truck (or Light Duty Diesel Vehicle: LDDV) are referred and the obtained average emissions from the combustion of a litre biodiesel are 2.78 kg CO_2, 7.5 g CO, 29 g NO_x and 1.3 g Particulate Matter (PM) (Pleanjai 2008). However, as CO_2 emissions from combustion of biodiesel are largely considered carbon neutral because they are of biogenic origin, this is not so for diesel. Thus, in the study, only CO_2 emissions from the part of biodiesel that is from the nonbiogenic methanol contributing around 5.6 % of the total carbon content in the biodiesel are considered to contribute to global warming. Therefore, the use phase GHG emission of biodiesel would be only 0.15 kg CO_2/L biodiesel.

2.4 Environmental Sustainability of Palm Biodiesel

2.4.1 GHG Performance

Based on global warming potential factors of IPCC (2007), life-cycle GHG emissions per MJ of palm biodiesel in various scenarios of waste treatments at the oil mills as defined in Table 2.2 can be examined. The results reveal that the differences in measures for managing EFB and POME at the mills lead to different GHG emissions of palm biodiesel i.e., from 22 to 45 g CO_2 eq/MJ biodiesel as shown in Fig. 2.3. Base case scenario of biodiesel system in Thailand in which EFB is dumped and POME is treated in the open ponds with methane leakage brings about the highest GHG emissions. Two major sources of GHG emissions are N-fertilizer application at the plantation stage and methane generated from the treatment of POME in open ponds at the milling stage sharing about 38 and 40 % of the total GHG emissions of biodiesel, respectively.

Nevertheless, the biogas production from POME nowadays has been recognized in Thailand due to the enhancement of the CDM opportunities. This is a great opportunity to improve GHG performance of biodiesel by installing the anaerobic treatment and biogas recovery system. Then, the emitted methane would be avoided and the energy credits from biogas could be accounted to the GHG performance of biodiesel. In scenario 1, the GHG emissions of biodiesel could be reduced by 46 % as compared to base case. In addition, POME can also be used for co-composting with EFB as in scenario 2 and this will bring about 43 % GHG

Table 2.2 GHG performances for various biodiesel systems in Thailand

	GHG emissions (g CO_2-eq/MJ biodiesel) Range	% Net avoided GHG emissions when comparing with diesel[a] Range
Excluding LUC	22[b]–45[c]	42[c]–71 %[b]
Including dLUC		
Forest land—oil palm	261[b]–283[c]	(−240[b])–(−269 %)[c, d]
Cropland—oil palm	3[b]–25[c]	67[c]–96 %[b]
Grassland—oil palm	11[b]–34[c]	56[c]–85 %[b]
Rubber—oil palm	7[b]–29[c]	62[c]–91 %[b]

[a] % Net avoided GHG emissions are estimated based on diesel fuel-cycle GHG emissions = 76.8 g CO_2 eq/MJ (Silalertruksa and Gheewala 2012)

[b] Referring to biodiesel system (scenario 3) in which POME is treated to produce biogas before co-composting with EFB and returning the produced compost to the plantation

[c] Referring to biodiesel system (base case) in which EFB is dumped in the plantation and POME is treated in the open ponds with CH_4 leakage

[d] (-) means GHG emissions increased as compared to diesel

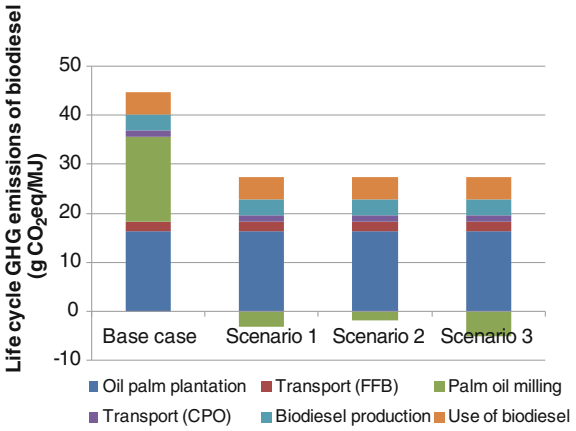

Fig. 2.3 Life-cycle GHG emissions of palm biodiesel produced from different production systems (g CO_2 eq./MJ biodiesel)

emissions reductions. However, the most efficient system found in scenario 3 i.e., POME after biogas production was returned to produce compost which would result in 50 % GHG emissions reduction.

2.4.2 Direct Land-Use Changes and GHG Consequences

One of the most concerns from the rapidly increasing demand for biodiesel is the unregulated conversion of lands and/or cropping systems into oil palm (or called "land-use change"). This in turn may cause the drawbacks to the ecosystems and society such as the substantial release of CO_2 into the atmosphere from carbon stock change and food-fuel conflicts. So far, land-use change has not yet been much relevant for palm biodiesel production in Thailand as biodiesel demand is small scale and FFB used currently originate from the old palm plantations. However, to satisfy the future anticipated CPO demand for food and palm biodiesel production, both oil palm productivity improvement and expansion of palm plantations are necessary and land-use change impacts would become important.

In the study, four possible direct land-use changes (dLUCs) originate into oil palm in Thailand including (1) tropical forest land in the northeast, (2) cropland such as cassava plantation in the northeast or the east, (3) grassland which is assumed to represent the available set-aside land in Thailand, and (4) old rubber fields in the south are considered in the analyses. Those land-use change scenarios are from the onsite surveys and/or interviews with farmers (Siangjaeo et al. 2011; JGSEE 2010) accompanied with the policy to encourage the new oil palm plantations of the RTG which specified in the palm oil industry and oil palm development plan (years 2008–2012) (NCGEB 2009). Based on the IPCC guidelines for calculating GHG emissions caused by dLUCs (IPCC 2006), the GHG performances of biodiesel after accounting for GHG consequences of dLUCs are estimated as shown in Table 2.2.

The results indicate that there is a wide range of GHG performance of biodiesel if dLUC is taken into account in the system boundary. The worst case found was for the conversion of tropical forest land to oil palm which would increase the released GHG of palm biodiesel production around 5–8 times as compared to the case where LUC is excluded, due to loss of biomass carbon stock. This in turn will cause biodiesel to have higher GHG emissions than conventional diesel by around 240–269 %. Nevertheless, it must be noted that forest land conversion is unlikely to occur in Thailand as it is illegal and restricted by government. The conversions of field crop (e.g. cassava), old rubber field, and set-aside land to oil palm on the other hand would bring about GHG benefits i.e., the gain of biomass carbon stock and/or the increase in soil organic carbon stock. The life-cycle GHG emissions of those three scenarios range from 3 to 29 g CO_2 eq/MJ and the net avoided GHG emissions compared to diesel are ranged 56–91 %. Therefore, policy measures to regulate the new oil palm plantations by encouraging only the suitable land types are important to maintain the environmental sustainability of palm biodiesel production in the future.

2.4.3 Other Environmental Impacts

Apart from life-cycle GHG emissions, Table 2.3 shows the other three environmental impact potentials including acidification potential (AP) which is expressed as g SO_2 eq., eutrophication potential (EP) which is measured in g PO_4^{3-} eq., and photochemical ozone creation potential (POCP) is measured relative to ethylene as expressed in g C_2H_4 eq. which is determined based on the impact potential factors of CML method (Heijungs et al. 1992). As per MJ of fuels, palm biodiesel has lower AP but higher EP as compared to diesel. However, there is no significant difference of POCP between them. In addition, the assessment also shows that utilization of EFB and POME in all scenarios (i.e. scenario 1–3) could help

Table 2.3 Potential environmental impacts of palm biodiesel compared to diesel (per MJ of fuels)

Environmental impacts	Environmental impact potentials[a]		% net avoided impact potentials compared to diesel
	Palm biodiesel	Diesel[b]	
Acidification (g SO_2 eq.)	~0.40–0.41	~0.44	~7–9 %
Photochemical oxidation (g C_2H_4 eq.)	~0.03	~0.03	–
Eutrophication (g PO_4^{3-}eq.)	~0.11	~0.09	~(−11)–(−12 %)

[a] Environmental impacts entire the life-cycle production and use of fuels

[b] Environmental impacts of diesel production are evaluated based on LCI data of Ecoinvent database (2007) and tailpipe emissions of diesel in LDDV (Pleanjai 2008)

improve the environmental performance of palm biodiesel in all impact categories (Silalertruksa and Gheewala 2012).

For example, oil palm plantation is the main contributor to AP and this originates from the SO_2 and NO_x generated during production of N-P-K fertilizers. Therefore, substitution of chemical fertilizers used at the plantation by the compost from the co-composting of EFB and POME would be an opportunity to mitigate the environmental burdens of palm biodiesel (Stichnothe and Schuchardt 2010). POME is found to be the major source of eutrophication impact followed by fertilizers used in the oil palm plantation stage and methanol in the biodiesel conversion stage, respectively. Therefore, apart from good management of POME at the palm oil mills, the effective use of fertilizers in agriculture could be one of the measures in practice to reduce this impact. Additionally, the proper management of POME would also help reduce POCP as this impact associated with the emissions of NO_x, CH_4, and CO; methane emissions from open ponds at the oil mills are the major contributor in high POCP of palm biodiesel.

2.5 Socioeconomic Impacts of Biodiesel Production

2.5.1 Employment Effects

Table 2.4 shows the estimated direct and indirect employment induced by biodiesel production in Thailand based on the "hybrid approach" i.e., direct employment in agriculture is estimated from the labor costs in FFB production (OAE 2010) and the annual wage of labor for agriculture in Thailand (NSO 2010); while, for palm oil milling and biodiesel production, which have the exact number of producers, direct employment was collected from direct surveys with 17 palm oil mills and 5 biodiesel plant producers, respectively. Indirect employment which is generated in the industries that produce intermediate deliveries to the agriculture and biodiesel processing sectors is estimated from the inverse matrix from the aggregated 2005 Thai IO tables in new format (50 × 50 major sectors) relevant to biofuels production and the direct employment coefficient derived from the

Table 2.4 Socioeconomic effects of palm biodiesel in Thailand

		Per ml biodiesel			Per TJ biodiesel		
		Direct	Indirect	Total	Direct	Indirect	Total
Employment	Employed persons (person-years)	74	54	128	2.0	1.5	3.5
GDP effects	GDP (M$)	0.54	0.19	0.73	0.015	0.005	0.020
National trade balance	Import (M$)	0.22	0.39	0.61	0.006	0.011	0.017

number of employed persons in each economic sector recorded by the National Statistical Office (NSO) of Thailand (Silalertruksa and Gheewala 2011).

The results show that production of 1 million litre of palm biodiesel generates employment around 128 person-years or around 3 person-years/TJ of biodiesel. In comparison with diesel (based on the average ex-refinery price of high speed diesel during 2006–2008 i.e., 19.4424 THB/L), producing biodiesel requires about 10 times more persons than diesel per joule of energy content. Direct employment in agriculture (i.e., oil palm plantation) is the major employment benefit generated from palm biodiesel production contributing around 54 % of the total employment generation followed by the indirect employment induced in the agricultural sector and in biodiesel production. The significant employment generation in agriculture implies that the promotion of biodiesel in Thailand could help spur rural development. Nevertheless, two major reasons of the huge numbers of employed persons in agriculture are identified as follows: (1) agricultural is the most labor-intensive sector in the Thai economy, because the farmers are generally small scale with manual operation; and (2) low productivities of agriculture due to lack of good agricultural practices.

2.5.2 Impacts of Biodiesel on GDP Development

The study also determines the effects of biodiesel production on the total value added or gross domestic product GDP of Thai economy. Even though the GDP of a country is an indicator to measure economic performance and the size of the economy, measuring the changes in GDP can imply the amount of income generated and retained in the country itself (Wicke et al. 2009). Based on final demand approach in IO analysis, the assessment reveals that producing 1 ml of palm biodiesel contributes around 0.73 M$ to the national GDP as shown in Table 2.4. The main contributor to the changes in GDP is the direct impact from agriculture followed by the indirect impacts from energy and chemicals consumptions, respectively. This is because feedstock cost (i.e. CPO) is the largest production cost component of biodiesel contributing around 62–73 % of direct impacts or 29–55 % of total impacts on GDP. Methanol is the second largest contributor to GDP development. However, the study did not consider the induced impacts of increased use biodiesel to the decrease in operation of refinery sectors because biodiesel used in Thailand is in the blended form. The new biodiesel production sector, therefore, assumes to have no effect to the refineries in the views of products competition. To provide the extent of the socioeconomic impacts of biodiesel policy in Thailand, if the target of producing about 4.5 ml palm biodiesel per day was achieved in 2022, the estimated overall impacts of biodiesel production in Thailand are 1,193 M$ additional GDP.

2.5.3 Impacts of Biodiesel Production on the National Trade Balance

Balance of trade is one of the essential aspects for a country as it measures a country's dependence on other countries and the country's possibilities for generating income from selling to other countries (Wicke et al. 2009). The study, therefore, determines the amount of imports needs for biodiesel production in Thailand comparing with the case of diesel production. IO analysis has been used in the same way as the analyses of impacts on GDP and the results show that producing 1 TJ of palm biodiesel will result in the increase of total imports around 0.017 M$. Nevertheless, if compared to the cases of producing diesel at the same energy performance i.e. 1 TJ, production of biodiesel to substitute petroleum fuels could decrease the country's import around 0.053 M$ per TJ of biodiesel. The largest contributor to increase imports is the indirect impacts of chemicals used in biodiesel conversion stage followed by the indirect impacts from energy consumed. Thus, if the targets of producing about 4.5 ml/day (or equivalent to 55,024 TJ/day) were achieved in 2022, the biodiesel production could help reduce imports around 2,849 M$/year.

2.6 Recommendations Toward Sustainable Palm Biodiesel Production in Thailand

Several recommendations for improving the environmental and cost performance of palm biodiesel along with the sustainability of palm oil and palm biodiesel industry in Thailand are as follows:

2.6.1 Increasing Environmental and Cost Efficiency of the Existing Palm Biodiesel Systems

1. Utilizing all byproducts and wastes generated at the palm oil milling stage is essential to enhance environmental and cost performance of palm biodiesel (Silalertruksa et al. 2012). For example, recovery of biogas and nutrients from POME should be encouraged using CDM opportunities. EFB, which is posing a disposal problem to many mills, especially the difficulty to manage it by mulching during the peak crop inducing high cost for transportation and distribution in the plantations, could be used for other purposes e.g., used as fuel for boilers, used as substrate for straw mushroom cultivation, or mixing with POME to make co-compost due to its high N-P-K content (APC 2007). Additionally, the surplus shells from in-house boilers could be used to produce activated carbon or as a source of fuel for cement and brick factories

(Chavalparit et al. (2006). The credits of substituted materials from waste recovery such as diesel for operating power generator replaced by biogas, chemical fertilizers replaced by co-compost of POME and EFB, fossil fuels for grid-power generation replaced by biomass fuels such as shells and EFB of the Small Power Providers (SPP) would induce externality savings and help improve the economic and environmental performance of palm oil and palm biodiesel industries in Thailand (Silalertruksa et al. 2012). In addition, at the plantations, biomass residues such as fronds and trunks are available and may be used as biomass for energy. Nevertheless, to avoid erosion and wash-out of soil in case of heavy rains especially if the plantation is located in mountainous areas, the suitable percentages for removing fronds and trunks for energy are 35 and 85 %, respectively for mountainous areas and 67 and 98 %, respectively for flat territory plantation (APC 2007).

2. To secure the availability of CPO supply for future biodiesel production, both increase in FFB yield and oil extraction rates are required. The good agricultural practices (GAP) for oil palm as suggested by Department of Agriculture to farmers needs to be encouraged to smallholders especially knowledge about the suitable time-period of harvest which would result in high content and good quality of oil in FFB (DOA 2009). In addition, using co-compost derived from EFB and POME which contain nutrients as organic fertilizers would be another measure to improve soil quality and to reduce chemical fertilizer consumption. Moreover, the quality standards and quality pricing for FFB should be established for supporting the FFB and oil extraction rate improvement program. The good practices could help increase FFB yield from 17.5 to 22 Mg/ha or even to reach the genetic potential of oil palm varieties i.e. 31.3 Mg/ha (NCGEB 2009).
3. Specification of the land suitable for future oil palm plantation expansion needs to be performed by the government. Unregulated expansion of palm plantation would result in low FFB productivity and would lead to adverse impacts on the environment e.g., increase in GHG emissions. The palm biodiesel systems based on land that were previously tropical forest have such large GHG emissions that they cannot meet the GHG emission reduction target. Based on the assessment results, conversion of set-aside land to oil palm would be the suitable way for social and environmental sustainability of future palm oil and palm biodiesel industry as compared to the conversion of the other lands.

2.6.2 To Encourage "Palm-Based Biorefinery" and "Cascade Utilization" of Biomass Residues and Wastes Generated from Palm Biodiesel System to Maximize the Sustainability Benefits

Concerns about the ongoing increase in prices of fossil resources, especially crude oil stimulates interest in the potentiality of using biomass as an alternative to fossil

resources for production of the marketable products (materials/chemicals) and energy products e.g., transportation fuels. The concept of "biorefinery" is therefore introduced similar to the petroleum refinery in which a single source feedstock (crude petroleum) can be processed to multiple products (Goh and Lee 2010). However, in biorefineries, biomass is considered as feedstock and will be processed into a spectrum of marketable products and energy (IEA 2012). A wide range of technologies (e.g., biochemical conversion, thermal-chemical conversion) able to separate biomass resources (e.g., wood, energy crops, and agricultural residues) into their building blocks (e.g. carbohydrates, proteins, and triglycerides) which can be converted to value added products, biofuels, and chemical, will be considered and integrated into the biorefinery system (Cherubini 2010). Due to substantial amounts of biomass residues and wastes being generated in oil palm plantations and at palm oil mills, this concept, therefore, has a potential to be exploited for palm industry.

Figure 2.4 shows the examples of two scenarios of palm-based biorefinery systems that are able to integrate into the normal system of palm biodiesel production in Thailand for more fully utilizing biomass residues and wastes generated from palm industry. The two scenarios proposed are i.e., (1) palm-based biofuel refinery (PBR) and (2) palm-based biorefinery along with the concept of "cascade utilization". Palm-based biofuel refinery scenario (PBR) refers to the system where the CPO is still processed into biodiesel but the primary biomass residues i.e., fronds and trunks from oil palm plantations and secondary biomass residues released in the mills i.e. fiber, EFB and shells will be collected and crushed into smaller size for pretreatment, and subsequently hydrolysis to obtain fermentable sugar and eventually bioethanol (Goh and Lee 2010). The lignin will also be separated from lignocelluloses and can be used as fuel for power generation. The ash from combustion of lignin can be returned to the plantations to improve soil quality. Glycerol, byproduct of transesterification process, can also be considered as fuel for co-generation.

The scenario of "palm-based biorefinery" along with the concept of "cascade utilization" of biomass resource is also shown in Fig. 2.4. Cascade utilization is the sequential use of biomass as feedstock to produce materials and energy (i.e., preferring materials production before energetic utilization) (Steubing et al. 2010; Raschka and Carus 2012; UNIDO 2007). The study proposes an example of cascade utilization concept for improving palm biodiesel system in Thailand by applying it for biodiesel glycerol i.e., biodiesel glycerol will be used sequentially for materials and then for energy (i.e., epichlorohydrin for epoxy resins, burning) (Pagliaro et al. 2007; Raschka and Carus 2012). Recently, a $157 million biobased chemical plant utilizing glycerol from biodiesel for epichlorohydrin production in Thailand has been successfully commissioned in Map Ta Phut, Thailand by Solvay, an international chemical group, and its Thai affiliate Vinythai. The plant uses Solvay's epicerol technology and the production capacity is about 100,000 M t/year (Voegele 2012).

In addition, biomass wastes from palm oil can also be converted by other conversion technologies to produce hydrogen-rich gas which is expected to

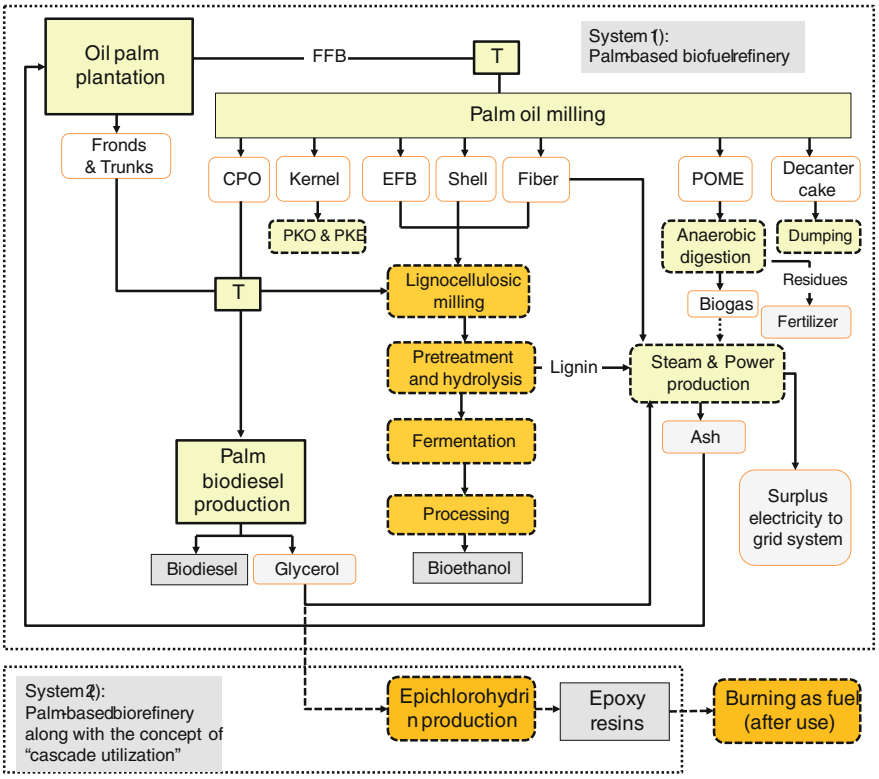

Fig. 2.4 The schematic diagram of the proposed palm-based refinery systems

become one of the major sources of energy in the future due to its cleanliness and its high calorific value fuel (Mohammed et al. 2011) or other components e.g., acetone-butanol-ethanol (ABE) that can be used as "green chemical" and bio-plastics e.g., polylactic acid (PLA), polyhydroxyalkanoate (PHA), etc. (IEA 2012; JIE 2008). This integrated approach of using biorefineries for the co-production of both value-added products and biofuels from biomass resources is the promising approach to reduce production costs of conventional biofuels production and would help create a sustainable market of biofuels in the future if the financial support for biofuels provided by government such as tax reduction were put off (IEA 2012). Thus, the palm-based biorefinery will not only provide biodiesel production cost reduction but it can enhance energy security and mitigate climate change as palm biomass residues are efficiently used. Moreover, the emerging of biorefinery industry would induce job opportunities in the future.

2.7 Conclusion

To address the sustainability of palm biodiesel production and use in Thailand, the study applies the decision supporting tools i.e., (LCA) and IO analysis for determining the environmental and socioeconomic impacts of palm biodiesel from various systems compared to conventional diesel that being substituted. The results indicate that policy to promote biodiesel in a developing country as Thailand has a significant effect to the economy as it could result in various positive externalities to the economy such as GHG emissions reduction, acidification reduction, employment generation, GDP development, and trade balance improvement. This raises the attractiveness of biodiesel and makes it competitive to diesel in terms of net social benefits. However, there are also the environmental drawbacks from biodiesel production that the policy makers need to consider such as the increase in the eutrophication impact to the environment of biodiesel as compared to diesel. In addition, an unregulated expansion of oil palm plantations in the future such as the invasion of tropical forest for oil palm could lead to the substantial increase of GHG emissions of palm biodiesel and it in turn would cause biodiesel to have higher GHG emissions than conventional diesel. Several recommendations, therefore, have been identified for improving the sustainability of palm oil and palm biodiesel industry in the future in both environmental and economic dimensions. For the short-term improvement, GAP for oil palm cultivation, methodologies to treatment of byproducts, and wastes generated at the mills such as biogas recovery from POME, co-composting of EFB and POME, and utilization of biomass residues for fuel should be introduced nationwide. However, for the long-term improvement, the study recommends that palm-based biorefinery systems along with the concept of "cascade utilization" need to be investigated in order to fully utilize biomass residues and wastes generated from palm oil and palm biodiesel industry.

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Part II
Pretreatment Technologies

Chapter 3
Progress in Physical and Chemical Pretreatment of Lignocellulosic Biomass

Keikhosor Karimi, Marzieh Shafiei and Rajeev Kumar

Abstract Lignocelluloses are abundant and nonfood-based materials that are considered as the most suitable feedstocks for the future energy production. However, these materials have naturally evolved to resist against physical and biological attacks. Thus, the conversion yield of lignocellulosic materials without a preprocessing step, called pretreatment, is not typically high enough for a process to be commercially viable. However, in the last decade or so, continued worldwide research efforts resulted in a significant improvement in the understanding of the biomass characteristics that influence subsequent biological conversions. The cell wall composition, characteristics, components distribution, and linkage between different parts are some of the factors that have been shown to have significant effects on biological conversion of lignocelluloses. In this chapter, different aspects of the parameters affecting the pretreatment and progress in the characteristic modification of lignocelluloses are reviewed. Furthermore, the challenges and conflicts in the related researches are discussed and some suggestions with concluding remarks are presented. Moreover, the most important processes, including pretreatment with acid, alkali, and cellulosic solvents are presented. The fundamental reactions and biomass structural changes in the processes imparted by these leading pretreatments, as well as recent progresses, are also reviewed.

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3.1 Introduction

Lignocellulosic materials are the only renewable sources that have great potential to produce appreciable amounts of fuels to replace fossil fuels. Different types of lignocelluloses from a variety of resources, e.g., agricultural residue, forest residues, and municipal solid wastes, are abundantly available at considerably low prices (Wyman 1996). However, these materials are very recalcitrant to microbial and enzymatic conversions. Thus, for an efficient conversion, prior to any biological conversion, their structure should be opened up by a process called pretreatment. This upstream processing is to render the downstream microbial and enzymatic processing (Zhu and Pan 2010). It is also used for improvement in digestion of these materials by animals (Castro et al. 1993).

The pretreatment is the focus of a large number of researches and is considered as a key for unlocking the bioethanol production from lignocelluloses. It is less investigated for improvement of biogas, animal feed processing, and other metabolite production. The basis of improvement in all these pretreatments seems to be the same, but when we come to details, it can be significantly different. For instance, a suitable pretreatment method for bioethanol application may not improve biogas production (Jeihanipour 2011). In the enzymatic hydrolysis, it is a mixture of enzymes that should enter the biomass pores, while in the biogas production and in animal digestion, it is the hydrolytic microorganisms that should enter the pores. On the other hand, in biogas production and animal digestions, a highly efficient and active multienzyme complex, including different cellulases and hemicellulases, are involved.

In this chapter, first, the lignocelluloses features that should be considered for modification in the pretreatment are reviewed. Then, the effective parameters in the pretreatment are discussed. Finally, the basis and progresses in the acid, alkali, and cellulose solvent-based pretreatments are presented and discussed.

3.2 Lignocellulosic Materials

There are several types of lignocelluloses including agricultural residues, herbaceous crops, and woody tree species (hardwoods and softwoods). Lignocelluloses are mainly composed of the following components:

- Carbohydrates (cellulose and hemicellulose)
- Lignin
- Extraneous materials

Since this chapter discusses the pretreatment of lignocelluloses, a brief introduction to the components of the lignocelluloses is presented here.

3.2.1 Carbohydrates

The carbohydrate portion of lignocellulose includes cellulose and hemicelluloses.

3.2.1.1 Cellulose

Cellulose is one of the main components of wood and many other lignocellulosic materials that typically represent 40–50 % of dry weight. Cellulose with $(C_6H_{10}O_5)_n$ formula is a polysaccharide consisting of a linear chain of D-glucose units connected by $\beta(1 \rightarrow 4)$ glycosidic bonds. Since the glucose residue is tilted toward its neighbors by 180°, it would be more accurate to consider the cellulose as a polymer of cellobiose, and not glucose (Fengel and Wegener 1984; Lee et al. 1994; Pérez and Samain 2010).

Cellulose has a crystalline structure (45–96 % crystallinity) organized into compacted crystallite microfibrils (about 35 * 40 A in width and about 500 A in length) with high average degree of polymerization (DP) (defined as molecular weight of cellulose/molecular weight of one glucose unit, 7,000–15,000 for plant cellulose) (Chang et al. 1981; Fengel and Wegener 1984). The high tendency to form both intra- and intermolecular hydrogen bonds by the hydroxyl groups is responsible for the strength and crystallinity of cellulose (Chang et al. 1981).

The stabilization and crystallinity of cellulose mainly originate from the presence of hydroxyl groups (OH) (Festucci-Buselli et al. 2007). Three of these functional groups are available in each glucose molecule (Fig. 3.1). Thus, the inner and outer surfaces of cellulose are covered by OH-groups, which are able to make H-bond with other OH-groups and other groups available in lignocelluloses, such as O-, N-, and S-groups (Pérez and Samain 2010). The linkage energy of van der Waals in water is only 0.15, while the H-bond in water and cellulose is 15 and 28 kJ/mol, respectively. The water adsorption by cellulose can take place by forming H-bond between water's-OH and cellulose free OH-groups, which are not linked with each other. This is the base of swelling of cellulose by water (Fengel and Wegener 1984).

The cellulose molecules in lignocelluloses are in the form of fibrils, where the fibrils are composed of microfibrils. Microfibrils in turn are composed of elementary fibrils that are further associated with hemicellulose and lignin. Each microfibril is suggested to contain approximately 36 glucose chains (Ding and

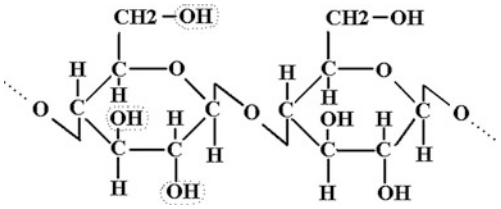

Fig. 3.1 One cellulose unit

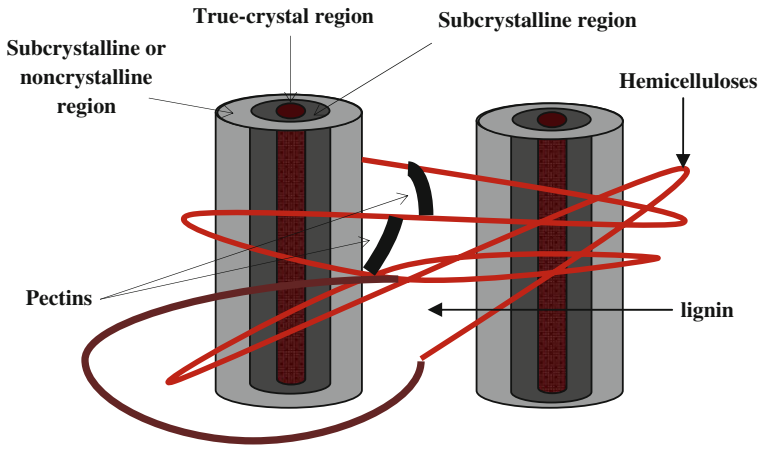

Fig. 3.2 Different crystalline parts of cellulose microfibrils

Himmel 2006). The microfibrils consist of three groups of glucose chains; true-crystal chains (core chains), subcrystalline chains (transition chains), and "subcrystalline or noncrystalline" chains (surface chains) (Fig. 3.2) (Ding and Himmel 2006; Festucci-Buselli et al. 2007). The true-crystal chains are the most resistant part of cellulose for chemical and biological hydrolysis.

3.2.1.2 Hemicellulose

Hemicellulose, also called polyoses, a heteropolymer of polysaccharides and polyuronides, is available in almost all lignocellulosic materials along with cellulose. The polysaccharide part of hemicellulose contains different polymers of hexosans (mannan, glucosan, galactan, and rhamnan) and pentosans (xylan and araban). Polyuronides parts contain hexuronic acids and methoxyl, acetyl, and free carboxylic groups. Polyuronides are more sensitive to chemical and biological attacks than polysaccharide (Norman 1934; Billa and Monties 1991).

Xyloglucan, galactoglucomannans, arabinoglucuronoxylan, xylan, glucuronoxylan, arabinoxylan, mannan, and glucomannan are the main polymers in hemicelluloses. The dominant monomeric sugar in softwoods hemicelluloses is mannose which is highly acetylated and contains galactose side groups. While xylose is dominant in hardwoods and agriculture residues which is less acetylated and contains arabinose side groups. Other side chains such as fucose may also be present on the hemicelluloses backbone (Fry 1989).

Due to the higher content of methylglucuronic acid side groups, the hydrolysis of xylan in softwoods is more difficult than xylan in hardwoods (Teleman et al. 1995, 2002). The xylose to methylglucuronic acid ratios in softwood and hardwood is approximately 5:1 and 10:1, respectively. Methylglucuronic acid branches

may also be protected during dilute acid pretreatment and remain in the fiber (Czirnich and Patt 1976; McCarter et al. 2002).

Therefore, mixture of different types of sugar monomers and acids including xylose, mannose, glucose, galactose, arabinose, rhamnose, and acetic acid can be present in hemicellulose hydrolysates (Wyman 1996; Peng et al. 2012).

Unlike cellulose, which is crystalline and recalcitrant to hydrolysis, hemicellulose has an amorphous structure with short chain polymers as a side chain (Girio et al. 2010; Peng et al. 2012). In spite of its complex structure, hemicellulose can be easily hydrolyzed by acid treatment as well as hemicellulase enzymes (Girio et al. 2010). It is also a more digestible part of lignocelluloses in biogas production and digestion by animals (Keys et al. 1969; Keys and DeBarthe 1974).

3.2.1.3 Lignin

Lignin, a very complex polymer, playing a cementing role to connect cells, increases the mechanical strength properties, and makes plant resistant against diseases and biodegradation by microorganisms. Lignin is sometimes referred as glue between hemicellulose and cellulose components; while sometimes the hemicellulose is referred as glue between lignin and cellulose. Anyway, hemicellulose and lignin are known to cover the surface of cellulose which adds structural strength to the cellulose matrix (Pérez and Samain 2010). Softwoods (25–40 %) contain higher lignin than hardwoods (18–25 %) and agriculture residues (10–20 %) (Fengel and Wegener 1984; McMillan 1992); however, the lignin content is not the only difference between softwoods and other lignocelluloses. The main distinction is originated from the difference in monomeric units and linkage types in lignin. This dissimilarity in the lignin content may result in significant differences in susceptibility of various pretreatment techniques between hardwoods and softwoods. Pretreatment of hardwoods and agriculture residues is usually less harsh than softwoods. The reason is the presence of higher number of vessels in the hardwoods and agriculture residues which permit greater heat and mass transfer into the biomass matrix (Cochard and Tyree 1990; Hepworth et al. 2002; Kim et al. 2011). Generally, easier penetration of chemicals, enzymes, and heat makes the hardwoods and agriculture residues easier for pretreatment than softwoods.

Lignin is a cross-linked polymer of hydroxyphenylpropanoid units connected by C–C and C–O–C linkages, in which over 10 inter-phenylpropane linkage types have been detected. There are several monomeric units and linkage types in lignin. There are two major classes of lignin, guaiacyl lignins (G-lignin) and syringyl lignins (S-lignin). They contain guaiacyl (G), Syringyl (S), and hydroxybenzaldehyde (H) units (Lewis and Yamamoto 1990). Different lignocellulose materials with different age and cultivation conditions have different ratios of G, S, and H. The lower accessibility of plant vessels is partially the result of the occurrence of guaiacyl lignin type in the vessel walls. Therefore, not only the amount of lignin,

but also the guaiacyl to syringyl ratio in lignin can affect the swelling of the cellulosic residue (Ramos et al. 1992). The principal structural elements in lignins have been largely investigated; however, many aspects of the lignin chemistry are still unclear.

For lignin synthesis in woody materials, a series of secondary reactions are recognized leading to cross-linking between lignin and hemicelluloses (Lee 1997). Biodegradation of lignin is a secondary metabolic process, occurring only under low levels of nitrogen (Lee 1997).

During plant biosynthesis, it is believed that lignin is not simply deposited between cellulose and hemicellulose, but is linked with at least part of them. These linkages are termed as lignin-polysaccharide complex (LPC) or lignin-carbohydrate complex (LCC) (Chesson 1988). Thus, as a result of these linkages, it is almost impossible to completely separate or purify cellulose or hemicellulose from lignin, and to have lignin free of polysaccharides. Furthermore, lignin has a tendency of recondensation during delignification processes (Kim et al. 2003). Not only are van der Waals and H-bond involved, but chemical bonds such as covalent bonds are also detected between lignin and polysaccharides (Besombes and Mazeau 2005).

3.2.1.4 Extraneous Materials

A large number of compounds are available in lignocelluloses, known as extraneous materials, which can be extracted by means of polar and nonpolar solvents. The composition and content of these materials vary among lignocelluloses species. Based on their solubility in water, extraneous materials are divided into two categories: extractives or nonextractives (Fan et al. 1982). The most important parts of extractive components are resins (fats, fatty acids, resin acids, and phytosterols), terpenes (isoprene alcohols and ketones), and phenols (residue and byproducts of lignin biosynthesis) (Fan et al. 1982; Fengel and Wegener 1984).

The inorganic components such as alkali earth carbonates, oxalates, starches, pectins, and proteins make the nonextractives part of extraneous materials. In some types of grasses and straws, there is also a significant level of nonextractive silica crystals (Fan et al. 1982). Particularly, rice straw is covered by a layer of silica, which results in a different behavior in the pretreatments compared with other similar biomass, e.g., wheat straw (Binod et al. 2010).

Usually, the extraneous parts are not considered to play much of a role in lowering cellulosic biomass biological conversions, and fewer attempts have been made in this direction. Although it might be intriguing, it would be difficult to see the effect of these components, since they are in large numbers and mostly present in low amounts.

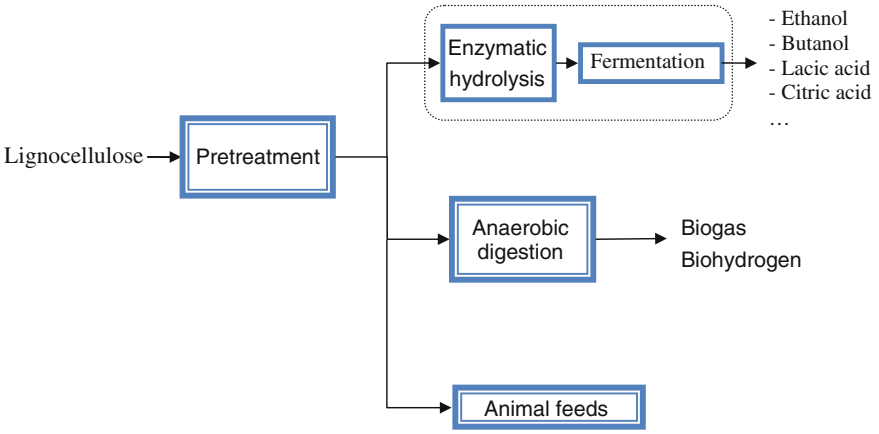

Fig. 3.3 Pretreatment of lignocelluloses for different proposes

3.3 Pretreatment

The main purpose of lignocellulosic materials pretreatment is to improve their biological conversion in the subsequent bioprocessing (Figs. 3.3 and 3.4). This process is known as a key for economically feasible production of different chemicals, e.g., ethanol, butanol, lactic acid, and biogas, as well as animal feed (Cameron et al. 1990, 1991; Shah et al. 1991; Castro et al. 1993; Deschamps et al. 1996; Wang et al. 2004; Yang and Wyman 2008; Taherzadeh and Karimi 2008; Aad et al. 2010; Teghammar et al. 2010).

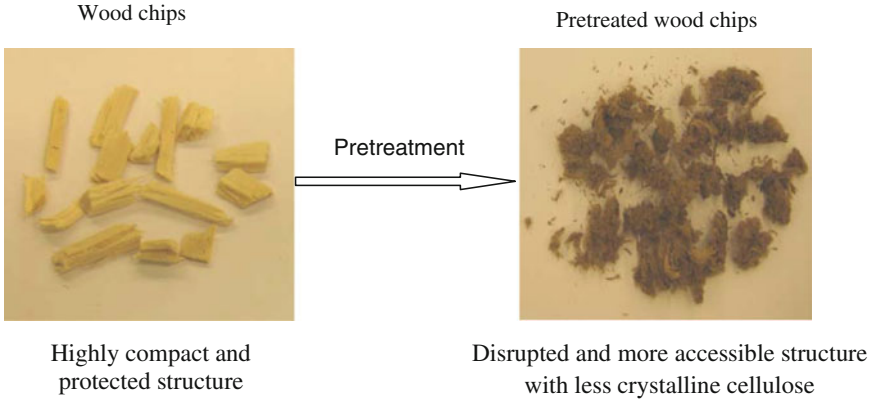

Fig. 3.4 The effects of pretreatment on biomass [Unpublished data (Shafiei et al. 2012)]

3.3.1 Effective Parameters in Pretreatment

Pretreatment is generally considered as a process for disruption of the naturally resistant structure of lignocellulose that limits the hydrolysis of carbohydrates, i.e., cellulose and hemicellulose (Yang and Wyman 2008). In other words, pretreatment is a process to remove the lignocelluloses recalcitrance for its biological conversion via microbial or enzymatic processing (Zhu and Pan 2010). The most effective parameters in the biological conversion of lignocelluloses are believed to be cellulose crystallinity, accessible surface area, lignin and hemicellulose protection, cellulose DP, degree of hemicelluloses acetylation, cellulase adsorption and desorption, and the biomass swelling capacity (Wyman 1996; Taherzadeh and Karimi 2008; Hendriks and Zeeman 2009).

In spite of a number of efforts, it is still not possible to correlate the lignocellulose properties to the effectiveness of enzymatic hydrolysis. For instance, when a pretreatment reduces the lignin content, it cannot be firmly concluded that the improvement in hydrolysis yields was exclusively due to lignin. On the other hand, when one specific characteristic of lignocellulose changes as a result of a pretreatment process, it is not possible to keep others unchanged; thus, it is not easily possible to see the effect of a single parameter.

To date, there are limited efforts toward the development of useful methods for evaluation of lignocelluloses characteristics that affect their biological degradation by microbial or enzymatic hydrolysis (Chandra et al. 2008). In spite of different efforts, it is difficult to link the effective parameters to their hydrolysis properties (Chandra et al. 2008). The effective parameters that presumably affect cellulose enzymatic hydrolysis are briefly presented and discussed here.

3.3.1.1 Cellulose Crystallinity

In enzymatic hydrolysis of lignocelluloses, cellulose crystallinity plays a major role in the biological conversion of cellulosic materials. For example, cotton fibers are almost pure cellulose, in which no lignin and hemicellulose are available. However, it is not possible to reach a high yield of enzymatic hydrolysis from natural cotton due to its high crystallinity (Jeihanipour 2011).

Generally, cellulosic materials contain different cellulose regions, categorized as crystalline and amorphous celluloses, in which the major part is in crystalline form. The crystallinity is often defined as the ratio of these two regions. Natural cellulose is mainly in the form of cellulose I, which is composed of cellulose I_α in cotton and dominantly I_β in the lignocelluloses. The molecules are organized in a parallel manner in cellulose I. Cellulose II, referred to as regenerated cellulose, is also a crystalline form of cellulose with a nonparallel arrangement of the molecules and is thermodynamically more stable than cellulose I. Cellulose II is more susceptible for chemical and biological conversions and can be obtained by chemical pretreatment of lignocelluloses (Hayashi et al. 1975; Chundawat et al. 2011a, b; Mittal et al. 2011).

Between the crystalline regions, there are some amorphous cellulose parts that can easily adsorb water and become soft and flexible (Ciolacu et al. 2011). The chemicals can typically penetrate more easily into the amorphous region of cellulose, where reactions can take place, while the crystalline region remains unattacked. The amorphous cellulose has a higher accessibility and higher enzyme binding capacity than crystalline cellulose (either I or II). Thus, the hydrolysis of the amorphous cellulose is much faster than the crystalline cellulose (Hong et al. 2007; Kumar and Wyman 2008). Highly amorphous cellulose can be obtained by different treatments, e.g., ball milling, alkaline treatments, and dissolution in some solvents such as concentrated phosphoric acid. However, the constructed amorphous cellulose is unstable in the presence of water and partly forms cellulose II (Isogai and Atalla 1991).

It is often reported that decreasing the cellulose crystallinity increases the rate and yield of lignocelluloses bioconversions (Bertran and Dale 1985; Jiang et al. 2007; Bak et al. 2010; Jeihanipour et al. 2010b). In contrast, several investigations showed more digestibility of more crystalline cellulose (Grethelin 1985; Lin et al. 1985; Wyman 1996). The conflict appears when the limiting factor is solely not crystallinity, while the other factors are more important. In some cases, no relationship was found between the rate of hydrolysis and crystallinity. For instance, it was shown that the crystallinity of cellulose in pretreatment of some types of wood by mild acid treatment is not an effective parameter, while pore size distribution contributed more to the improved enzymatic hydrolysis (Grethelin 1985). In some other cases, increase in crystallinity resulted in higher hydrolysis yields (Kim and Holtzapple 2005, 2006). For instance, when the amorphous fractions of corn stover were removed by lime pretreatment, the crystallinity of the treated materials increased, while increasing the pore sizes positively affected the hydrolysis. Therefore, in this case increasing the crystallinity increased the yield and rate of hydrolysis (Kim and Holtzapple 2006).

Therefore, one should consider the crystallinity as an important factor in biological conversion of lignocelluloses; however, it cannot be considered as the sole effective parameter in all cases but should be considered together with other factors.

It should be noted that crystallinity may influence the initial rate of hydrolysis, rather than the final sugar yields (Chang and Holtzapple 2000). Furthermore, the susceptibility of hydrolysis by pretreatment, as a result of crystallinity reduction, cannot be easily distinguished from the changes made by other parameters such as cellulose DP (Yang et al. 2011).

3.3.1.2 Accessible Surface Area

Increasing the accessible area is among the main objectives of all pretreatment processes (Mosier et al. 2005; Rollin et al. 2011); although in most studies, it is correlated with lignin or hemicellulose removal and not considered as an

individual factor (Taherzadeh and Karimi 2008). Enzymatic hydrolysis consists of three steps (Sun and Cheng 2002):

1. Adsorption of hydrolytic enzymes onto the cellulose surface;
2. Hydrolysis of cellulose to its oligomers and cellobiose; and
3. Desorption of the enzymes to the liquid phase.

Direct physical contact between the enzymes and cellulose is one of the main requirements for enzymatic hydrolysis; thus, the biomass accessible surface area is among the main effective parameters for enzymatic hydrolysis.

The mechanism in bacterial hydrolysis of cellulose, e.g., in biogas production processes, is completely different. In these processes, it is the microorganisms that need to reach the cellulose surface. Reaching the surface, they produce a highly efficient multi-functional complex enzyme, called cellulosome, and hydrolyze the cellulose and hemicellulose fractions. Therefore, in bacterial hydrolysis, there are no free enzymes and also the hydrolysis products are subsequently channeled to the cells through some produced fibrous corridors (Lamed et al. 1985; Morag et al. 1990; Lynd et al. 2002; Vazana et al. 2010). Thus, the biomass pores for bacterial cellulose degradation are more important than in the enzymatic hydrolysis.

Lignocelluloses have external and internal surface areas. The size and shape of the particles are related to the external surface area, whereas the fiber porosity or capillary structure in cellulosic fibers is related to the internal surface area. Typically, natural dried lignocelluloses have a very small internal surface and a large external surface area. The external surface area can be increased by size reduction. Suitable pretreatment is expected to significantly increase both the surface areas, especially the internal surface area (Fan et al. 1980).

The accessible surface area sharply increases in the initial stages of hydrolysis of a part of fibers. However, the rate of hydrolysis usually decreases in the latter stages, in spite of higher surface area for the enzymes. This indicates that area is not the major limiting factor for hydrolysis in the latter stages. The faster hydrolysis in the beginning of the process is related to hydrolysis of amorphous cellulose parts and the slowdown in the latter is related to higher crystallinity of the residual cellulose (Fan et al. 1980). Therefore, the effects of accessible surface area should be considered together with the other effective parameters in the hydrolysis.

The solute exclusion (SE) technique was used for measurement of accessible surface area (Stone et al. 1969; Grethelin 1985). The initial rate of enzymatic hydrolysis of cotton linters was compared with the accessibility of the substrate to molecules with 40 Å sizes. A linear relationship was observed between the hydrolysis rate and the accessible surface area (Stone et al. 1969). The initial hydrolysis rate for hard- and softwood using cellulase was also linearly correlated with the pore size of the substrate accessible to molecules with 51 Å diameter (the representative of cellulase molecule size) (Grethelin 1985). However, the method is time-consuming.

The Simons' Stain (SS) technique was formerly developed for evaluation of porosity in pulp fibers (Behrendt and Blanchette 1997). Since 2001, the SS method

has been used for estimation of pore distribution, an indication of accessible substrate area and correlated to the enzymatic susceptibility of lignocelluloses (Esteghlalian et al. 2001). This method is based on adsorption of two different dyes, a small and a large molecule. When adsorbed, they indicate the large and small pores of the fibers (Chandra et al. 2008). The method was recently modified and showed to rapidly predict the improvements made by pretreatments (Chandra et al. 2008, 2009).

The swelling capacity or water retention value (WRV), which is the degree to which a lignocellulosic material swells in the presence of water, is a simple and rapid method for assessing the swelling potential of lignocellulose. The increase in swelling is correlated with substrates' accessibility for subsequent hydrolysis by cellulases (Ogiwara and Arai 1968; Chandra et al. 2009). It was examined for substrates with different chemical composition and crystallinity, and linear relationship between the enzymatic hydrolysis rate and the WRV was found (Ogiwara and Arai 1968).

3.3.1.3 Lignin and Hemicellulose Protection

The cellulose is cemented and covered by lignin and hemicellulose. Since it is responsible for rigidity and prevention of water, chemical, and enzyme diffusion through the lignocelluloses, lignin composition, content, and distribution are recognized to play a major role in recalcitrance of lignocellulosic materials. Lignin, as a physical barrier, limits the cellulose enzymatic and bacterial accessibility and consequently its degradability. It is often shown that delignification can significantly improve the hydrolysis of lignocelluloses (Kim and Lee 2006; Martin et al. 2008; Ko et al. 2009; Hallac et al. 2010; Kuhad et al. 2010; Wu et al. 2011a, b; Chen et al. 2012).

Hemicellulose is also a physical barrier and reduces the accessibility of enzymes and bacteria to the cellulose fibers. Similar to lignin, hemicelluloses removal is often reported to improve the enzymatic hydrolysis of lignocelluloses (Tosun 1995; Kim et al. 2001; Um et al. 2003; Zhu et al. 2005; Canilha et al. 2011; Haverty et al. 2012; Pei et al. 2012).

However, it is difficult to follow the individual effects of lignin removal, since in most delignification methods a part of the hemicellulose is also removed, and vice versa. Hydrolysis improvement by both lignin and hemicellulose removal appears to be related to increase in the accessible surface area.

3.3.1.4 Degree of Polymerization of Cellulose

High DP of cellulose has been presented as a key parameter contributing in its low susceptibility to hydrolysis (Puri 1984; Kuo and Lee 2009a; Hallac and Ragauskas 2011). Longer glucan chains (higher DP) associated with higher number of hydrogen bonding consequently limit the accessibility of enzymes and bacteria to

the cellulose surfaces. Decreasing the DP resulted in higher saccharification to a certain extent (Puri 1984). In some cases, it was shown that cellulose DP plays a more important role in the hydrolysis compared to its crystallinity (Puri 1984). As expected, the shorter cellulose chains (lower DP) are more susceptible to hydrolysis than longer chains (Zhang and Lynd 2005).

However, it is not easily possible to measure the cellulose DP without changes in its properties. Isolation and dissolution of cellulose techniques come with significant changes in the DP of cellulose. An ideal DP measurement technique should be able to fractionate cellulose, dissolve it, and analyze its DP values without its derivatization; however, such a method has not been developed yet (Hallac and Ragauskas 2011).

3.3.1.5 Hemicellulose Acetylation

Deacetylation of lignocellulosic materials, specially the extensively acetylated lignocelluloses, was reported to improve the digestibility (Teixeira et al. 2000; Kim and Holtzapple 2006; Kumar and Wyman 2009a; Zhao and Liu 2011). Acetyl groups of the hemicellulose are attached to lignin and hinder the cellulose and hemicellulose hydrolysis. Like lignin, the acetyl groups are also considered as one of the barriers for enzymatic hydrolysis (Chang and Holtzapple 2000).

It was shown that deacetylation by dilute acid treatment resulted in five to seven times improvement in digestibility of aspen wood and wheat straw (Grohmann et al. 1986a, b). On the other hand, in some studies, acetyl content removal had minor effects on the digestibility, whereas lignin content and crystallinity had the greatest effects (Chang and Holtzapple 2000).

On the other hand, acetyl groups might inhibit the formation of hydrogen bonds between cellulose and enzymes (Chang and Holtzapple 2000; Teixeira et al. 2000). Increase in diameter of cellulose and change in the enzymes hydrophobicity were also suggested as reasons for inhibition of enzymatic degradation by acetyl groups (Pan et al. 2006b). Therefore, it was concluded that increasing the accessible surface area is not enough to make cellulose ready for efficient conversion, and deacetylation is also necessary (Yang et al. 2011).

Alternatively, it was shown that acetyl content or even cellulose crystallinity does not have negative effects when the biomass contains low amount of lignin. However, deacetylation is an important factor in digestibility of highly crystalline cellulose with moderate lignin content lignocelluloses (Chang and Holtzapple 2000). Nevertheless, this is not the case in hydrolysis of pure cellulose, e.g., cotton.

In acid or alkaline treatments, acetyl groups removal is accompanied with changes in the remaining lignin and hemicelluloses; thus, it is not possible to study the effects of deacetylation alone by general pretreatment and fractionation processes. The acetyl groups can be selectively removed by pretreatment with NMMO. Over 88 % of acetyl content of oak and spruce woods can be removed by NMMO pretreatment, while the lignin and hemicellulose contents were not changed. This pretreatment process resulted in significant improvement in

enzymatic hydrolysis (Shafiei et al. 2010). However, the effect of deacetylation was not analyzed in the previous studies and the improvement was related to lower crystallinity of the treated cellulose.

3.3.1.6 Cellulase Adsorption and Desorption

Hydrolysis of cellulose requires physical contact between cellulase and cellulose. The hydrolysis starts with adsorption of cellulases to the cellulose molecules; therefore, as expected, the hydrolysis rate is shown to be controlled by enzymes adsorption onto cellulose (Kumar and Wyman 2009a; Khodaverdi et al. 2012). A strong linear relation was found between cellulase adsorption and enzymatic hydrolysis rate (Karlsson et al. 1999; Kumar and Wyman 2009b).

The maximum adsorption capacity (σ) of cellulase that can be adsorbed to a biomass is shown to be a controlling factor for efficient hydrolysis. This parameter directly relates to the enzyme accessibility to active sites of cellulose in a substrate (Kumar and Wyman 2008). On the other hand, after the catalytic actions, the enzyme should desorb from the substrate, adsorb on another active part of cellulose, and start the hydrolytic actions again (Wald et al. 1984).

It is frequently reported that lignin is responsible for irreversible adsorption of hydrolytic enzymes on the lignocelluloses (Yang and Wyman 2006; Qing et al. 2010; Heiss-Blanquet et al. 2011). However, the amount of cellulase desorbed did not correlate with the residual lignin (Kumar and Wyman 2009a).

It is expected to be a relation between lignin and hemicellulose removal and the enzyme adsorption, since they are correlated to the accessible surface area. However, no clear correlation of hemicellulose and lignin removal with cellulase adsorption was found. It is suggested that the lignin-carbohydrates linkages removal plays the major role in increasing the accessible area for cellulase rather than hemicellulose and lignin removal (Kumar and Wyman 2009a).

Concluding Remarks and Suggestions About the Effective Parameters

The following points should be considered in the analyses of the effective parameters for lignocellulose biological conversion:

- More than one parameter is changed during pretreatment, and, therefore, several parameters should be taken into consideration to understand the most effective one. For instance, it is not enough to just analyze the crystallinity and show that it is reduced, since it might be one of the effective parameters, but not the dominant one.
- Lignin and hemicellulose contents should be analyzed. It can be related to the enzyme accessible surface area, water retention value, and cellulase adsorption.
- Acetyl group removal should be analyzed, since the improvement may be related to the inhibition of enzymes that form hydrogen bonds with cellulose.

- Analysis of DP by traditional methods may not be useful, since they change the DP.
- At least one of the analyses indicating the effects of accessible surface area should be conducted. The swelling capacity or water retention value is the easiest one, while the SS is more accurate.
- The crystallinity should be measured by X-ray or FTIR and related to the initial rate of hydrolysis.
- FTIR analysis can be used for analysis of biomass changes during the pretreatments. Changes of cellulose I to II and also the acetyl and lignin bonds in the biomass can be analyzed by FTIR; however, unfortunately, the results for lignocelluloses are not usually reproducible.
- SEM and macroscopic (Fig. 3.4) analyses are useful to provide qualitative observations about the changes. However, it is not possible to relate them to the effective parameters, e.g., to crystallinity and enzyme adsorption.

Finally, to be sure whether the pretreatment is effective, hydrolysis should be performed. The analyses above just help to understand the causes for the improvements.

3.4 Pretreatment Processes

A number of processes have been studied for pretreatment of lignocellulosic materials prior to enzymatic hydrolysis. A few were also suggested for improvement of bacterial and animal feed digestions. The methods are usually categorized into "Physical pretreatment", "Chemical pretreatment", "Biological pretreatment", and their combinations, and are summarized in Table 3.1.

The following is a list of the desirable pretreatment aspects (da Costa Sousa et al. 2009; Wyman et al. 2005; Yang and Wyman 2008; Taherzadeh and Karimi 2008; Chundawat et al. 2011a):

1. High product yields in subsequent enzymatic hydrolysis at low enzyme loadings or fermentation operations with minimal treatment cost.
2. Low or minimal chemical consumption for pretreatment, neutralization, and subsequent conditioning.
3. Possibility of recycling and reusing of the chemical used in the pretreatment.
4. Minimal waste production.
5. Limited size reduction needs since milling is an energy intensive and expensive process.
6. Fast reactions and/noncorrosive chemicals to minimize pretreatment reactor cost.
7. The concentration of hemicellulose sugars from pretreatment should be above 10 % to keep fermentation reactor size and reasonable level and facilitate downstream recovery.

Table 3.1 Different processes for pretreatment of lignocellulosic materials

Pretreatment method	Process
Physical treatment	Milling
	Sonication
	Irradiation
	Hydrothermal
	High pressure steaming
	Expansion
	Extrusion
	Pyrolysis
Chemical and physicochemical pretreatments	Alkali (*Sodium hydroxide, Liquid ammonia, and Ammonium sulfite*)
	Acid (*Sulfuric acid, Hydrochloric acid, Phosphoric acid*)
	Explosions [*Steam explosion, Ammonia fiber explosion* (*AFEX*), *CO_2 explosion, and SO_2 explosion*]
	Gas (*Chlorine dioxide, Nitrogen dioxide, Sulfur dioxide*)
	Oxidizing agents (*Hydrogen peroxide, Wet oxidation, and Ozonation*)
	Solvent extraction of lignin (*Ethanol–water extraction, Benzene-water extraction, Ethylene glycol extraction, and Butanol-water extraction*)
	Solvent (*Ionic liquids, Cellulosic solvents*)
Biological pretreatments	Fungi, actinomycetes, and bacteria

Taherzadeh and Karimi (2008, 2007)

8. Hydrolysate conditioning in preparation for subsequent biological steps should not form products that have processing or disposal challenges.
9. Pretreatment should facilitate recovery of lignin for conversion to valuable byproducts.

3.4.1 Physical Pretreatments

Changing the structure of biomass, typically increasing the enzyme accessible surface area, and reducing the degrees of polymerization of biomass, are possible by physical pretreatments such as size reduction (Zhu and Pan 2010; Harun et al. 2011). Different types of milling (e.g., ball milling, hammer milling, colloid milling, two-roll milling, and vibro energy milling), irradiations (e.g., by microwaves, gamma rays, electron beams, and ultrasonications), and extrusion (subjecting the biomass to heating, mixing, and shearing) are used for this propose (Taherzadeh and Karimi 2008; Zheng et al. 2009).

Modification of biomass structure with a single physical treatment is typically not enough for efficient enzymatic hydrolysis, although it can be enough for improvements in biogas production. Thus, the physical treatments are used prior to (or together with) chemical and biological treatments (Taherzadeh and Karimi 2008; Yu et al. 2009).

Size reduction is used prior to most chemical pretreatments. Although it can affect the efficiency of the process, it heavily impacts process economy. Most chemical pretreatments are not successful without size reduction. However, in explosive, organosolv, and solvent processes large particles may be used (Zhu and Pan 2010; Shafiei et al. 2012). Explosive pretreatments, such as steam explosions, need less energy than mechanical size reduction. However, the explosive pretreatments are not easily possible in laboratory investigations and there are some limitations in their scalability. Furthermore, they are not much effective for softwoods (Zhu and Pan 2010). In some organosolv processes, such as ethanol organosolv pretreatment, which is one of the most effective methods, size reduction is not necessary. The process is also effective for softwoods (Pan et al. 2005, 2006a, c, 2007a, b). However, the process is not yet considered as an alternative process for large-scale pretreatment of lignocelluloses.

Size reduction can also be performed after chemical pretreatment, as refereed post-chemical pretreatment size reduction. Post-chemical pretreatment size reduction has different advantages compared to that before chemical pretreatments. Besides more effectiveness, the main advantage is lower mechanical energy consumption. On the other hand, without pre-size reduction, it is possible to work with denser solids and consequently higher solid per liquid ratio in the process, resulting in more concentrated hemicellulose sugar liquid. Furthermore, separation of fibers from the pretreated mixture is easier after pretreatment (Zhu and Pan 2010). However, post size reduction is not applicable in all pretreatments.

Different irradiation processes have also been shown to improve the digestibility of lignocelluloses (Fernandez-Cegri et al. 2012). Treatment of biomass with high energy irradiation can modify the structure (Bak et al. 2009). However, its application is limited to less recalcitrant biomass such as rice straw (Bak et al. 2009). Ultrasonication, on the other hand, has been used at large scale for the improvement of digestibility of different organic material and sludge resulting in higher yield of biogas and lower amounts of residual sludge (Pham et al. 2009; Elbeshbishy et al. 2011).

Combined irradiation (mainly ultrasonic and microwave) and chemical pretreatments also have been shown to improve digestibility than a single chemical pretreatment. The irradiations can work in conjunction with NaOH pretreatment (Rodrigues et al. 2011; Singh et al. 2011), ionic liquid pretreatment (Ha et al. 2011; Ninomiya et al. 2012), and ammonia pretreatment (Chen et al. 2012).

3.4.2 Chemical Pretreatments

Chemical pretreatment is treatment of the lignocelluloses using chemicals to modify the crystalline structure of cellulose and remove and/or modify hemicelluloses and lignin (Taherzadeh and Karimi 2008; Zhu and Pan 2010). There are several reviews and chapter books which present and compare chemical pretreatments (McMillan 1994; Wyman 1996; Galbe and Zacchi 2007; Taherzadeh

and Karimi 2008; Alvira et al. 2010; Zhu et al. 2010; Zhu and Pan 2010; Mora-Pale et al. 2011; Hendriks and Zeeman 2009).

There are also some pretreatments referred to as "physico-chemical pretreatments", where a chemical pretreatment is combined with a physical treatment. The physical treatment is typically a mechanical treatment (mainly size reduction) or explosion. The explosion can play a size reduction role in these processes. Here, details and progresses in the most effective and widely used processes, i.e., alkali, acid, and solvent pretreatments, are reviewed. However, this does not mean that other processes are less important. Understanding the mechanism and the details of the reactions may help for further improvement of the processes.

3.4.2.1 Alkaline Pretreatment

NaOH alkali pretreatment for improving in vitro digestibility of straws by ruminants are found at least since 1919 (Millett et al. 1976). Alkali pretreatment refers to the application of an alkaline solution such as NaOH, $Ca(OH)_2$, or ammonia to modify the structure and composition of lignocelluloses (Deschamps et al. 1996; Zhao et al. 2008b; Cheng et al. 2008; Glaus and Van Loon 2008; Martin et al. 2008; Mirahmadi et al. 2010).

Alkaline processes are among the most efficient pretreatment processes, especially for hardwoods and agricultural residues. The alkaline pretreatment can remove or modify lignin and hemicellulose and increase the porosity of lignocelluloses (Tarkow and Feist 1969). Nevertheless, the treatment is a very complicated process. It involves different reactions, dissolution of polysaccharides, and hydrolysis and decomposition of polysaccharides (Fengel and Wegener 1984). The alkali treatment of cellulosic materials is an old process, which was the focus of the pulp and paper industries and research, so-called mercerization (Takai and Colvin 1978).

These treatments can result in low amounts of residual lignin, along with reduced crystallinity. Intraparticle porosity and channel size are also increased by alkali pretreatment (McMillan 1992).

The efficacy of dilute NaOH pretreatment is higher for straws, lower for hardwoods, and very low for softwoods. This is related to the lignin type and content in these materials (McMillan 1992). The alkali pretreatment can also reduce the cellulose DP (Fengel and Wegener 1984).

Alkali treatments are among the pretreatments that increase the swelling capacity of lignocelluloses (McMillan 1992), which can be due to lignin removal. On the other hand, alkali treatment (e.g., by NaOH) enhances the polyionic character of the pretreated lignocelluloses, which is related to the diffusion of sodium ions into the lignocellulose. These ions remain in the lignocellulose and act as a countercharge to carboxylate ions. This polyionic character of the pretreated materials promotes swelling. This property can be incorporated even by acid washing in which the sodium ions are displaced by protons (McMillan 1992). However, the ammonia pretreatment makes lower swelling characteristics than

NaOH, because of reduced polyionic character. Nevertheless, chemically combined nitrogen or residual ammonia contribute to overall nitrogen content, which improves the value of lignocelluloses as a ruminant feed (McMillan 1992).

NaOH pretreatment processes can be classified into severe and moderate conditions processes. In severe conditions processes, the concentration of NaOH is low (0.5–4 %), but a high temperature and pressure is used, and recycling of NaOH is not usually possible (Cheng et al. 2008; Mirahmadi et al. 2010). Its mechanism is reactive destruction and solubilization of lignin and hemicellulose. However, at moderate conditions, high NaOH concentration (6–20 %) is applied at ambient pressure and low temperatures (e.g., at 0 °C) (Mirahmadi et al. 2010; Wu et al. 2011a, b). Dissolution of cellulose at these conditions is the main phenomena (Zhao et al. 2008b). This dissolution needs high concentration of NaOH (at least 6–8 % depending on temperature). The processes employing high sodium hydroxide concentrations do not remove much lignin, but the NaOH solution may be reused. However, the pretreatment is not very effective on softwoods (Mirahmadi et al. 2010).

Effect of Alkali Treatments on Polysaccharides

Partial degradation of cellulose and hemicellulose is possible during alkali pretreatment. A large number of reactions may take place at elevated temperature in the alkali conditions. The most important reactions are (Sjostrom 1977; Fengel and Wegener 1984; Pérez and Samain 2010):

- Dissolution of nondegraded polysaccharides;
- Formation of alkali-stable end-groups referred to as peeling reactions of end groups (peeling-off);
- Hydrolysis of glycosidic bonds and acetyl groups;
- Decomposition of dissolved polysaccharides.

While the most important reactions are peeling at temperatures about 100 °C, the alkali hydrolytic reactions are considerable at temperatures above 150 °C. Similar to acid pretreatment, hydrolysis and degradation of hemicelluloses are much faster than cellulose in alkali treatment. However, xylans are more stable than glucomannans. The easy cleavage of arabinose side groups in softwood xylans has a stabling effect against many alkali reactions, since with loss of the side groups an alkali-stable end groups, mainly metasaccharinic acid end group, is formed (Fengel and Wegener 1984). The endwise peeling continues to degradation until a competing reaction, so-called stopping reactions, takes place that terminates the degradation. The major stopping reactions start with hydroxyl elimination from the degrading intermediates and convert the intermediate into alkali-stable metasaccharinic acid end group. Without the stopping reaction, whole cellulose and hemicellulose may be decomposed by peeling (Henderson 1970; Yoneda et al. 2008).

While the alkali treatment can liberate mainly acetic acid (acetyl groups from hemicellulose backbone) and some small amounts of other dicarboxylic acids, in

severe conditions the degradation can result in formation of formic acid as an end product (Henderson 1970). However, the rate of hydrolysis in alkali pretreatment is much slower than acid pretreatment (Fengel and Wegener 1984).

Overall, during alkali pretreatment, two main reactions take place (Fengel and Wegener 1984; Henderson 1970):

- Hydrolytic cleavage of polysaccharides, which can produce new reducing end groups that can be easily degraded, and
- formation of alkali-stable end groups.

Alkali treatment is among the most widely used methods for pulping. Polysaccharide stabilization during the alkali treatment is important in pulping since this can increase the pulp yield. It was found that some additives, such as polysulfides, sodium borohydride, hydrogen sulfide, and anthraquinone, could stabilize polysaccharides against alkali peeling (Procter and Wiekenkamp 1969; Biermann 1996; Aravamuthan 2004; Biswas et al. 2011). Effects of these additives, however, have yet not been investigated in lignocelluloses pretreatment.

The released sugars, e.g., glucose and xylose, may result in degradation products under alkali pretreatment. The treatment of xylose and glucose with 2.5 % NaOH at 96 °C under nitrogen atmosphere resulted in several aliphatic degradation products together with several cyclic enols and phenolic compounds (Forsskahl et al. 1976). The alkali pretreatment temperature can be a very important parameter. It can also affect the molecular weights of resultant hemicellulosic polymers. For instance, pretreatment with 4 % NaOH solution for 18 h was more effective at 40 °C than 20 °C for releasing mannose, arabinose, rhamnose, and uronic acids from bagasse hemicellulose. However, more xylose, glucose, and galactose formed at lower temperature (20 °C) compared to higher temperature (40 °C) (Xu et al. 2006). The average molecular weight of hemicellulosic polymers was also reduced more when lower temperature was used; thus, mild alkali pretreatment can efficiently reduce the molecular weight of hemicellulose (Xu et al. 2006).

Cellulose partly degrades during alkali pretreatments. The reason can be either chemical or physical stopping. Chemical stopping is the formation of a nonreactive metasaccharinic end group by reactive interim products. On the other hand, when the degradation reaches crystalline regions of the polysaccharide chains, the peeling reaction cannot proceed further for steric reasons, referred to as physical stopping (Glaus and Van Loon 2008).

Effect of Alkali Treatment on Cellulose Structure

The changes in cellulose structure by NaOH treatment are (Mansour et al. 1972):

- Change in crystallinity by mercerization;
- Changes in distribution of crystalline and amorphous regions;
- Depolymerization.

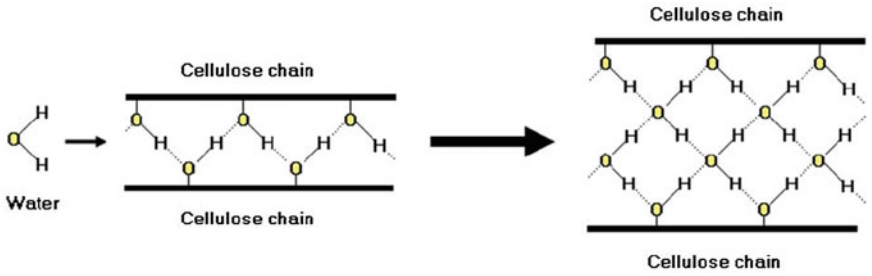

Fig. 3.5 Swelling of cellulose

Effects of cold and hot NaOH pretreatments on crystallinity depend on the substrate. Cold alkali pretreatment was shown to be more effective in reducing crystallinity index of Rag and Kraft paper wood pulp than hot treatment (Mansour et al. 1972).

The crystallinity of cellulose changes in alkali treatments. When cellulose is treated with alkali solutions, the cellulose swells to various extents depending on the temperature, alkali concentrations, and pretreatment retention time (Fig. 3.5). By treatment in the presence of Na^+ cations in NaOH pretreatment, 300 % cellulose swelling was observed in the form of $(C_6H_{10}O_5)_6(NaOH)(H_2O)_3$, while LiOH and KOH increased the swelling up to 250 % (Fengel and Wegener 1984). Na^+ cations are small ions that are able to enter the smallest pores in the cellulose and move through the cellulose (Deshpande et al. 2008). Complete de-crystallization of cellulose is possible for high crystalline celluloses.

On the other hand, in NaOH pretreatment, complete transformation of cellulose I into cellulose II is possible (Fig. 3.6). Meanwhile, only partial or nontransformation may occur in the cellulose by pretreatment with other alkali solutions (Pérez and Samain 2010). Cellulose II can also be obtained from cellulose I by the processes of regeneration and mercerization (Festucci-Buselli et al. 2007). In strong alkali treatment process, cellulose I can highly swell due to the formation of alkali–cellulose complexes, and recrystallize after washing with water to form the cellulose II.

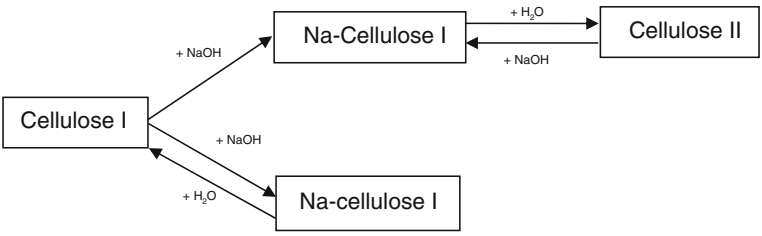

Fig. 3.6 Transformation of cellulose in NaOH treatment [adapted from Fengel and Wegener (1984)]

Strong alkali treatment (mercerization) has been investigated thoroughly for conversion of native cellulose (cellulose I) into cellulose II (regenerated cellulose) (Takai and Colvin 1978).

Re-crystallization, transformation of cellulose I into cellulose II, is observed in many pretreatment methods. Several studies have noted increases in crystallinity of cellulosic materials during pretreatment with alkali, steam, and acid chlorite (Gümüskaya and Usta 2006). Transformation of cellulose I into cellulose II in the residue cellulose is observed in supercritical water treatment, in spite of considerable degradation (Ago et al. 2004). Transformation of cellulose I into cellulose II is also possible by milling. When cellulose containing more than 30–50 wt % water is ball milled, cellulose I was transformed into cellulose II (Ago et al. 2004). Although the cellulose II is also a crystalline form of cellulose, it is much easier to hydrolyze than cellulose I.

Effect of Alkali Treatment on Lignin

Hydroxyl ions are responsible for delignification in alkali pretreatment. Hydrogen sulfide can also be involved if available in the reaction media (sulfate pulping). In alkali treatment of lignocelluloses, three groups of reactions can take place: fragmentations, degradation and dissolution, and condensations of lignin. Condensations refer to the reactions of lignin units to fragments with increased molecular weight and low solubility (Patt et al. 2011). The effects and reactions in alkali treatment on lignin are complicated because of the different stability and behavior of different types of linkages and structural elements in this hetroploymer. A small portion of lignin may degrade by cleavage of less frequent linkages, leading to a reduction or total elimination of side chains. Small molecules, e.g., methanol and formaldehyde, can be the products of alkali delignification (Fengel and Wegener 1984).

Combination of Alkali Pretreatment with Other Methods

Recent studies showed that a combination of alkali pretreatments with other treatments can significantly improve hydrolysis. A combination of steam explosion, dilute acid, oxidative treatments, wet milling, microwave, and ultrasonication with alkali treatment resulted in better enzymatic hydrolysis sugar yields than alkali treatment alone (He et al. 2010; Keshwani and Cheng 2010; Rodrigues et al. 2011).

Since the alkali treatment is mainly used to remove lignin, it can be combined with other methods for other effects or improvements. For instance, alkali treatment followed by explosion is more effective than alkali treatments alone. The explosion cannot change the crystallinity, but it is effective in increasing the surface area and reducing the degree of polymerization of cellulose (Puri and Pearce 1986). The steam explosion of sugarcane bagasse followed by alkaline delignification resulted in higher lignin (91 %) and hemicellulose (72 %) removal (Rocha et al. 2012).

Dilute sulfuric acid pretreatment of rice hulls followed by delignification with NaOH led to a major lignin removal, while the cellulose content increased to 68.5–70.5 %. The combined process resulted in more than nine times improvement in the enzymatic hydrolysis (Martin et al. 2008). In another study, dilute acid pretreatment of empty palm fruit bunch fiber followed by NaOH pretreatment resulted in a novel substrate for enzymatic digestibility (Kim et al. 2012).

Alkaline/oxidative (e.g., NaOH/H_2O_2) mixtures are effective for oxidizing and pretreatments with these mixtures are able to selectively remove lignin. They can effectively enhance the enzymatic hydrolysis of biomass (Cheng et al. 2008). Curreli et al. (1997) presented a mild alkaline/oxidative pretreatment method at low temperature (25–40 °C). They first subjected the biomass to an alkaline (1 % NaOH for 24 h at 40 °C) process for mainly solubilizing hemicellulose and rendering the lignocellulose more accessible to further pretreatment. In the second stage, alkaline/oxidative pretreatment (1 % NaOH and 0.3 % H_2O_2 for 24 h at room temperature) was performed for solubilizing and oxidizing lignin to minor inhibitory compounds. About 90 % cellulose recovery with 81 % degradation of lignin was obtained.

An efficient and environmentally friendly process for pretreatment of corn stalk with active oxygen (O_2 and H_2O_2) followed by a treatment with a recoverable solid alkali (MgO) effectively removed lignin and extractives, and increased the accessible surface area (Pang et al. 2012).

3.4.2.2 Acid Pretreatments

Acid-catalyzed treatment is known to be one of the most effective methods for lignocelluloses pretreatment (McMillan 1994; Wyman 1996; Taherzadeh and Karimi 2008). This is the most applied method for cleavage of glycosidic bonds in hemicellulose (Jacobsen and Wyman 2000). The cleavage of the glycosidic and glucuronosyl linkages can occur under relatively mild conditions while decomposition of the liberated monosaccharides can only take place at severe conditions (Bertaud et al. 2002).

In an acidic media at elevated temperatures, the cellulose structure becomes unstable due to the breakage of hydrogen bonding, which is the primary force that holds the cellulose chains together. After disruption of the cellulose crystalline structure, the acid molecules can penetrate into the cellulose. The acid-soluble lignin, forming a lignin-carbohydrate complex, can also appear in the liquid phase (Xiang et al. 2003). Dilute acid hydrolysis can efficiently break hemicellulose-lignin linkages and hydrolyze glycosidic bonds in hemicellulose (Grohmann et al. 1986b). After the treatment, the liquid solution mainly contains hemicelluloses and the solid part is mainly composed of cellulose and lignin. One problem in acid hydrolysis is partial degradation of sugars, mainly xylose to furfural. A part of xylose remains in the residue (Grohmann et al. 1986a, b).

The remaining cellulose after dilute acid hydrolysis of lignocelluloses, called hydrocellulose, contains lower DP but higher crystallinity in many lignocellulosic materials (Fengel and Wegener 1984). However, this increase in crystallinity

depends on the substrate and on the applied conditions. For instance, for cotton, it showed a decrease in the crystallinity (Mugnolo et al. 1988).

The acid used in the process is usually sulfuric acid (Harris et al. 1984; Torget et al. 1991; Lis et al. 2000; Nguyen et al. 2000; Kim and Lee 2002; Soderstrom et al. 2003; Um et al. 2003; Saha et al. 2005; Sun and Cheng 2005; Ewanick et al. 2007; Sassner et al. 2008), while other acids such as hydrochloric (Titchener and Guha 1981; Higgins and Ho 1982), phosphoric (Um et al. 2003), nitric (Fengel and Wegener 1984), and trifluoroacetic (Fengel et al. 1978; Fengel and Wegener 1979) acid have also been used both for pretreatment before enzymatic hydrolysis and for saccharification itself.

Effective parameters in dilute acid pretreatment are (Wyman 1996; Kim and Lee 2002; Xiang et al. 2003):

1. Acid concentration;
2. Type of acid;
3. Temperature (and correspondingly pressure at high temperatures);
4. Retention time (heat up time, retention time at maximum temperature, and depressurizing time) during the process;
5. The temperature profile;
6. Properties of lignocellulose (the physical state of the cellulose);
7. Intra-particle acid diffusion within lignocellulosic biomass particles;
8. Presoaking of lignocelluloses with acid.

Once cellulose is hydrolyzed to glucose, three reactions can occur that can reduce the glucose yield (Jacobsen and Wyman 2000; Xiang et al. 2003):

- Glucose–lignin interaction (formation of complex with acid-soluble lignin);
- Decomposition of glucose; and
- Reversion reactions.

The disappearance of glucose during acid hydrolysis of lignocellulosic biomass is higher than expected. The suggested reason is recombination of glucose with acid soluble lignin in the hydrolyzates (Xiang et al. 2003):

(Acid Soluble Lignin) + Glucose → (Acid Soluble Lignin)-Glucose

At elevated temperatures, the presence of protons causes the formation of intermediate carbonium ions that have a high nucleophilic reaction affinity. Therefore, lignin fragmentation or lignin condensation reactions may occur. These acid soluble lignin active sites can result in condensation reactions between acid soluble lignin and glucose (Xiang et al. 2003).

Different types of reactors are used for dilute acid treatments including batch, plug flow, percolation, countercurrent, and shrinking-bed reactors (Taherzadeh and Karimi 2007).

In spite of the wide application of dilute acid pretreatments, the process has several serous limitations (Yang and Wyman 2008):

- Corrosive environment and expensive material of construction;
- Degradation reaction products such as furfural and HMF;

- Necessity of liquid phase detoxification for fermentation;
- Acid neutralization and the gypsum formed present difficulties in downstream processing;
- Although the sulfuric acid is cheap, neutralization cost and $CaSO_4$ disposal cost must be included;
- Remainder of lignin in the substrate and binding of enzymes to lignin needs high enzyme use and costs;
- High substrate concentrations are impossible (it was found that complete hydrolysis is not possible at high substrate concentrations, because of the redistribution of lignin within the substrate and the structural modifications (Ramos et al. 1992).

Dilute Acid Pretreatment Followed by Explosion

In some pretreatment processes, it is found that explosive decompression (sudden reduction of pressure) exerts a mechanical shear on the lignocelluloses, which may increase the specific surface area of the biomass by defibrating individual cellulose microfibrils or by expanding the lignocellulose matrix (McMillan 1992; Brownell et al. 1986; Shimizu 1988; Ballesteros et al. 2000). On the other hand, it was also shown that explosion is unnecessary in steam explosion pretreatment. Brownell et al. (1986) showed that the explosion part of steam explosion pretreatment contributes nothing to the enzymes accessibility, and consequently had no effect on fermentation. Morjanoff and Gray (1987) found no significant effect of explosion for dilute acid pretreatment of sugarcane bagasse prior to enzymatic saccharification. The pressure usually does not have any significant effects on the liquid chemical reactions. However, when the pressure suddenly decreases, the water in the biomass changes to vapor, rashly escapes, and disrupts the biomass structure. However, in a severe acid treatment, the process is so effective that the pretreated biomass does not need further disruptions.

Effect of Acid Pretreatment on Cellulose, Hemicellulose, and Lignin

In mild pretreatments, the acid can solubilize some of the amorphous cellulose and increase the pore sizes in the residue. The acid treatment can affect the crystallinity up to a certain level (Kumar et al. 2009). However, recrystallization and increase in crystallinity can also happen in a severe acid treatment. Although the crystalline regions of cellulose resist the attack of acid in dilute acid treatment, the process can reduce the DP up to a certain level, called the leveling-off DP (Fengel and Wegener 1984; Knappert et al. 1980).

Possibility of high hemicellulosic sugars recovery, 80–90 %, is one of the major advantages of dilute acid pretreatment (Yang and Wyman 2008). The extent of acetyl ester removal is quite high (e.g., 90 %) besides little disruption of other

fractions (i.e., lignin) (Grohmann et al. 1989). As xylan becomes deacetylated, the cellulose becomes more accessible and more digestible (Knappert et al. 1980).

Only little lignin dissolves in dilute acid process, mainly acid soluble lignin. However, the residual lignin is disrupted and significant effects have been observed in increasing the enzymatic susceptibility of cellulose (Foston and Ragauskas 2010; Pingali et al. 2010). Therefore, it is believed that acid pretreatment dose not dissolve lignin but some condensation reactions may occur (Fengel and Wegener 1984). Addition of acid in steam treatment for batch pretreatment showed reduced lignin removal, although it increased cellulose digestibility (Yang and Wyman 2004). Since dilute acid pretreatment can effectively enhance the enzymatic hydrolysis without efficient removal of lignin, it is suggested that lignin removal is not necessary for effective conversion of cellulose (Donohoe et al. 2008). Besides partial depolymerization, repolymerization of lignin has also been claimed during hemicellulose hydrolysis (Yang and Wyman 2004).

A large fraction of lignin reacts to soluble products of hydrolyzate, and if left in the reactor, reacts further to form insoluble species. Both lignin dissolution and precipitation can take place in acidic conditions. In dilute acid pretreatment, the lignin can condense onto the biomass surface, and this should be avoided in pretreatment (Yang and Wyman 2004).

Rate of Hydrolysis of Carbohydrates and Decomposition of Sugars

Five carbon sugars acid hydrolysis rate, e.g., xylose, is faster than hexoses. The relative acid hydrolysis rates of different sugars are shown in Table 3.2. As can be seen in the table, the hydrolysis rate of hemicellulosic polymer constituent sugars, xylose, mannose, galactose, and arabinose are much higher than glucose (Saeman 1945, 1949).

Dehydration of produced sugars is unavoidable in acidic hydrolysis conditions that can result in loss of sugars. A series of degradation products can form. The main degradation products are furfural produced from pentoses and uronic acids and hydroxymethylfurfural furfural (HMF) from hexoses. HMF can also convert into levulinic and formic acid (Taherzadeh and Karimi 2007).

Different models have been proposed for hydrolysis of hemicellulose in dilute acid treatments. The models are summarized in Fig. 3.7. A simple basic model (Model A) considered depolymerization of pentosan to xylose and then to

Table 3.2 Relative rate of hydrolysis (Shafizadeh 1963)

Monomer	Rate of hydrolysis compared to mannose
Mannose	1
Glucose	0.4
Galactose	3.9
Xylose	3.8
Arabinose	3.8

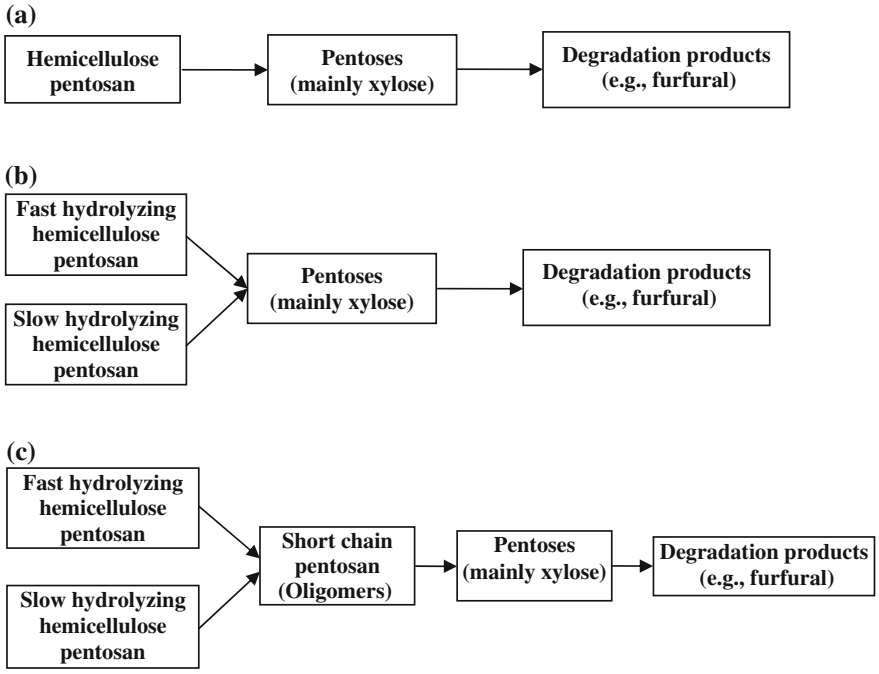

Fig. 3.7 Different models for acid hydrolysis of pentosans in hemicelluloses

degradation products (Saeman 1945). Kobayashi and Sakai (1956) observed that the rate of hydrolysis reaction decreased significantly after conversion of 70 % of xylose (Model B). The fast and slow hydrolyzing fractions differ in different substrates, typically in the range of 65 and 35 %, respectively, for most materials (Shen and Wyman 2011). However, they do not consider the intermediates between hemicellulose to xylose formation in this model (Model B). The model was further modified by considering the oligomers formation and breakdown to xylose monomers (Model C). This model seems to be more realistic which includes the role of oligomers (Jacobsen and Wyman 2000).

Different models were also proposed for dilute acid hydrolysis of cellulose (Jacobsen and Wyman 2000). However, the main purpose in dilute acid pretreatment is hemicellulose removal and less cellulose degradations; thus, the treatment conditions should be severe enough to hydrolyze hemicellulose fraction but not to hydrolyze the cellulose fraction.

3.4.2.3 Pretreatment with Solvents

Solvent pretreatments are based on either modification of cellulose crystalline structure or reducing the lignin content of the lignocellulosic materials. Using cellulose solvents such as phosphoric acid, NaOH/urea, N-methylmorpholine-N-

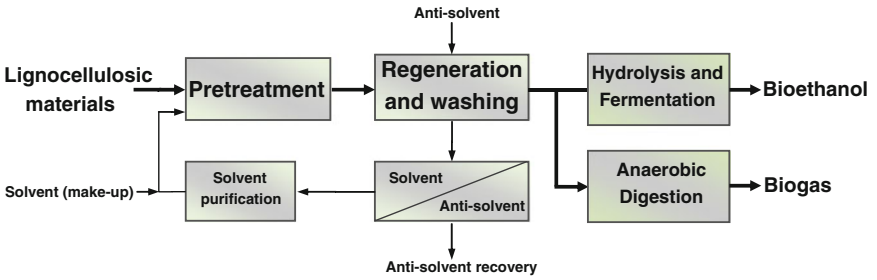

Fig. 3.8 A schematic process of biofuels production from lignocellulosic materials using cellulose solvents pretreatment

oxide (NMMO), and certain ionic liquids (ILs) for pretreatment showed significant improvements in the enzymatic hydrolysis of lignocelluloses. One of the main advantages of the solvent pretreatment is minimum destruction of fermentable sugars. On the other hand, use of strong acid or base results in production of toxic pollutants to the environment. However, NMMO and a number of ILs are green solvents that were recently introduced for pretreatment of lignocelluloses (Dogan and Hilmioglu 2009; Kuo and Lee 2009b; Jeihanipour et al. 2010a, b; Li et al. 2010a, b; Lucas et al. 2010; Nguyen et al. 2010; Qi et al. 2010; Shafiei et al. 2010; Fu and Mazza 2011; Mora-Pale et al. 2011; Sant'Ana da Silva et al. 2011; Shafiei et al. 2011; Shill et al. 2011; Bose et al. 2012; Geng and Henderson 2012; Hong et al. 2012; Khodaverdi et al. 2012; Lynam et al. 2012). These efficient cellulose solvents are considered as "green" chemicals because of their low toxicity to humans and to the environment (Meister and Wechsler 1998; Rosenau et al. 2001; Mora-Pale et al. 2011). Pretreatment with these solvents has the advantage of moderate conditions, no requirement for neutralization, and low production of inhibitory compounds. Furthermore, the ability to recycle and reuse NMMO in industrial scale and for ILs in the bench scale have been proven (Li et al. 2009; Jeihanipour et al. 2010a, b). In pretreatment with solvents, the materials are treated with cellulose solvents and then an antisolvent, which can be water or alcohol, is added to regenerate the materials. Therefore, the solvents can be separated from solid and reused in the process (Fig. 3.8).

Ionic Liquids

Recently, different ILs have been extensively used for pretreatment of lignocellulosic materials. Ionic liquids are organic salts composed of organic cations and either organic or inorganic anions. Four groups of cations are mainly used for categorizing ILs: quaternary ammonium, N-alkylpyridinium, N-alkyl-isoquinolinium, and 1-alkyl-3-methylimidazolium (Liu et al. 2012). Besides being a powerful solvent for cellulose, ILs have unique properties such as low vapor

pressure and high thermal and chemical stability. The desired property is adjustable by the selection of proper cation and anion.

Shortly after pioneer investigations on the capability of ILs for cellulose dissolution (Swatloski et al. 2001), ILs were introduced for pretreatment of cellulose. About a 50-fold increase in the initial rate of hydrolysis of pure cellulose by [BMIM][Cl] opened a new research area in the pretreatment (Dadi et al. 2006).

[BMIM][Cl] and [EMIM][Ac] are among the most efficient ILs for pretreatment of lignocellulosic materials. Pretreatment is usually performed at 3–5 % solid loading, temperature of 110–160 °C, duration of 15 min to 5 h, and at atmospheric pressure. A high yield of cellulose saccharification (80–96 %) has been reported for IL pretreatment of switchgrass, triticale straw, maple wood, corn stover, kenaf, bagasse, and eucalyptus (Table 3.3).

Increase in pretreatment temperature (up to 130 °C) and time (up to about 5 h) improved the cellulose hydrolysis yield (Liu and Chen 2006; Lee et al. 2009; Li et al. 2009). However, pretreatment at severe conditions, i.e., at high temperature or for a very long time, showed no significant increase in the yield (Dadi et al. 2006), but reduced the amount of recovered carbohydrates (Lee et al. 2009; Fu and Mazza 2011).

Certain ILs have the ability of cellulose dissolution, structural modification, and even its direct hydrolysis (Liu et al. 2012). Partial conversion of cellulose I into cellulose II and reduction in cellulose crystallinity after pretreatment was reported (Dadi et al. 2006; Kuo and Lee 2009a; Zhao et al. 2010; Fu and Mazza 2011; Jeihanipour 2011; Silva et al. 2011; Khodaverdi et al. 2012). Increase in the accessible surface area for the hydrolytic enzymes adsorption was also observed (Zhao et al. 2010; Silva et al. 2011).

Depending on the nature of anion, ILs can dissolve lignin as well (Pu et al. 2007). Lignin removal is reported as another mechanism for enhanced enzymatic hydrolysis by some ILs together with reduction of the crystallinity (Lee et al. 2009; Zhao et al. 2010; Fu and Mazza 2011).

Effect of IL Pretreatment on Cellulose

Since the pretreatment with ILs is based on dissolution of cellulose, the ability of IL for dissolution of cellulose and lignocelluloses may relate to the efficiency of the pretreatment. Factors affecting the ability of IL for cellulose dissolution are the size of the anions and cations. Similar ILs with larger cations have less ability to form hydrogen bonds with cellulose (Zhao et al. 2008a; Mäki-Arvela et al. 2010). Thermochemical radii of halogen anions showed the same effect on the cellulose solubility in halogen containing anions. Therefore, [BMIM][Cl] is able to dissolve cellulose; while, [BMIM][PF6] and [BMIM][BF4] are unable to dissolve cellulose (Mäki-Arvela et al. 2010). However, despite the ion size, combination of the proper cation and anion and their interactions may result in a more powerful solvent. For example, [EMIM][Ac] is a better cellulose solvent than [EMIM][Cl] (Zavrel et al. 2009). Hydrogen bonds basicity, which is measured as β-parameter

Table 3.3 Pretreatment of some lignocellulosic materials with ILs

Ionic liquid	Conditions[a]	Raw material	Enzymatic hydrolysis	References
[BMIM][Cl]	5 %, 130–150 °C, 10 min–3 h	Cellulose	Similar results in all conditions, over 85 % conversion	Dadi et al. (2006)
[EMIM][DEP][b]	4 %, 130 °C, 30 min	Wheat straw	55 % yield over 53 % after 5 reuses of IL	Li et al. (2009)
[BMIM][Cl]	5 %, 130 °C, 20 min	Cotton	About 90 %	Kuo and Lee (2009a)
[EMIM][Ac]	5 %, 50–130 °C, 0.5–32 h	Maple wood	95 % (130 °C and 90 min) 97 % (90 °C and 32 h)	Lee et al. (2009)
[Me(OEt)3-Et3 N][Ac][b]	1 %, 110 °C, 15 min	Switchgrass	96 %	Zhao et al. (2010)
[EMIM][Ac]	3 %, 160 °C, 3 h	Switchgrass	96 %	Li et al. (2010a, b, c)
Ammonia+[EMIM][Ac][c]	5 %, 130 °C, 24 h	Rice straw	97 %	Nguyen et al. (2010)
50 % [EMIM][Ac] solution[d]	3 %, 150 °C, 90 min	Triticale straw	81 %	Fu and Mazza (2011)
[EMIM][Ac]	3 %, 160 °C, 3 h	Corn stover	Over 90 %	Li et al. (2011)
[EMIM][Ac]	5 %, 120 °C, 2 h	Sugarcane bagasse	Over 90 %	Sant'Ana da Silva et al. (2011)
[EMIM][Ac]+Ultrasonic[b]	5 %, 25 °C, 120 min	Kenaf powder	86 %	Ninomiya et al. (2012)
[EMIM][Ac]	5 %, 120 °C, 10 min	Bagasse and Eucalyptus	Over 70 %	Uju et al. (2012)

[a] The numbers are solid loading (wt %), temperature, and pretreatment time

[b] The IL presented is the most efficient among several studied ILs

[c] Pretreatment was conducted in two steps. The material (10 %) was first treated with 10 % ammonia for 6 h at 100 °C. The washed and dried materials were then treated with IL

[d] Pure IL to 5 % solution of IL was examined. Fifty percent solution was the most efficient one

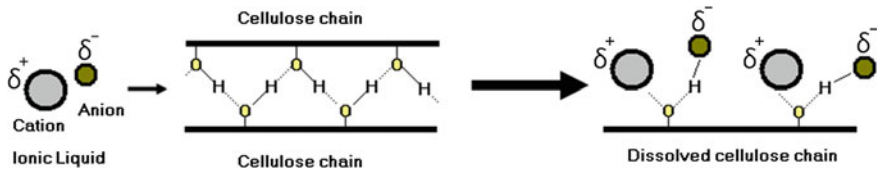

Fig. 3.9 Dissolution mechanism of cellulose in IL

in Kamlet-Taft equation, and Hildebrand solubility parameter are other factors affecting the ability of ILs in cellulose dissolution (Mäki-Arvela et al. 2010).

Although it has not been thoroughly investigated yet, the plausible mechanism of cellulose dissolution with ILs is presented in Fig. 3.9. NMR relaxation measurements confirmed the hydrogen bonding between the carbohydrates hydroxyl group and chloride ion from [BMIM][Cl], which lead to disruption of the hydrogen bond network in the dissolved cellulose (Moulthrop et al. 2005; Remsing et al. 2006). Hydrogen bonding between cellobiose hydroxyl groups and both cation and anion of [EMIM][Ac] were also confirmed by NMR studies (Zhang et al. 2010). Molecular dynamic studies showed similar hydrogen bonding between cellulose and acetate anion, and hydrophobic interaction between cellulose and the cation of [EMIM][Ac] (Liu et al. 2010).

Other factors to be considered when choosing an IL for pretreatment are the price, physical properties, availability, toxicity, corrosivity, biodegradability, and water tolerance (Mäki-Arvela et al. 2010). Among the ILs, [EMIM][Ac] and [BMIM][Cl], which are mostly used for pretreatment of lignocellulosic materials, are efficient solvents for cellulose and the former is an efficient solvent for lignocelluloses (Mäki-Arvela et al. 2010). Acetate-based ILs, e.g., [EMIM][Ac], exhibit less toxicity and corrosivity compared to halogen containing ILs, e.g., [BMIM][Cl]. [EMIM][Ac] is considered as a biodegradable solvent (Zavrel et al. 2009; Liebert 2010).

Several investigations on direct hydrolysis of cellulose or lignocelluloses with ILs are reported. These studies were conducted with or without addition of acid or metal chlorides as catalyst and resulted in 64–97 % yield of reducing sugars (Li and Zhao 2007; Li et al. 2008; Sievers et al. 2009; Binder and Raines 2010; Tao et al. 2010; Zhang et al. 2010).

Drawbacks of Pretreatment with ILs

Very high price of ILs is the main disadvantage of using these chemicals for pretreatment. The pretreatment with ILs is not industrially feasible if efficient (over 99 %) reuse is not possible. Besides, as they are recycled and reused, the efficiency of the ILs for pretreatment decreases. The presence of dissolved lignocellulosic compounds in the recycled ILs might contribute to the reduction in the efficiency of ILs; however, the mechanism is not clear yet (Li et al. 2010c; Shill et al. 2011).

On the other hand, ILs have negative effects on cellulase activity and affect the final yield of cellulose hydrolysis. Therefore, efficient removal of these compounds from pretreated materials is necessary (Zhao et al. 2009). However, ILs trapped inside the treated material reduces the washing efficiency and increases the amount of water required. Thus, reusing the water is also necessary. Otherwise, it consumes a huge amount of water and produces a huge amount of wastewater as well.

Pretreatment with NMMO

N-methylmorpholine-N-oxide (NMMO) is a cellulose solvent industrially used in the Lyocell process. In this modern and environmental friendly process, cellulose is spun into fiber using 85 % NMMO solution. The basic research on using NMMO for cellulose dissolution and fiber making was conducted during the 1970s, and since the 1990s it is being used in commercial scale Lyocell processes (Fink et al. 2001). However, since 2009, NMMO has been investigated for efficient pretreatment of lignocelluloses (Kuo and Lee 2009b), and in some cases, it has been shown to be more effective than ionic liquids (Poornejad et al. 2012).

NMMO with 83–87 % purity is able to dissolve cellulose, while increase in water content results in swelling and ballooning of cellulose at NMMO concentration of 79 and 73 %, respectively (Biganska and Navard 2003; Jeihanipour et al. 2010b). In the case of ethanol production, the pretreatment method employing 85 % NMMO, which is able to dissolve cellulose, was found to be more efficient while lower concentration of NMMO (ballooning and swelling mode) was found to be suitable for improvement of biogas production (Jeihanipour et al. 2010b).

The pretreatment conditions usually are: temperature 120–130 °C under atmospheric pressure (Table 3.4). Depending on the raw material and its size, the optimum pretreatment time would vary from 20 min to 5 h. Generally, pure cellulose or cellulose pulp requires less pretreatment time (Kuo and Lee 2009a; Wang et al. 2011) compared to wood and wood chips (Shafiei et al. 2010, 2011).

NMMO pretreatment was also used for improvement of biogas production (Jeihanipour et al. 2010b; Jeihanipour 2011; Teghammar et al. 2012. The pretreatment efficiently improved the methane yield of softwood spruce chips, milled spruce, rice straw, and triticale straw to 49, 95, 79, and 89 % of the theoretical yield, respectively, which was equal to 400–1,200 % improvement compared to methane from untreated materials. Furthermore, the digestion time was significantly decreased (Teghammar et al. 2012). Pretreatment of highly crystalline cellulose could also improve the methane yield. The pretreatment removed the rate limiting step from hydrolysis to the acetogenesis/methanogenesis phase. However, at high cellulose loadings (over 25 g/l), faster hydrolysis led to inhibition of biogas production, accumulation of volatile fatty acids, decrease in pH, and finally inhibition of the hydrolysis/acidogenesis phase. In order to overcome these problems, a two-stage digestion process was suggested (Jeihanipour 2011).

Table 3.4 Pretreatment of some lignocellulosic materials with NMMO

Conditions[a]	Raw material	Enzymatic hydrolysis and fermentation	References
1 %, 120 °C, 0.5–15 h	Highly crystaline cellulose	2.5 h pretreatment: 100 % saccharification yield, over 82 % ethanol yield	Jeihanipour et al. (2010b)
6 %, 90–130 °C, 1–3 h	Spruce and oak	83 and 72 % saccharification yield for Spruce and oakwood, 89 and 79 % ethanol yield	Shafiei et al. (2010)
5 %, 130 °C, 20 min	Cotton	Over 85 % saccharification yield	Kuo and Lee (2009a)
6 %, 130 °C, 1–5 h	Spruce and birch chips	88 and 92 % saccharification yield for Spruce and birch, 195 and 175 mg ethanol/g wood after 5 h pretreatment	Lennartsson et al. (2011)
5 %, 130 °C, 30 min	Cellulose pulp from populus tormentose	Over 80 % saccharification yield	Wang et al. (2011)

[a] The numbers are solid loading (wt %), temperature, and pretreatment time

Effect of NMMO Pretreatment on Cellulose

The pretreatment mechanism employing NMMO is based on the formation of strong hydrogen bonds between NMMO and cellulose (Fink et al. 2001; Zhao et al. 2008a). After addition of water, the hydrogen bonds between water and NMMO are stronger than NMMO cellulose, and therefore the dissolved cellulose is regenerated. The regenerated cellulose is less crystalline and more accessible to cellulolytic enzymes (Cuissinat and Navard 2006; Zhao et al. 2008a). In a study on pretreatment of pine wood with NMMO, linear correlation between overall glucan conversion rate and cellulose accessibility was found. However, reduction in crystallinity was linearly related to the initial hydrolysis rate rather than the overall glucan yield of hydrolysis. Structural studies confirmed reduction of lignin on the surface of wood flour after pretreatment as well as positive effects of increasing pretreatment time on cellulose accessibility (Liu et al. 2011). In pretreatment of pure cotton, increase in the amorphous structure was said to be the main reason for enhancement of enzymatic hydrolysis (Kuo and Lee 2009a).

Advantages and Disadvantages of Pretreatment with NMMO

The main advantage of NMMO-pretreatment is its high efficiency (over 80 % saccharification yield), minor degradation of raw materials, and negligible production of inhibitory compounds (Shafiei et al. 2010, 2011; Poornejad et al. 2012). NMMO is an environmentally friendly chemical and a wastewater containing

NMMO can be treated in conventional wastewater treatment plants (Meister and Wechsler 1998).

However, NMMO in concentrations above 5 and 25 g/l showed inhibition effects on enzymatic hydrolysis and fermentation by *S. cerevisiae,* respectively (Jeihanipour et al. 2010b; Shafiei et al. 2010). Therefore, the treated materials must be washed before enzymatic hydrolysis.

Techno-economic studies showed that efficient recycling of NMMO is required in order to have an economically feasible process for pretreatment of lignocellulosic materials with NMMO. However, certain side reactions, which are present in the Lyocell process, might affect the pretreatment system as well and lead to decomposition of NMMO and increase in stabilizer consumption (Rosenau et al. 2001). On the other hand, efficient removal of NMMO from the treated material by washing requires high amounts of water. This huge amount of water must be evaporated in an energy consuming process before reusing NMMO which is an energy intensive process (Shafiei et al. 2011).

3.5 Conclusion

Dilute acid treatment is among the most investigated pretreatment processes and is one of the leading options for application in industrial scales. Some of the drawbacks of the process can be minimized by optimization of reactors and effective parameters. However, the environmental impacts of the process, particularly the waste disposal, are unavoidable.

Alkaline processes are also very efficient for pretreatment. However, they are typically effective for hardwoods and agricultural residues. They can significantly change and modify the structure of lignocelluloses and render their hydrolysis. They are able to reduce lignin, hemicellulose, cellulose DP, and crystallinity, and increase the accessible surface area, swelling capacity, and enzyme adsorptions. However, the treatment is complicated and the effective parameters should carefully be optimized, otherwise they result in adverse effects. Furthermore, these processes typically need high amounts of water for washing the residue, and the chemical recycling and reuse is also difficult.

Pretreatment with cellulosic solvents is probably the most effective method. High carbohydrate recoveries, no toxic pollutants production, environmentally friendly, no neutralization requirement, and performing under moderate conditions are among the advantages of these pretreatments. However, the prices of these solvents are high and they are economically feasible only when they are almost completely recycled and reused, whereas separation of these solvents from cellulose is very difficult and needs large amounts of water. After separation of the solvent from the biomass, it consumes a considerable amount of energy for the solvent recovery from the dilute solvent–water solutions.

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Chapter 4
Acid Pre-treatment Technologies and SEM Analysis of Treated Grass Biomass in Biofuel Processing

Anthonia O'Donovan, Vijai K. Gupta, Jessica M. Coyne and Maria G. Tuohy

Abstract Currently, ethanol is the most important renewable fuel in terms of volume and market value. It is produced from sugar- and starch-based materials such as sugarcane and corn, which is unsustainable. The second generation production of ethanol derived from lignocellulosic materials is now the prime target of biofuel production. Hydrolysis of lignocellulosic materials is the first step for either digestion to biogas (methane) or fermentation to ethanol. Enzymatic hydrolysis of lignocelluloses without pre-treatment is not effective because of the high stability of lignocellulose materials to enzymatic or bacterial attacks. Pre-treatment by physical, chemical or biological means are essential processes for ethanol production from lignocellulosic materials. Pre-treatment enhances the bio-digestibility of the wastes for ethanol and biogas production and increases accessibility of the enzymes to the materials. It results in enrichment of the difficult biodegradable materials, and improves the yield of ethanol or biogas. A detailed understanding of the composition of the lignocellulosic waste is essential to develop and optimize the mechanistic model for its conversion. This model primarily includes pre-treatment processes which help integrate waste streams into the raw materials for ethanol plants, for improved production of ethanol (Taherzadeh and Karimi 2008). This chapter discusses in detail the composition and chemical constituents of the grass cell wall which contributes to agricultural waste residues, a plentiful and sustainable biofuel feedstock. Pre-treatment methods are discussed with a focus on mild acid pre-treatments and scanning electron microscopy analysis (SEM) of post-treatment biomass residuals is reviewed.

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4.1 Introduction

Lignocelluloses comprise a large fraction of crop residues, forest residues, dedicated energy crops, animal manures or municipal solid waste. Grass lignocelluloses are a major resource in the emerging cellulose to ethanol strategy for biofuels. The potential bioconversion of carbohydrates in this potential resource is limited by the constituents of the grass plant cell wall. Lignocelluloses are composed of cellulose, hemicellulose, lignin, extractives and several inorganic materials. The following sections detail the composition of the plant cell wall which explains the difficulties in utilising grass in the production of biofuel (Taherzadeh and Karimi 2008).

4.1.1 Plant Cell Wall

Lignocellulosic biomass refers to plant biomass. All plant cells are surrounded by an extracellular matrix known as the cell wall, a polysaccharide-rich matrix which is a major component of terrestrial plants. The plant cell wall is a composite structure and is divided into three layers; the middle lamella, the primary wall and the secondary wall (Carpita and Gibeaut 1993; Somerville et al. 2004) (Figs. 4.1 and 4.2). The middle lamella is the most external of all three layers and acts as a separating panel between two cells (Heredia et al. 1995). It is composed mainly of pectic substances and is the first boundary component to be formed by the cell during cytoplasmic division (Heredia et al. 1995; Hernon et al. 2010). The primary and secondary cell walls differ in function and in composition.

4.1.2 The Primary Cell Wall

The major polysaccharides in the primary wall are cellulose, hemicellulose and pectin. During cell expansion, the middle lamella is impregnated with cellulose, hemicellulose, pectin and glycoproteins to form the primary wall (Reiter 2002; Somerville et al. 2004). The primary wall is found at the junction of cells and at the outer edges of secondary walls. It is the first wall laid down and surrounds growing and dividing plant cells. These walls provide mechanical strength but must also expand to allow the cell to grow and divide. The wall derives its strength from long cellulose fibres held together by an amorphous matrix of protein and polysaccharide, which creates a composite structure that is highly resistant to compression. The polysaccharide-rich primary wall is composed of ~90 % carbohydrate and ~10 % protein with trace amounts of auxiliary substances. Of the major carbohydrates cellulose is the most abundant, constituting about 20–30 % of primary cell walls. The matrix is composed predominantly of two other

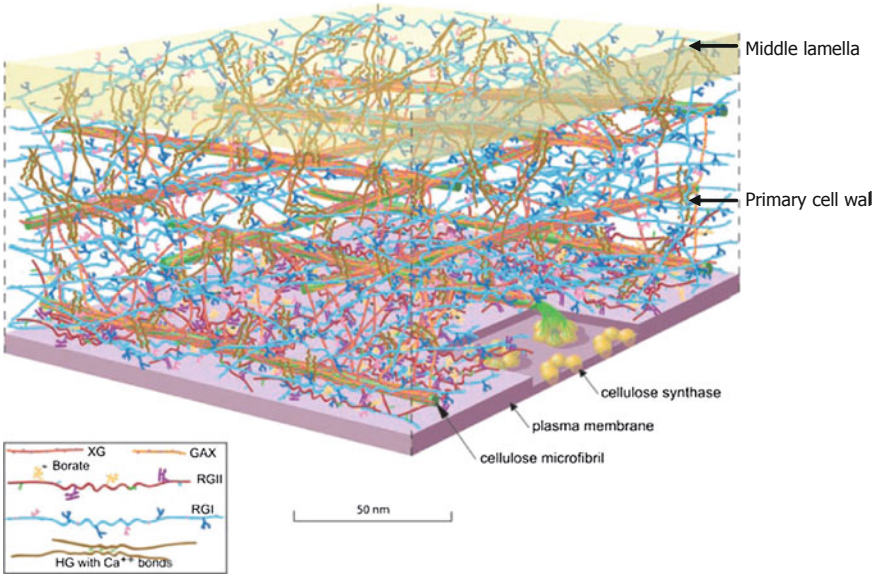

Fig. 4.1 Schematic representation of the structure of the primary cell wall. Xyloglucan (*XG*), glucoronoarabinoxylan (*GAX*), rhamnogalacturonan I (*RGI*), rhamnogalacturonan II (*RGII*) and homogalacturonan (*HG*) (Somerville et al. 2004)

types of polysaccharide, hemicellulose and pectin, together with structural glycoproteins. The most common hemicellulose in the primary cell wall is xyloglucan. In grass cell walls, however, xyloglucan and pectin are reduced in abundance and partially replaced by glucuronarabinoxylan. The fibres and matrix molecules are connected by a combination of covalent cross-links and non-covalent forces into a highly complex structure (Figs. 4.1 and 4.2) (Alberts et al. 1989).

The primary cell wall defines the rate of growth of plant cells, as well as their size and shape. It acts as a barrier to pathogens, while fragments of its polysaccharides have specific regulatory functions. Other functions of the primary cell wall include:

- Structural and mechanical support.
- Resist internal turgor pressure of cell.
- Ultimately responsible for plant architecture and form.
- Regulate diffusion of material through the apoplast.
- Carbohydrate storage—walls of seeds may be metabolised.
- Protect against pathogens, dehydration and other environmental factors.
- Source of biologically active signalling molecules.
- Cell–cell interactions.

(http://www.ccrc.uga.edu)

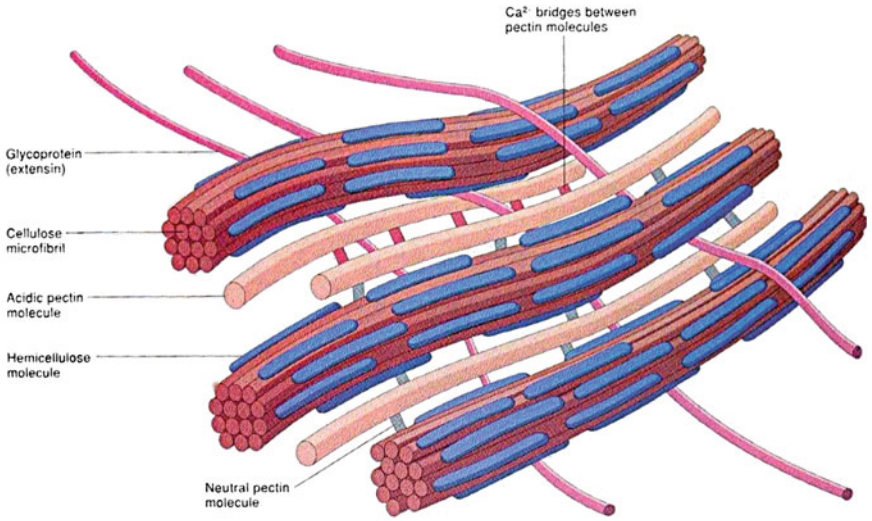

Fig. 4.2 Interactions between the major components of the primary cell wall. Hemicellulose molecules such as the xyloglucans are linked by hydrogen bonds to the surface of the cellulose microfibrils. The xyloglucans can be cross-linked to acidic pectin molecules, such as the rhamnogalacturonans, through short neutral polysaccharide components of pectins, e.g. arabinogalactans. Cell wall glycoproteins are woven tightly into the cell wall to complete the matrix (Alberts et al. 1989)

4.1.2.1 Type I and Type II Primary Walls

The primary cell walls of plants are classified into two major groups: Type I walls (in dicots e.g. all flower plants) and Type II walls (in monocots e.g. grass) with respect to the chemical structures of components, wall architecture and their biosynthetic processes. They vary in several ways; in the complex glycans that interlace and cross-link the cellulose microfibrils to form a strong framework, in the nature of the gel matrix surrounding this framework and they also vary in the types of aromatic substances and structural proteins that covalently cross-link the primary and secondary walls and lock cells into shape (Carpita 1996).

Type I walls are characterised by a cellulose–xyloglucan framework with approximately equal amounts of cellulose and cross-linking xyloglucans with various minor amounts of arabinoxylans, glucomannans and galacto-glucomannans (Nishitani 1997). Most type I wall xyloglucans share a repeating heptasaccharide unit structure subject to further derivatisation by additional galactosyl, fucosyl and arabinosyl units. Xyloglucans occur in two distinct locations in the wall. They bind tightly to exposed faces of glucan chains in the cellulose microfibrils, and they span the distance between adjacent microfibrils or they simply twin with other xyloglucans to lock the microfibrils into place (http://cellwall.genomics.purdue.edu).

The cellulose–xyloglucan framework of Type I walls is typically embedded in a matrix of pectic polysaccharides. The major pectins comprise principally homogalacturonans (HGA) and rhamnogalacturonan I and II (RG-I and RG-II). Some models suggest that these three components are covalently linked to one another to form the pectic network (Willats et al. 2001; Ridley et al. 2001). Some HGAs and RGs are cross-linked by ester linkages to pectins or other polymers held more tightly in the wall matrix. Neutral polymers composed of arabinose or galactose residues are branched to the rhamnosyl residues of RG-I (RG I backbone) in the pectic polysaccharides. Some of these side chains are further cross-linked by ester linkages to other pectic components or to non-pectic polymers through coumaroyl and feruloyl residues. In the pectic network, calcium ions serve as cross-links between the de-esterified carboxylic acid groups in the HGA and RG-I domains, whereas borate di-ester bridges cross-link the RG-II domains (Kobayashi et al. 1996; Ishii 1999; O'Neill et al. 2001). RG II has the richest diversity of sugars and linkage structures known. Some Type I walls also contain several types of structural proteins that may interact with the pectic network. The various structural proteins can form intermolecular bridges with other proteins without necessarily binding to the polysaccharide components (http://cellwall.genomics.purdue.edu).

Type II walls are found only in commelinoid monocotyledons, which include cereals such as rice (*Oryza sativa*), oats, barley and the Poales which represent families of plants such as grasses (Poaceae). Type II walls differ from type I walls primarily as they have less xyloglucan than cellulose. They contain cellulose microfibrils of the same structure as those of the Type I wall, but the predominant glycans that cross-link the cellulose microfibrils in cereals are glucuronoarabinoxylan (GAX) (Nishitani and Nevins 1991) and β1,3:β1,4 mixed glucans (Kato et al. 1982). Grasses have typical type II walls that are rich in GAX and β1,3/β1,4 glucan (Ebringerová et al. 2005). When grass cells begin to elongate, they accumulate mixed-linked β-glucans in addition to GAX. This type of β-glucan is unique to the Poales and is a rare example of a cell expansion specific. Unbranched GAXs can hydrogen bond to cellulose or to each other. The attachment of arabinose and glucuronic acid side groups to the xylan backbone of GAXs prevents the formation of hydrogen bonds, diminishing the extent of cross-linking between two unbranched GAX chains or GAX to cellulose (http://cellwall.genomics.purdue.edu).

Type II walls have small amounts of XyG, but these XyGs contain neither arabinose nor fucose. Compared with the pectin-abundant type I wall, the type II wall contains less pectin. In general, grasses are pectin-poor. With the exception of the lack of fucose, grass pectins are similar in structure to those of dicots. Grasses, which have very little structural protein compared with dicots and non-commelinoid monocots, have higher amounts of phenylpropanoids, which form extensive interconnecting networks primarily when cells stop expanding (Iiyama et al. 1990). In the non-lignified type II walls the principal hydroxycinnamate is ferulic acid. In the lignified walls both ferulic and p-coumaric acid are found. Sinapic acid, 5-hydroxyferulic acid and caffeic acid have also been reported, although these are much less abundant. Ferulic acid is esterfied to the C5 of the arabinosyl side chains of arabinoxylans (Nishitani and Nevins

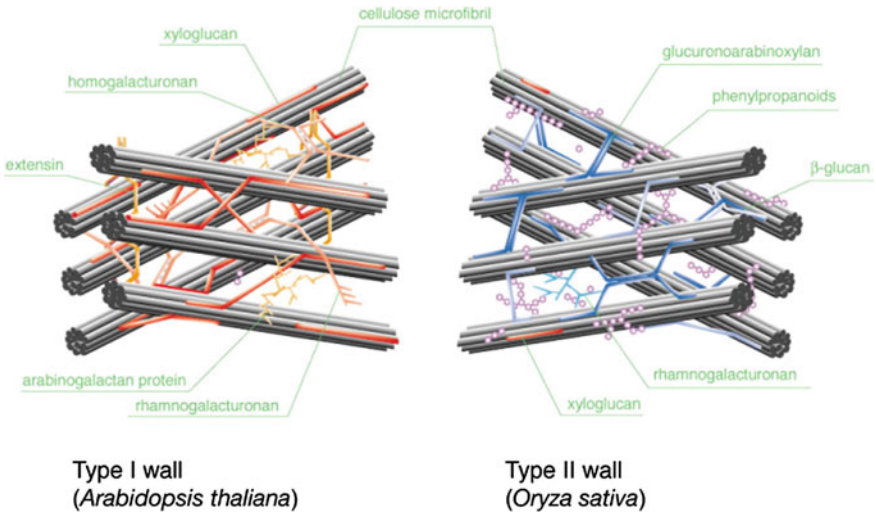

Fig. 4.3 The structural differences between type I and type II cell-wall types, as represented by *Arabidopsis* and rice. Based on the model of Carpita and McCann 2000

1989). Ferulic acid can undergo oxidative dimerisation forming arabinoxylan networks (http://cellwall.genomics.purdue.edu) (Fig. 4.3).

The organisation and interactions of primary wall components are not known with certainty and there is still considerable debate about how wall organisation is modified to allow cells to expand and grow. The covalently cross-linked model, the tether model, the diffuse layer model and the stratified layer model are among the models proposed to account for the mechanical properties of the wall (refer to http://www.ccrc.uga.edu for further details of these models). Much research is still required to provide a complete description of the primary wall at the molecular level which is difficult as evidence shows primary walls are dynamic structures whose composition and architecture changes during plant growth and development.

4.1.3 Secondary Cell Walls

The much thicker and stronger secondary wall accounts for most of the carbohydrate in biomass and is deposited once the cell has ceased to grow. It is deposited by the protoplast inside the primary wall (Hernon et al. 2010).The secondary walls surrounded cells that differentiate to form specialised functions and are particularly important in specialised cells that require great mechanical strength and structural reinforcement (e.g. wood cells) (Cosgrove 2005).

The secondary walls of woody tissue and grasses are composed predominantly of cellulose, lignin, and hemicellulose (xylan, glucuronoxylan, arabinoxylan, or glucomannan). Cellulose makes up ~43 % of the secondary wall. Therefore,

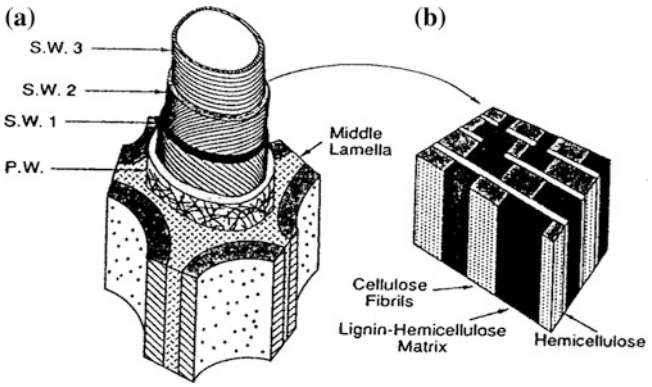

Fig. 4.4 **a** Schematic representation depicting the organisation of the cell wall layers composing woody fibres. **b** Probable relationship of lignin and hemicellulose to the cellulose microfibrils in the secondary cell walls. Primary cell wall (*P.W.*); secondary cell walls (*S.W.*1–*S.W.*3) (Béguin and Aubert 2000)

cellulose is more abundant in the secondary wall than in the primary wall which means the secondary wall is rigid and not readily stretched.

The cellulose fibrils are embedded in a network of hemicellulose and lignin. Cross-linking of this network is believed to result in the elimination of water from the wall and the formation of a hydrophobic composite that limits accessibility of hydrolytic enzymes and is a major contributor to the structural characteristics of secondary walls (http://www.ccrc.uga.edu). Xylans are one of the major hemicelluloses in secondary cell walls of dicots and all walls of grasses. Xylan, which accounts for up to 30 % of the mass of the secondary walls in grasses, contributes to the recalcitrance of these walls to enzymatic degradation.

Generally, the secondary wall consists of three sub-layers, denoted S1, S2 and S3, from outside to inside, which are distinguished by differences in the orientation of their cellulose microfibrils (Heredia et al. 1995) (Fig. 4.4).

4.1.4 A Closer Look at the Plant Cell Wall Polysaccharides

Polysaccharides are the largest carbohydrate molecules and may contain thousands of monosaccharides joined by glycosidic bonds. Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They can be assigned to three main groups: cellulose, hemicelluloses and pectins (Table 4.1).

4.1.4.1 Cellulose

The world's most abundant biopolymer cellulose or β-1-4-glucan is a linear polysaccharide polymer of cellobiose units (repeating units of glucose). Cellulose is the major constituent of cell wall polysaccharides. Individual glucan chains of

Table 4.1 Matrix components of the cell wall (Brett and Waldron 1996)

Phase	Components	
Microfibrillar	Cellulose (β 1,4-glucan)	
Matrix	Pectins	Rhamnogalacturaonan I
		Arabinan
		Galactan
		Arabinogalactan I
		Homogalacturonan
		Rhamnogalacturaonan II
	Hemicelluloses	Xylan
		Glucomannan
		Mannan
		Galactomannan
		Glucuronnmannan
		Xyloglucan
		Callose (β 1,3-glucan)
		β 1,3- β 1,4-glucan
		Arabinogalactan II
	Proteins	Extensin
		Arabinogalactan-proteins
		Others, including enzymes
	Phenolics	Lignin
		Ferulic acid
		Others, e.g. coumeric acid & truxillic acid

8,000–12,000 D-glucose residues linked by β-1,4-glycosidic bonds associate via H-bonds and align parallel to each other to form microfibrils that are largely crystalline. Each microfibril can consist of up to 250 cellulose-rich chains and is twisted in a right-handed manner at intervals along the microfibril. These fibrils are attached to each other by hemicelluloses, amorphous polymers of different sugars as well as other polymers such as pectin, and covered by lignin. The microfibrils are often associated in the form of bundles or macrofibrils (Endler and Persson 2011). These rigid cellulose structures form inert, insoluble fibres of great strength, which are characteristic of cellulose molecules present in the primary and secondary cell walls of higher plants. Each cellulose crystal contains numerous tens of polymeric chains in a parallel orientation. This special and complicated structure make cellulose resistant to both chemical and biological treatments.

The structure of cellulose is not uniform. It contains both highly crystalline and less ordered amorphous regions. During its biosynthesis, chains of varying lengths are created. Therefore, an individual chain may contribute to one or more crystalline regions, leading to the occurrence of occasional chain ends even within the highly ordered crystalline regions (Teeri 1997). The less ordered amorphous regions have a reduced level of H-bonding so they are more susceptible to hydrolysis (Cowling 1974; Gharpuray et al. 1983; Zhao et al. 2007) (Fig. 4.5).

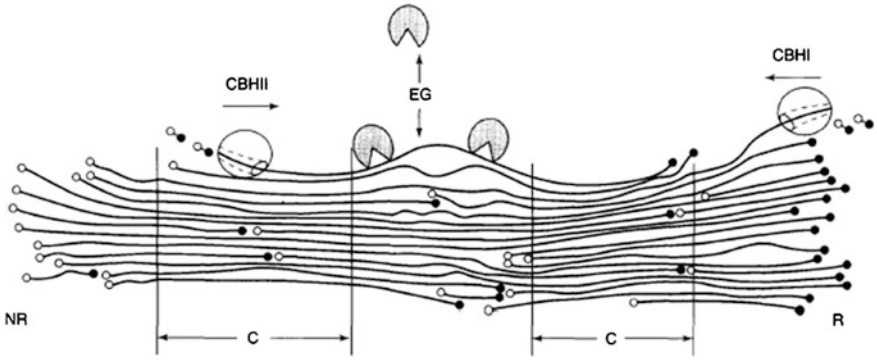

Fig. 4.5 Schematic representation of the degradation of crystalline cellulose by the cellulase degrading system of *Tr. reesei*, reducing end (*R*), non-reducing end (*NR*), highly ordered crystalline regions (*C*), cellobiohydrolase I (*CBHI*), cellobiohydrolase II (*CBHII*), endoglucanase (*EG*), (Teeri 1997)

Investigations of the structure of the maize primary cell wall, cellulose microfibril and its biosynthesis suggest sequential fibre synthesis, i.e. elementary fibril → microfibril → macrofibril. In this model, rosettes containing 36 CesA proteins (cellulose synthase complex) produce 36 β-D-glucan chains that assemble through hydrogen bonding and van der Waals forces to form an elementary fibril. This elementary fibril is thought to be a heterogenous structure containing a crystalline core and a sub-crystalline shell structure. When formed the elementary fibrils coalesce to form a microfibril, which in turn disperses at the distal ends to form parallel-arranged macrofibrils (Fig. 4.6). Polysaccharides such as hemicellulose and pectin are later deposited on the surface of the microfibril (Ding and Himmel 2006; http://www.ccrc.uga.edu).

4.1.4.2 Hemicellulose

Hemicelluloses are defined as low-molecular weight polysaccharides, soluble in alkali and are closely associated with cellulose and lignin in the plant cell wall. Hemicelluloses are aligned along the surface of the cellulose microfibrils and act as a physiological 'glue' in the plant cell wall. Hemicellulosic polymers can also form links between each other which can connect cellulose microfibrils together. In addition, they can also act as a lubricant to prevent direct microfibril–microfibril contact (Heredia et al. 1995).

Unlike cellulose, which is composed exclusively of glucose units, hemicelluloses are heteroglycans composed of a combination of pentoses (C5 sugars) and hexoses (C6 sugars), that include, D-xylose, L-arabinose, D-glucose, D-mannose, D-galactose, with smaller amounts of L-rhamnose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid and D-galacturonic acid. Individual plant species contain

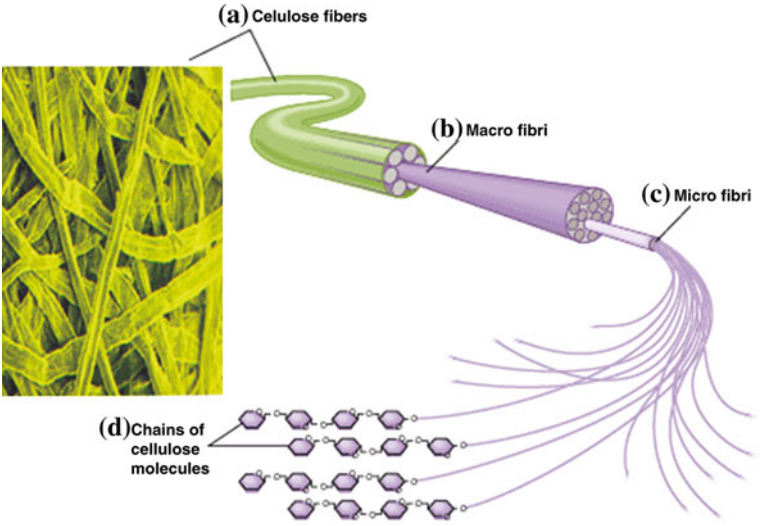

Fig. 4.6 Schematic representation of the organisation of cellulose chains within a microfibril (http://nutrition.jbpub.com)

hemicelluloses of varying composition. The dominant sugars in hemicelluloses are mannose in softwoods and xylose in hardwoods and agriculture residues including grass. As hemicelluloses are branched, these heteropolymers do not form crystalline structures or microfibrils. Hemicellulose chains are far shorter in length than cellulose and have a relatively low degree of polymerisation (Timell 1964 and Tsmousis 1991). Although hemicelluloses are not organised into crystalline arrays, their organisation in the cell wall is not random. Spectroscopy studies show their preferred orientation appears to be parallel to the cellulose microfibrils (Morikawa et al. 1978). The branched structures of hemicelluloses have little resistance to hydrolysis, and are easily hydrolysed by acids to their monomer components. Several different plant cell wall polymers are known as hemicelluloses; xyloglucan, glucuronoarabinoxylan, xylan, mixed linkage glucans, mannans and galactomannans (Hernon et al. 2010; http://www.ccrc.uga.edu).

Although xyloglucan is the major hemicellulose in most flowering plant primary walls, xylan is the principle type of hemicellulose that is found in the primary walls of cereals, softwoods and hardwoods. Xylans normally occur as heteropolysaccharides, containing different substituent groups in the side chains that are covalently attached to the β1,4-D-xylose backbone. All plant xylans contain a β-1,4-linked D-xylose backbone, which can be substituted by different side groups (Fig. 4.7). Residues commonly substituted on the xylan backbone include acetyl, arabinosyl and glucuronosyl groups (Hernon et al. 2010).

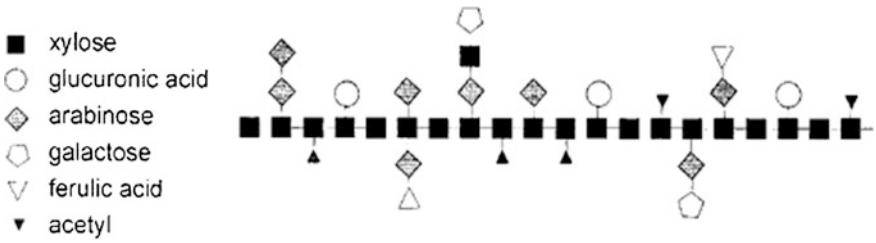

Fig. 4.7 Schematic representation of a hypothetical xylan (de Vries and Visser 2001)

4.1.4.3 Pectins

Pectin is a family of complex carbohydrates found in all plant primary walls and in the middle lamella of terrestrial plants. All higher plants except the grass family have walls of 30–35 % pectin. The walls of the grass family contain ~10 % pectin. The structures of pectins vary with species and pectins isolated from the same source may also display structural and compositional differences, depending on the location within the cell wall (Faik 2010). Pectins studied to-date have been isolated from only a small fraction of the total number of flowering plants and little is known about the pectins from other non-plant sources (Ridley et al. 2001).

Three classes of pectic polysaccharides have been characterised: homogalacturonans, rhamnogalacturonans, and substituted galacturonans (Faik 2010). Pectin polysaccharides all contain 1,4-linked α-D-galacturonic acid and homogalacturonan is the most abundant pectic polysaccharide.

The 'smooth' regions of pectins contain linear sequences of α-1,4-linked galacturonic acid units or homogalacturonan. The 'hairy' regions contain highly branched rhamnogalacturonan polysaccharides which display significant heterogeneity with respect to monosaccharide and structural composition (de Vries and Visser 2001). The term 'pectic substances' covers a range of colloidal polysaccharides that can be extracted from the cell wall, including xylogalacturonan, arabinan, galactan and type I arabinogalactan, as well as homogalacturonan and rhamnogalacturonan (Heredia et al. 1995).

Pectin forms gels in the presence of divalent cations (e.g. Ca++) or in acidic conditions in the presence of high solute concentrations (e.g. sucrose). Oligosaccharides that are released from homogalacturonan by endopolygalacturonases, induce plant defence responses and regulate plant growth and development.

Proposed functions of pectins in plants include:

- Cell wall structure/Assembly
- Cell–cell adhesion
- Cell expansion
- Cell wall porosity
- Ion, growth factors, enzyme binding
- Biomechanics: regulation of water flow
- Reservoir of biologically active oligosaccharides

- Pollen tube growth
- Seed hydration
- Leaf abscission
- Fruit development

(http://www.ccrc.uga.edu)

4.1.4.4 Starch

After cellulose and hemicellulose, starch is the most abundant carbohydrate found in nature. Grasses were first domesticated for their starch-rich seeds (grains or kernels) and the starch content has subsequently been increased by breeding. Starch accounts for 70–80 % of the dry weight of the mature maize kernel (Comparot-Moss and Denyer 2009; Dinges 2001).

Starch is a major food reserve and energy store found in the cytoplasm of plants and it is composed of α-glucose residues that are linked together by α-1,4 and/or α-1,6 glycosidic bonds (Bertoldo and Antranikian 2002). Starch is present in two main forms, amylose and amylopectin and these two forms represent 98–99 % of the dry weight of starch granules; the distribution of these forms can vary from one plant species to another. Starches are generally composed of 75–80 % amylopectin, however, the exact proportion varies from species to species. Some crops including barley and maize contain very little amylose and are almost entirely made up of amylopectin (Richardson and Gorton 2003).

4.1.4.5 Lignin

Lignin is a very complex molecule and is constructed of phenylpropane units linked in a three-dimensional structure which is particularly difficult to biodegrade. Lignin is the most recalcitrant component of the plant cell wall, and the higher the proportion of lignin, the higher the resistance to chemical and enzymatic degradation. The basic function of lignin is to cement cells together. Generally, softwoods contain more lignin (up to 30 % of the dry weight of softwoods) than hardwoods and most of the agriculture residues. It gives plants rigidity and protects them from microbial attack. White and brown-rot fungi and bacteria can degrade lignin.

Approximately, 20 % of the grass secondary cell wall is lignin which essentially fills the pores between the polysaccharides. Grass lignin shares similarities with dicot lignin in that it is primarily composed of syringyl, derived from sinapyl alcohol (∼40–61 %) and guaiacyl (∼35–49 %) units. Grass lignin also contains ∼4–15 % of r-hydroxyphenyl units of which there are only trace amounts in dicot lignin. The assembly of the monolignols appears to be similar in dicots and grasses. Grasses also differ from dicots in that grass lignin contains substantial

amounts of ferulic acid and r-coumaric acid. Ferulic acid residues attached to GAX may serve as nucleation sites for lignin formation (Vogel 2008).

Lignin is insoluble in most organic solvents as it is a highly polymerised amorphous material. The lignin content of lignocellulose is one of the drawbacks of using these feedstock materials in fermentation, as it makes lignocellulose resistant to chemical and biological degradation (Taherzadeh and Karimi 2008).

4.2 Grass

Grasses are known to be amongst the most important crops throughout the world and provide the majority of calories consumed by humans either by directly through the consumption of grains or indirectly through animals that are fed on grains and forage. Furthermore, grass is poised to become a significant source of renewable energy, because the sugars locked in the polysaccharides of the cell wall can be converted into liquid fuel (e.g. ethanol, butanol) (Vogel 2008).

The sugars in grass lignocellulose exist mostly as the polysaccharides cellulose and hemicellulose. These polysaccharides are not readily available as lignin and other aromatics covalently link with, and at times physically mask, plant carbohydrates, thus protecting these potential substrates from saccharification. Pre-treatment is required to free the carbohydrates for bioconversion to ethanol. Most often, the suggested pre-treatment is chemical (Anderson and Akin 2008).

4.3 Ethanol Production

Ethanol production from lignocellulose involves a number of processes that must occur in sequence. First, the lignocellulose is appropriately pre-treated to prepare it for enzymatic hydrolysis which releases monomeric sugars from the complex carbohydrate polymers. This sugar rich hydrolysate then becomes the substrate in a subsequent fermentation step which yields ethanol which has to be recovered by distillation and purified from the liquid fraction post fermentation. The pre-treatment is necessary to improve the rate of production and the total yield of simple sugars in the hydrolysis step (Hendriks and Zeeman 2009).

4.4 Factors Limiting Lignocellulose Hydrolysis

The hydrolysis of lignocellulose is limited by several factors. Primarily, the inherent properties of native lignocellulosic materials make them resistant to enzymatic attack. The aim of pre-treatment is to change these properties in order to prepare the materials for enzymatic degradation. Since, the composition of

lignocellulosic material is very complicated as described in earlier sections, their pre-treatment is not simple either.

The inherent properties of lignocellulose including the crystallinity of cellulose, its accessible surface area and protection by lignin and hemicellulose, degree of cellulose polymerisation and degree of acetylation of hemicelluloses are the main factors considered as affecting the rate of biological degradation of lignocelluloses by enzymes (Hendriks and Zeeman 2009; Taherzadeh and Karimi 2008).

4.5 Pre-treatments

A vast amount of pre-treatment processes for lignocellulosic residues are well documented. These pre-treatments range from mechanical or physical pre-treatments such as milling or irradiation, chemical and physiochemical pre-treatments such as alkali or acid treatments, liquid hot-water treatment, microwave-chemical treatment, steam, ammonia fibre or CO_2 explosion and solvent extraction treatments to biological treatments by microorganisms or enzymes. For more in-depth information on these pre-treatments see the review by Taherzadeh and Karimi (2008) (Fig. 4.8).

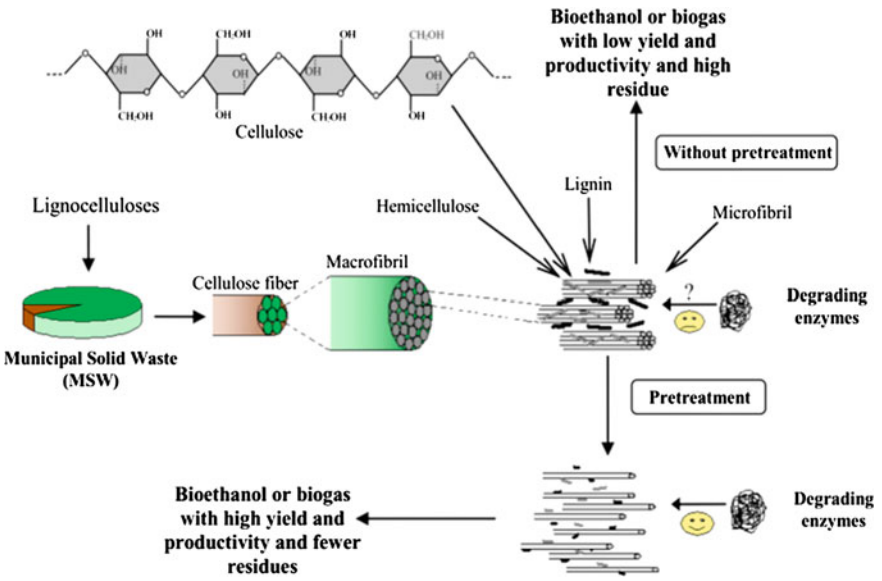

Fig. 4.8 The effect of pre-treatment on the accessibility of degrading enzymes (Taherzadeh and Karimi 2008)

4.5.1 Acid Hydrolysis Pre-treatment

Acid hydrolysis has been described as early as the nineteenth century, with commercial applications from the beginning of the twentieth century. Acid acts to breakdown hemicellulose and opens the lignocellulose structure for subsequent enzymatic attack. The two main approaches utilised in acid pre-treatment are concentrated acid with low temperature or dilute acid with high temperature. Treatment conditions that result in monomeric sugar (xylose) release without degradation to furfural, hydroxymethyl furfural and other volatiles are favoured.

Concentrated-acid with lower operating temperature pre-treatment has a clear advantage compared to dilute-acid processes. Concentrated acid (~30–70 %) processes are generally reported to give greater sugar yields resulting in higher ethanol yields. However, high acid concentration makes the process extremely corrosive, dangerous and expensive. The acid recovery, which is necessary in the concentrated-acid process for economical reasons, is an energy-demanding process and the neutralisation process also has disadvantages. Additionally, strong acid pre-treatment for ethanol production is not attractive because of the risk of producing inhibitory compounds such as furans which are inhibitory to yeast during fermentation. This degradation of monomers can also occur with the use of mild acid treatments at high temperature (Kumar and Murthy 2011).

Dilute Acid Pretreatment Dilute-acid hydrolysis is probably the most commonly applied method among the chemical pretreatment methods. The National Renewable Energy Laboratory (NREL) directs the largest biomass ethanol development effort in the world. The NREL promotes dilute acid hydrolysis, primarily because 80–90 % of hemicellulose sugars are recoverable by dilute acid pretreatment (Yang and Wyman 2008). Sulfuric acid is the most applied acid, while other acids such as HCl and nitric acid are also reported (Taherzadeh and Karimi 2008). Figure 4.9 (research done by the authors A. O'Donovan and V. K. Gupta) shows sugar yields released from dried and milled perennial ryegrass after mild acid

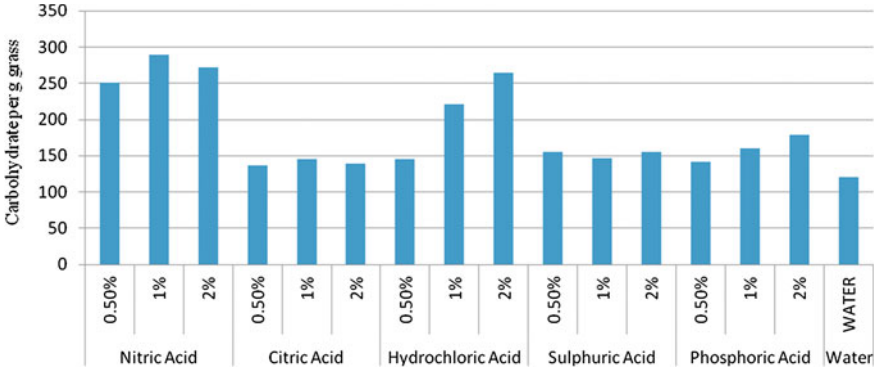

Fig. 4.9 Sugar yield in mild acid pretreatment hydrolysates of dried and milled perennial ryegrass

pre-treatment at 10 % solids loading which was performed at 121 °C for 30 min. The sugars released were determined using the principles of the DuBois method which measures the total carbohydrate present in the pre-treatment hydrolysate (DuBois et al. 1956). The results show mild pre-treatment using 1 % nitric acid was most effective in releasing sugars from the biomass. This is followed by 2 % HCL. Sulphuric, citric and phosphoric acids released similar sugar yields which were all lower than yields achieved using nitric acid and HCL. All acid pre-treatments showed higher sugar release compared to water pre-treatment alone.

During the mild acid pretreatment process biomass is treated at different acid concentrations (0.05–5 %) different combinations of temperatures (100–290 °C) and residence times (few seconds to several hours). Pre-treatments performed in short retention times (e.g. 5 min) is usually at high temperature (e.g. 180 °C), whereas pre-treatments performed in a relatively long retention time (e.g. 30–90 min) is at lower temperatures (e.g. 120 °C) (Taherzadeh and Karimi 2008). During pretreatment, most of the hemicellulose is solubilised and hydrolysed to sugar monomers. Some fraction of cellulose may be depolymerised into glucose. A quantity of the lignin fraction is dissolved and/or redistributed (Kumar and Murthy 2011).

However, the optimum conditions chosen for the highest sugar recovery after pre-treatment does not necessarily mean they are the most effective conditions for enzymatic hydrolysis. This was demonstrated in a 2007 study by C. Cara and co-workers. In this study, olive tree biomass was pre-treated using various acid conditions and subsequently hydrolysed enzymatically. The conditions of 170 °C and 1 % sulphuric acid which gave the greatest hemicellulose recovery in the pre-treatment step resulted in poor results in the enzymatic hydrolysis step. The conditions of 210 °C and 1.4 % acid gave the poorest sugar yield among all the pre-treatments performed but resulted in the maximum yield in enzymatic hydrolysis. In the same study, the researchers reported that the conditions that gave greatest sugar recovery overall were 180 °C and 1 % sulphuric acid (Cara et al. 2008). This indicates that the highest overall sugars, highest hemicellulose recovery and highest enzymatic hydrolysis yield can be achieved under different conditions (Taherzadeh and Karimi 2008).

Pre-treatment with acids such as nitric acid to remove lignin from lignocellulose is also well reported. This chapter deals with mild acid pre-treatment primarily for the removal of hemicellulose. For information in lignin removal refer to the review hydrolysis of lignocellulosic materials for ethanol production (Sun and Cheng 2002).

The major drawback of some acid pre-treatment methods is the formation of different types of inhibitors such as furans, carboxylic acids and phenolic compounds. Enzymatic hydrolysis may not be affected by these compounds, but they can exhibit inhibitory effects in microbial growth and fermentation, which results in less yield and productivity of ethanol or biogas (Taherzadeh and Karimi 2008). Therefore, pre-treatments at low pH should be selected properly in order to avoid or at least reduce the formation of these inhibitors.

4.6 Scanning Electron Microscopy

A scanning electron microscope (SEM) is an electron microscope that images a sample by scanning it with a beam of electrons in a raster scan pattern. The electrons bombard the atoms of the sample and the signal produced reflects information about the sample's surface topography, composition and other properties such as electrical conductivity.

For SEM analysis of grass biomass samples which were subjected to various mild acid pre-treatments as described earlier (a study done by the authors A. O'Donovan and V. K. Gupta), the treated grass biomass residues were oven dried at 60 °C and adhered onto a stainless steel specimen holder called a specimen stub with the aid of an adhesive carbon tab. As grass biomass is non-conductive it is coated with an ultrathin coating of electrically conducting material, deposited on the sample either by low-vacuum sputter coating or by high-vacuum evaporation. Non-conductive specimens tend to charge when scanned by the electron beam, and especially in secondary electron imaging mode, this causes scanning faults and other image artefacts. In the below images, the grass biomass was gold coated using a gold EM Scope SC500 Au coater but materials such as gold/palladium alloy, platinum, osmium, iridium, tungsten, chromium and graphite can also be used. The biomass must be electrically conductive, at least at the surface, and electrically grounded to prevent the accumulation of electrostatic charge at the surface. For additional information on SEM analysis refer to a review available on the Internet at http://serc.carleton.edu/research_education/geochemsheets/techniques/SEM.html. This online review also refers to the literature that further explores SEM.

SEM analysis is a useful tool to examine the effects of pre-treatments and enzymatic hydrolysis on the structure of the plant cell wall and has been used by several researchers for this purpose (Gomez et al. 2008; Jieben et al. 2011).

The gold coated biomass samples were analysed using a Hitachi S-570 SEM and suitably magnified images were recorded. Dilute acid pretreatment may affect biomass structure by solubilising or altering hemicelluloses, altering lignin structure and increasing the available surface area and pore volume of the substrate. The effects of various mild acid pretreatments are shown in images A to N, Fig. 4.10.

Images A and B show untreated grass. It is clear that the cells are well structured the fibres that make up the structure of the grass are connected very tightly. After treatment with just water (hot liquid pretreatment), the cells are generally still well structured and the fibres are still tightly connected (images C & D). After acid hydrolysis, the SEM images show the grass biomass has been affected by all acid pretreatments. In images E, F, G, H, K and L, the cells seem less structured and organised and the fibres seem less tightly connected. However, treatment with nitric acid seems to have had a very destructive effect on the grass biomass. This would correlate with the results of Fig. 4.9 which shows greatest sugar release was achieved by treatment with nitric acid. Image I shows how the grass fibres have completely come apart and in image J areas of the structure have become weakened, with pores starting to appear. Treatment with sulphuric acid also seems to

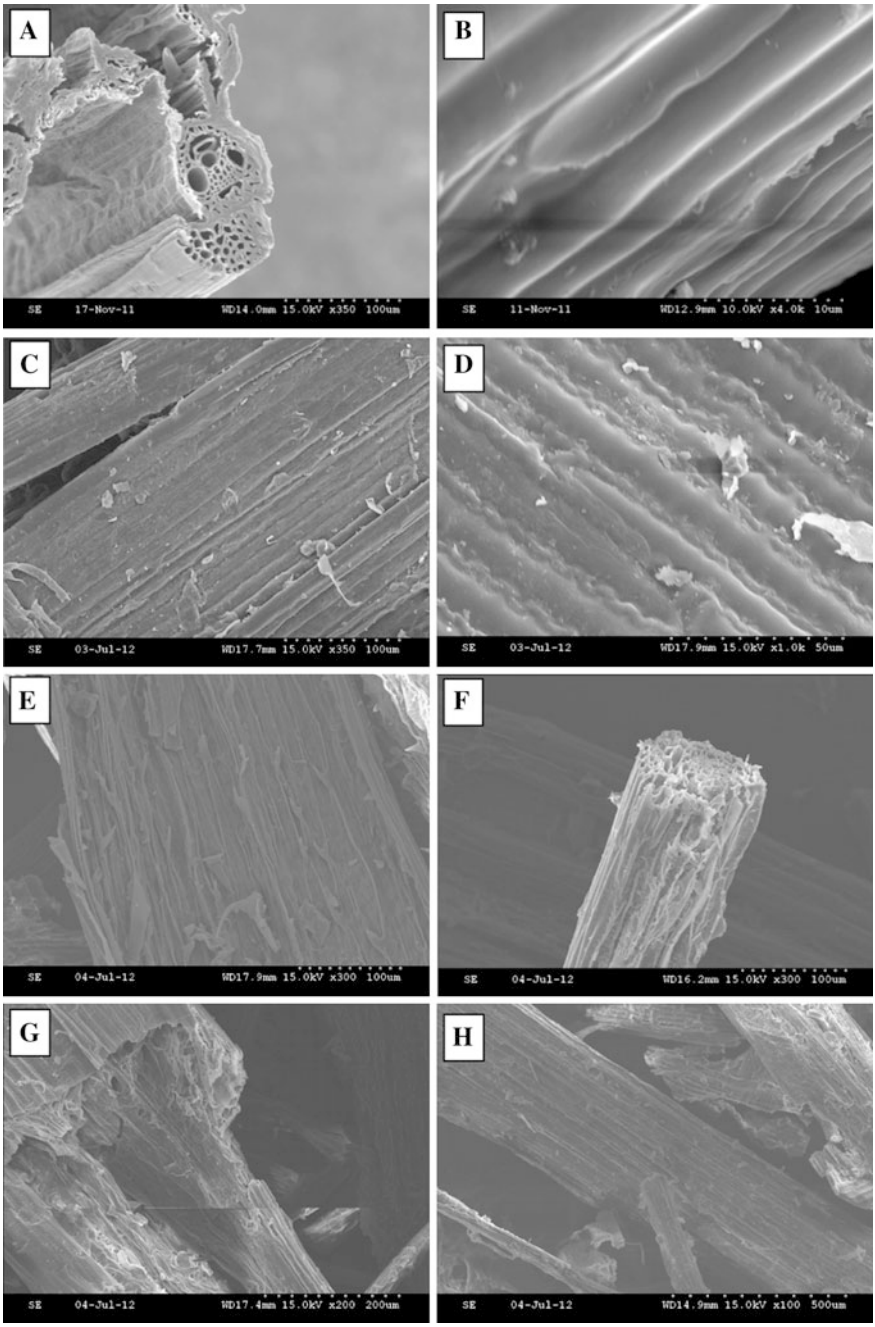

Fig. 4.10 Continued

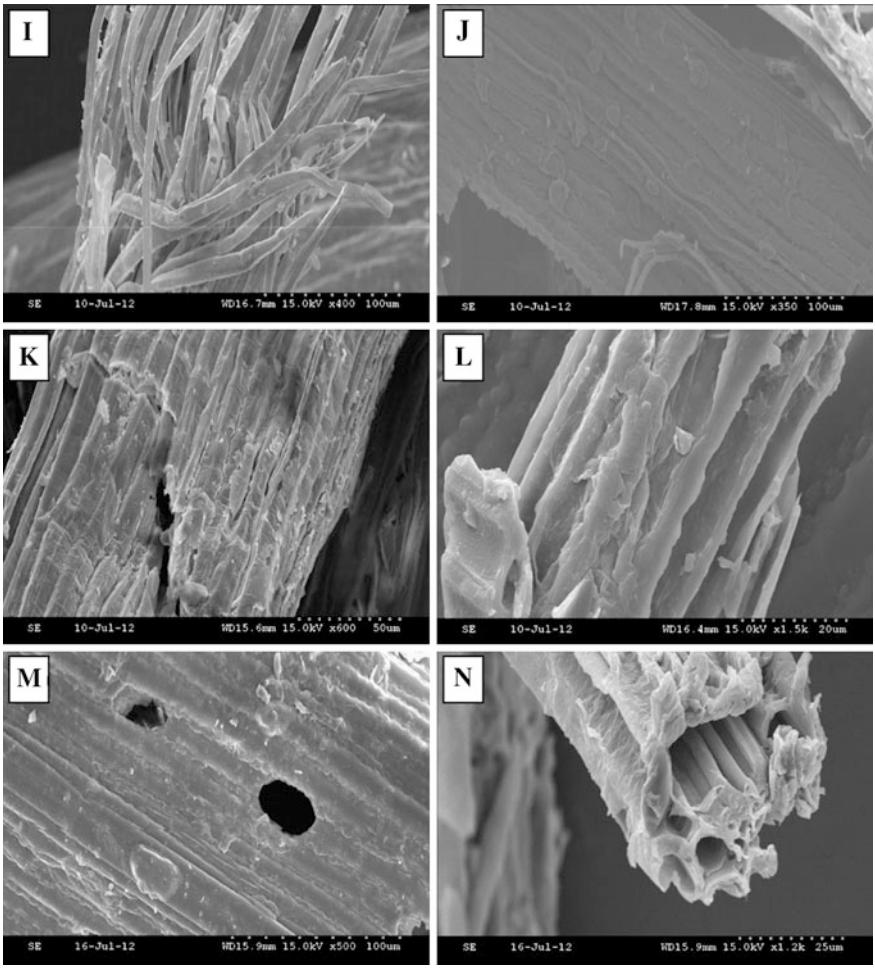

Fig. 4.10 The effects of various mild acid pretreatments are shown in images (**A**–**N**). The grass biomass was pretreated with a range of acids of different concentrations (0.5 and 2 %) at 10 % solids loading. The treatment conditions were 121 °C for 30 min. The biomass residue was separated from the pretreatment hydrolysate, oven dried and gold plated before SEM analysis

have been a very effective pretreatment as it is clear some cell tissues have been destroyed and very definite pores have appeared in the grass structure (images M and N). The destruction of the grass structure shown in these images may be attributed to the preferential degradation of the labile components such as hemicelluloses and acid soluble lignin.

Pre-treating grass biomass with dilute acid is a favorable process as it helps remove the hemicelluloses fraction and disrupt the grass structure which allows greater accessibility for the cellulase enzymes. It may also help lead to less hemicelluloses and lignin content in the cellulose preparation for the acid hydrolysed perennial rye grass.

4.7 Conclusion

The effect of pretreatments is very dependent on the biomass composition. This chapter focused on reviewing in detail the composition of the grass plant cell wall. Mild acid pre-treatments were reviewed and several mild acid pretreatment conditions were tested. The resulting acid hydrolysate sugar yields were noted and the pretreated biomass residues were subjected to SEM analysis to take a closer look at the effects of acid pretreatments, specifically on the plant cell wall. These pretreatment processes should make the lignocellulosic biomass more susceptible to enzymatic attack, where crystallinity of cellulose, its accessible surface area and protection by lignin and hemicellulose are the main factors in order to obtain an efficient hydrolysis.

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Part III
Conversion Technologies

Chapter 5
The Role of Fungal Enzymes in Global Biofuel Production Technologies

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Abstract The environmental impact of fossil fuels alongside the competition of agricultural land and water for the production of food versus that of first generation biofuels has led to great interest in improving second and third generation biofuel production. Lignocellulosic materials are the essential feedstock for second generation biofuels and vary according to the residing country and regions. At present, the biorefinery systems established to degrade the various lignocellulose feedstocks are expensive and inefficient. The stages in the biorefinery process include pre-treatment of the feedstock, acid or enzymatic hydrolysis followed by fermentation and possess various optimum temperatures and pH. Interest has turned to the role of fungi and various extracellular enzymes involved in the enzymatic hydrolysis of the lignocellulosic components, namely hemicellulose, cellulose and lignin. This review chapter discusses the leading enzymes involved in the production of biofuels, how they penetrate barriers within the biorefinery systems and their potential in the development of new production strategies.

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5.1 Introduction

Global concerns about the depletion of fossil fuel reserves, their detrimental effects on the environment through greenhouse gas (GHG) emissions, and the increase in crude oil prices have shifted the environmental and scientific focus onto the use of other forms of renewable energy. Over the past number of decades, multiple research groups have been extensively studying the potential of, and the processes involved in using, agricultural crops and biomass as feedstock for fuel and energy. Through these research clusters, biofuel and bioenergy production from various renewable resources, alongside the use of specific innovative technologies, has emerged as a solution to reduce detrimental environmental impacts.

The World Energy Council (WEC), in 2009, stated that the world primary energy consumption was calculated to be approximately 12 billion tonnes coal equivalent per year. According to the United Nations (UN), the world population is expected to increase to approximately 10 billion people by 2,050 which, in turn, will increase our consumption of energy to approximately 24 billion tonnes coal equivalent per year (Dashtban et al. 2009). These statistics give a clear indication of the growing need to convert to more sustainable forms of renewable energy in order to achieve these high-energy demands and to provide more 'greener' technologies to reduce the unwanted effects on our environment. Biorefinery systems have been implemented to convert renewable materials, for example wood or agricultural crops, into valuable products such as biofuels, feedstock chemicals and pharma compounds. The bioconversion process involved differs due to the feedstock used and products required by industry. With this concept in mind, different biorefining strategies have been implemented globally through the production of biofuels (and other valuable end products) and increasing its use annually compared to petrol and diesel.

In 2010, the WEC published a report on biofuels stating that the global biofuel production in 2007 had surpassed 34 million tonnes of oil equivalent (Mtoe) but only accounted for 1.5 % of the total consumption of transportation fuel (World Energy Council 2010). This percentage of biofuel consumption had increased to 2 % by 2010 and is expected to account for 5 % of the total transportation fuel consumption by 2030 (World Energy Council 2010). Some countries have been able to excel within the biorefinery industry while others struggle to produce the required volume of biofuels necessary to reach global standards. A report published by Taylor (2008) has indicated that 80 % of the 2.5 % of biofuel for transportation required in the UK were imported; the USA and Brazil were the main contributors, with biofuel produced from corn and sugarcane, respectively.

Taylor (2008) also commented on the Gallagher Review, published by the Renewable Energy Agency in 2008, which stressed that the harnessing of bioenergy and biofuel from agricultural crops was leading to the displacement of food crops and to a higher overall GHG emission than previously expected. The review establishes that biological resources other than agricultural crops need to become the prime feedstock for biofuels in order to combat the rising prices of food due to

competitive water and agricultural land use (Taylor 2008). Other biofuel resources are prevalent in the UK which do not compete for food, such as wheat straw where there is approximately 200,000 tonnes of wheat straw available per year (Taylor 2008).

5.2 Lignocellulose and the Plant Cell Wall

Lignocellulose is a renewable raw material used throughout the agricultural, forestry and food industries and is the structural component of all plants. This renewable material is composed of three main polymers: cellulose, hemicellulose and lignin (Dashtban et al. 2009; Martínez et al. 2005).

5.2.1 Cellulose

Cellulose is a linear, β-1, 4-linked polymer of D-glucose units and is the major component of lignocellulosic materials (Howard et al. 2003; Turner et al. 2007). The smallest repeating unit of cellulose is cellobiose, comprised of two β-1, 4-linked D-glucose units. The hydroxyl groups present in each cellobiose molecule allow for inter- and intra-chain hydrogen bonding within the cellulose polymer and between neighbouring polymers. This high degree of hydrogen bonding results in the formation of highly ordered crystalline structures, which in turn form ordered microfibril structures (Dashtban et al. 2009; Howard et al. 2003). The hydroxyl groups used within these hydrogen bonds determine the overall crystalline structure of the cellulose, and also gives the recalcitrant nature of cellulose towards hydrolysis (Harris and DeBolt 2010). There are six polymorphs of cellulose known to date: cellulose I, II, III_I, III_{II}, IV_I and IV_{II} (Festucci-Buselli et al. 2007). Cellulose I and II are the crystalline structures found in nature, whereas the other polymorphs are artificially created through chemical or heat treatments (Festucci-Buselli et al. 2007).

Cellulose I is the most abundant crystalline structure found in nature which is synthesised into two allomorphs: cellulose Iα and Iβ (Festucci-Buselli et al. 2007; Harris and DeBolt 2010). There are slight differences between these two allomorphs, the main difference resulting from the intra-chain hydrogen bonding between the OH group at position 2 and the OH group at position 6. The length of this bond distinguishes between the two crystalline forms; Iα has a shorter bond than Iβ (Festucci-Buselli et al. 2007).

Higher plants are able to synthesise both cellulose I allomorphs and as a result the cellulose present in plant cells has both crystalline and amorphous regions (Harris and DeBolt 2010). It has been suggested that cellulose Iα is the least stable of the two, with Iβ forming the core of the microfibril and a mixture of the two allomorphs surrounding this core creating amorphous regions (Festucci-Buselli

et al. 2007; Harris and DeBolt 2010). These amorphous regions are thought to allow associations between cellulose microfibrils, lignin and hemicellulose molecules, creating the structural framework of the plant cell wall (Harris and DeBolt 2010). This complex network system plays a part in the recalcitrance of the lignocellulosic material and causes difficulties in the hydrolysis of cellulose microfibrils into cellobiose and glucose molecules during lignocellulose bioconversion.

5.2.2 Hemicellulose

Hemicellulose is the second most abundant polymer found in lignocellulose. This polymer is a heterogeneous polysaccharide consisting of pentoses (D-xylose and D-arabinose), hexoses (D-mannose, D-glucose and D-galactose) and sugar acids (Chandel et al. 2011a; Dashtban et al. 2009; Saha 2003). They consist of both a linear β-1, 4-linked backbone polymer and branched polymers (Chandel et al. 2011a). Due to the variability in their sugar residues and their heterogeneity, the composition of hemicellulose is dependent upon the plant tissue and source; for example, hardwood hemicelluloses are mostly xylans, whereas softwood hemicelluloses are mostly glucomannans (Saha 2003).

Xylans are regarded as the most common hemicellulose within lignocellulosic materials. Other hemicelluloses include mannans, galactans and arabinans, which are found throughout the plant cell wall in varying percentages. Xylans contain a linear β-1, 4-D-xylopyranosyl backbone and branched polymers with residues such as L-arabinose, D-galactose or D-glucuronic acid attached in small quantities (Saha 2003). The polymers, as with all hemicelluloses, are found wrapped around each cellulose microfibril and can also be intertwined within the microfibrils during the crystallisation process (Arantes and Saddler 2011; Hu et al. 2011).

Xyloglucans, which are prevalent in the primary cell wall (PCW), are involved in the cross-linking of hemicellulose polymers to cellulose microfibrils indicating a structural role within the plant (Burton et al. 2010; Caffall and Mohnen 2009). This particular type of polymer possesses a β-1, 4-glucan backbone with heavily substitutions of xylose residues (Burton 2010). Mannans bear a similar structure to cellulose (Caffall and Mohnen 2009). Galactomannans are among the most studied plant cell wall polymers. They possess β-1, 4-mannan backbones with varying degrees of α-galactose substitutions and function as storage polysaccharides (Burton et al. 2010; Cosgrove 2005).

The backbone sugar units can be subjected to acetylation, methylation and the addition of phenolic groups (Burton et al. 2010; Turner et al. 2007). The degree of modification is also dependent upon the plant tissue and source. For example, the degree of acetylation of hardwood xylans is much higher than that of softwood xylans (Bastawde 1992). Deacetylation of xylan was originally carried out through alkali treatment of the lignocellulose but now can be carried out enzymatically through the addition of acetylxylan esterase (Bastawde 1992; Turner et al. 2007). The removal of acetyl groups allows for increased degradation of xylans to xylose

sugars during the pre-treatment of lignocellulose. These xylose monomers can be processed further to give xylitol, a pentose sugar alcohol that is a natural sweetener, which can be substituted for sugar in the diabetic diet and has the ability to reduce dental caries (Akpinar et al. 2011). Xylitol is just one example of the many valuable by-products that can be produced during the bioconversion of lignocellulose.

5.2.3 Pectins

Lignocellulosic materials contain varied quantities of other materials such as ash, proteins and pectins, depending on its source. Pectins are classed as the third major structural polysaccharide of the plant cell wall alongside cellulose and hemicellulose (Dashtban et al. 2009; Turner et al. 2007). They make up a large component of the PCW and can be used as an alternative storage macromolecules to starch in, for example, lupin seeds (Burton et al. 2010). This important polysaccharide also functions in determining the porosity and thickness of the cell wall and is regarded as a source of signalling molecules (Burton et al. 2010; Cosgrove 2005).

Pectins include homogalacturonan, xylogalacturonan and rhamnogalacturonan I and II (Cosgrove 2005; Harris and DeBolt 2010; Ridley et al. 2001). The backbone of the former polysaccharides is mainly comprised of α-1, 4-linked D-galacturonoic acid residues and, in the case of rhamnogalacturonans, L-rhamnose residues (Ridley et al. 2001). The polymers tend to have neutral sugar side chains such as L-rhamnose, arabinose, galactose and xylose attached to the backbone, and are often methylated and acetylated (Burton et al. 2010). In the case of xylogalacturonan pectins, the polymer contains branched xylose residues/chains (Cosgrove 2005).

Pectins have been found to be extremely beneficial in various industries. The polysaccharide has been used in the textile industry for many years and is used in the food industry primarily as a thickener, emulsifier and stabiliser (Turner et al. 2007). It has now come to light that pectins have a potential in drug delivery and may have beneficial cholesterol-lowering effects in the diet as well as increasing the yield of fermentable sugars for biorefinery systems such as biofuel production (Turner et al. 2007).

5.2.4 Lignin

Lignin, the third most abundant component of lignocellulose, has a structural role within plants and facilitates the vertical growth of land plants. This polymer also acts as a physical barrier against microorganisms and aids the transport of water through the xylem (Ferrer et al. 2008). It is normally found as a heterogeneous polymer composed of dimethoxylated, monomethoxylated and

non-methoxylated phenylpropanoid units, also known as syringyl (S), guaiacyl (G) and p-hydroxyphenyl (H) phenylpropanoid units, respectively (Campbell and Sederoff 1996; Dashtban et al. 2009; Martínez et al. 2005).

Due to the various intermolecular linkages that can be formed, the composition of lignin varies depending on the plant type. For example, angiosperms (hardwoods) are primarily composed of S and G units, whereas gymnosperms (softwoods) are mainly composed of G units (Martínez et al. 2005). In poplar, a chain of linear lignin is estimated to be between 13 and 20 units in length on average (Vanholme et al. 2010).

The phenylpropanoid units are derived from their corresponding aromatic alcohol precursors: sinapyl, coniferyl and p-coumaryl alcohol producing phenolic radicals through oxidation of the alcohols, which are coupled together oxidatively through ether and ester linkages (Ferrer et al. 2008; Harris and DeBolt 2010; Vanholme et al. 2010). The lignin polymers are extended through endwise coupling, consuming the phenolic radicals (Vanholme et al. 2010). The coupling of the phenylpropanoid units generally occurs at the β position creating β-O-4 (also known as β-aryl ether), β-5, β-β and β-1 linkages. Other linkages such as 5-5, 5-O-4 can also be created during lignin polymerisation (Harris and DeBolt 2010; Vanholme et al. 2010).

It has been noted that the β-aryl ether linkage is easily hydrolysed, whereas the other linkages are more recalcitrant (Harris and DeBolt 2010). This gives a varied degree of recalcitrance among the different types of lignocellulosic materials. For example, softwood lignin's are mainly composed of G units which contain the more recalcitrant β-5, 5-5 and 5-O-4 linkages, whereas angiosperms such as grasses (monocots) contain a mixture of G and S units which result in a mixture of β-aryl ether and other linkages therefore making the angiosperms slightly more acceptable to hydrolysis (Campbell and Sederoff 1996; Harris and DeBolt 2010; Isroi et al. 2011).

5.2.5 Plant Cell Wall

It has been documented that a plant consists of approximately 35 different cell types, with each cell being unique in its properties and characteristics (Cosgrove 2005). The plant cell wall is generally composed of three layers; the middle lamella, the PCW and the secondary cell wall (SCW), all of which consist of two phases; the microfibrillar phase and the matrix phase (Festucci-Buselli et al. 2007). The middle lamellae and PCWs are formed early during the growth and expansion of the cells while the SCWs are formed around most cells as the plant matures and ceases to grow further (Caffall and Mohnen 2009; Harris and DeBolt 2010).

There are two types of PCWs within angiosperms that are described in the literature; type I and type II (Harris and DeBolt 2010). Angiosperms are classified into two groups; monocots and dicots, distinctive of their number of seed leaves (cotyledons). Type I cell walls are normally found in dicots and lilioid monocots,

whereas type II cell walls are only seen in Poales, a type of commelinid monocot, and other related commelinid monocots (Harris and DeBolt 2010). The two PCW types can be distinguished by their differences in hemicellulose composition (Harris and DeBolt 2010). In type I walls, Xyloglucans are the main hemicellulose present in a pectinaceous matrix whilst type II walls largely consist of glucuronoarabinoxylans (GAXs) with little or no pectin present in the cell matrix (Harris and DeBolt 2010). The networking of these hemicellulose polymers within the plant cell wall leads to a reduced accessibility of the cellulose to hydrolytic enzymes and causing difficulties in the saccharification of all lignocellulose residues.

SCWs are primarily composed of celluloses, lignin's and hemicelluloses such as glucuronoxylans, in dicots and GAXs, in Poales (grasses), and are subdivided into three sub-layers; S1, S2 and S3 (Festucci-Buselli et al. 2007; Harris and DeBolt 2010). The SCW is not seen in every cell but aids in thickening the cell walls and providing additional structural support (Caffall and Mohnen 2009; Cosgrove 2005). The primary and SCWs differ in their composition of lignin, cellulose, hemicellulose and pectin. In poplar, the lignin content is between 19 and 21 % in the SCW but is absent in the PCW and the cellulose content is between 20 and 30 % in the PCW compared to 40–50 % in the SCW (Festucci-Buselli et al. 2007). Hemicelluloses are found in a much higher content within the SCWs than in the PCWs (Caffall and Mohnen 2009). The molecular structure and composition of the plant cell wall also differs depending on the lignocellulosic material in question. As a result, this can lead to variations in the percentage yields of fermentable sugars from the different lignocellulose sources (Arantes and Saddler 2011).

5.3 Lignocellulose and Biofuels

Biofuel produced from agricultural crops, e.g. cereals, maize, sugarcane, sugar beet and sweet sorghum, is referred to as first generation biofuels while that produced from lignocellulosic materials is referred to as second generation biofuels (Yuan et al. 2008). Biofuels can also be produced from microalgae which is termed third generation biofuels (Brennan and Owende 2010). Third generation biofuels are not discussed within this review as fungal enzymes are not required for its production.

The global production of first and second generation biofuels is dependent upon the lignocellulosic materials available for bioconversion for each individual country. In the United States, bioethanol is derived from starch-based feedstock such as corn, whereas in Brazil it is derived from bagasse, a by-product of the sugar cane industry (Dashtban et al. 2009; Howard et al. 2003; Simmons et al. 2008). These two countries alone produced approximately 90 % of the world's bioethanol fuel in 2008 according to Dashtban et al. (2009). The Iogen Corporation in Canada is also renowned for its bioethanol production with lignocellulose as feedstock. This company produces 0.52 million gallons of bioethanol per year

through the bioconversion of approximately 30 tonnes of wheat, oat and barley straw per day (Dashtban et al. 2009).

Over the past number of years, research into the production of first generation compared to second generation biofuels has indicated that the overall carbon dioxide emissions are greater from first generation biofuels and are reported to exceed the levels emitted by fossil fuel consumption (Yuan et al. 2008). This, along with the increases in food prices, has shifted the research focus onto second generation biofuels produced from various lignocellulosic materials and wastes such as bagasse, grasses and spent mushroom compost.

5.4 Biorefining

The bioconversion of lignocellulose is carried out in four major steps; pre-treatment, hydrolysis, fermentation and separation. The pre-treatment of lignocellulose materials is considered a key step in biorefining as it quickens the hydrolysis procedure, through enhancing the cellulose accessibility and increasing pore size, which, in theory, leads to higher sugar yields for fermentation. This is done through the removal of lignin and hemicellulose polymers through various treatment methods, which can be defined as chemical, physical or biological (Dashtban et al. 2009; Howard et al. 2003; Ong 2004).

5.4.1 Pretreatment

Physical pre-treatment methods include such as milling, irradiation and steam explosion. The latter consists of the lignocellulose being steamed at high pressure followed by either a rapid or slow reduction in pressure to dissolve the hemicelluloses into solution and allow the cellulose and lignin to remain as solids (Dashtban et al. 2009; Ong 2004). SO_2 or CO_2 can be used as catalysts although SO_2 is found to be highly toxic (Ong 2004).

Chemical pre-treatment methods include ammonium fibre explosion (AFEX), organosolv treatment and the addition of either acid or alkali (Dashtban et al. 2009; Isroi et al. 2011; Ong 2004). The use of acid as a catalyst, normally H_2SO_4, targets the hemicellulose to dissolve with lignin and cellulose remaining as solids, whereas the addition of alkali, normally NaOH, mainly targets lignin, leaving mainly cellulose as a solid with hemicelluloses (Dashtban et al. 2009; Ong 2004).

Although physical and chemical pre-treatment can effectively reduce the recalcitrance of lignocellulosic compounds within a shorter timeframe, they possess many environmental and cost concerns for industries. They require high-energy input alongside high pressure reactors and can produce toxic compounds and wastewater (Isroi et al. 2011).

Biological pre-treatment methods include the use of microorganisms in order to delignify the lignocellulose material (Dashtban et al. 2009). The enzymes produced by the microorganisms selectively disrupt the fibril and lignin structures of the plant cell wall and provide the advantages of lower energy demands, minimal waste production and reduced effects on the environment (Dashtban et al. 2009; Isroi et al. 2011).

The method chosen for pre-treatment is dependent upon the lignocellulosic material and the hydrolysis to be carried out afterwards. If the hydrolysis step is accompanied by fungal enzymes, which are optimised at a lower pH (approx. 4–5), the acidic pre-treatment is preferred as the first step in the bioconversion process (Dashtban et al. 2009).

5.4.2 Hydrolysis

Hydrolysis is the process in which the lignocellulose polymers are saccharified to yield fermentable sugars (hexoses and pentoses) (Harris and DeBolt 2010). There are two methods of hydrolysis used within the biorefining process; acid hydrolysis and enzymatic hydrolysis (Dashtban et al. 2009; Ong 2004).

Acid hydrolysis is the older method of the two and has been implemented on an industrial scale since World War I (Ong 2004). In this particular process, dilute or concentrated acid, normally H_2SO_4 as it is cheapest, is used to hydrolyse the cellulose with the reaction temperatures depending upon the molarity; dilute acids require high temperatures (above 200 °C) while concentrated acids require lower temperatures (Ong 2004). The acid hydrolysis approaches are less attractive due to the low yields with dilute acid and the recovery and environmental factors involved with concentrated acids (Ong 2004; Hernon et al. 2010).

In enzymatic hydrolysis, the lignocellulose is broken down into the corresponding monomeric sugars by specific enzymes produced from bacteria or fungi (Dashtban et al. 2009; Ong 2004). This approach is more complex, expensive and time consuming, as of this minute, in comparison to the acid hydrolysis approach but has the advantage of little or no by-products to dispose of at the end of biorefining process (Ong 2004).

5.4.3 Fermentation

Fermentation, the third step of bioconversion, converts the hydrolysates, mainly glucose, xylose, arabinose and mannose to bioethanol using microorganisms (Dashtban et al. 2009; Ong 2004). The hydrolysates are often detoxified before fermentation due to the production of inhibitory compounds, such as phenolic and furan derivatives, in the pre-treatment and hydrolysis steps (Dashtban et al. 2009; Ong 2004). *Saccharomyces cerevisiae* is the most commonly used microorganism

as it has a high fermentation rate and recombination techniques have created strains, for example TMB 3,400, capable of converting arabinose and xylose, as well as glucose, to bioethanol (Dashtban et al. 2009). This allows for the utilisation of a larger amount of the hydrolysates giving a higher percentage yield of bioethanol.

5.4.4 Combinations

Different combinatorial methods of the first three bioconversion steps have been under investigation in order to reduce production costs, increase end-product yield and to quicken the biorefinery process. Separate hydrolysis and fermentation (SHF) provides the opportunity of optimising each process separately, although it can result in the use of large amounts of enzymes such as β-glucosidase to overcome end-product inhibition during the hydrolysis making this a costly process (Dashtban et al. 2009). Simultaneous saccharification and fermentation (SSF) combines both steps into one reaction allowing for the direct fermentation of hydrolysates into bioethanol to reduce the enzyme costs involved but also compromises the conditions of both reactions and end-product yields (Dashtban et al. 2009; Ong 2004). Another method termed consolidated bioprocessing (CBP) can be used to combine all three steps into one with the use of one or many microorganisms (Dashtban et al. 2009). This particular process possesses the potential of lowering the bioethanol production costs to competitive fuel levels with more research into the microorganisms, enzymes and pH and temperature optima required.

5.5 Fungi in Biorefining

Microorganisms, especially fungi, have been established as important mediators in the production of biofuels and other valuable biorefinery products. In the literature, it is prevalent that fungi have been selected for industrial hydrolysis and pretreatment measures. This selection is based upon their ability to selectively or simultaneously degrade lignocellulosic materials, the high redox potential of their enzymes, their engineering capabilities and/or their thermostability.

Fungi are known to degrade lignocellulosic materials in nature; in soil, compost and forest litter. The method of degradation is dependent upon the genus of the fungus and has various end results. Basidiomycota and ascomycota are the main phyla which house these important lignocellulolytic fungi; basidiomycetes and ascomycetes, respectively. Ascomycota is the largest phylum distinguishable by the presence of asci within the fruiting body of the fungus while the basidiomycetes are characterised by the formation of basidia, a particular type of sporophore (Guarro et al. 1999). They are mainly responsible for the decay of wood and forest litter through the degradation and modification of lignocellulose (Martínez et al. 2005).

White-rot basidiomycetes are widely known as lignin degrading fungi but are not the only fungal type to have an impact on lignocellulose. Brown-rot basidiomycetes as well as some ascomycetous fungi, as previously mentioned, are capable of degrading the components of lignocellulose. The key to their degradative capabilities is the extracellular enzymes they secrete. White-rot basidiomycetes are capable of producing an array of enzymes including those with the potential to degrade all components of the lignocellulosic plant cell wall while brown-rots and ascomycetes produce a selection of these fungal enzymes.

There are key enzymatic systems produced by different fungi which attack each lignocellulose component separately. Lignin-modifying enzymes (LMEs) are capable of disrupting the recalcitrant lignin components, cellulase systems act on the cellulose microfibrils attempting to reduce the polymers into single glucose units, with the more diverse group of hemicelluloses cleaving the different backbone polymers and side chains of hemicelluloses.

5.5.1 Fungal Enzymes

The LMEs are a group of lignin degrading enzymes comprising laccase, lignin peroxidase, manganese peroxidase and versatile peroxidase but are not the only enzymes likely to be involved in lignin biodegradation. Phenol oxidase enzymes include the LMEs as well as tyrosinase, catechol oxidase and catalase-phenol oxidase which are all capable of oxidising various phenolic compounds (Sutay Kocabas et al. 2008). Cellulase systems are comprised of endo-1, 4-β-glucanase, cellobiohydrolase and β-glucosidases (Gao et al. 2012). Hemicelluloses include endo-1, 4-β-xylanases, β-xylosidases, endo-1, 4-β-mannanase and β-mannosidases. The upregulation of a particular group of hemicelluloses is dependent upon the nature of the lignocellulosic material. There are also accessory enzymes which aid the hemicelluloses in their degradation of hemicellulose polymers through the cleavage of side chain residues; these enzymes include α-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase, ferulic acid esterase and β-galactosidase (EC 3.2.1.23) (Saha 2003; Turner et al. 2007).

5.5.1.1 Laccase

Laccases (EC 1.10.3.2) are mainly extracellular glycoproteins belonging to the multi-copper oxidase (MCO) superfamily and are secreted by most white-rot basidiomycetes and some ascomycetes (Lundell et al. 2010). A number of brown-rot basidiomycetes are also capable of producing laccases in liquid cultures (Martínez et al. 2005). MCOs possess diverse roles and are reported to produce laccase activity in fungi, plants, bacteria and some insects (Kunamneni et al. 2008; Lundell et al. 2010).

The first molecular structures of fungal laccases produced by the basidiomycete Trametes versicolor and the ascomycete Melanocarpus albomyces were published in 2002 by Piontek et al. (2002) and Hakulinen et al. (2002), respectively. The typical fungal laccase has a molecular mass ranging from 60 to 80 kDa and an isoelectric point (pI) between 3 and 6 (Bonnen et al. 1994; Isroi et al. 2011; Lundell et al. 2010; Widiastuti 2008). These important oxidoreductase enzymes are comprised of three domains (D1, D2, and D3), to which four copper atoms are typically bound (Baldrian 2006; Martínez et al. 2005). Laccases are described in the literature as "blue", "yellow" or "white" according to the number of copper ions present within their active site (Baldrian 2006; Lundell et al. 2010; Martínez et al. 2005). The "blue" laccases are typically described as 'true' laccases due to the presence of all four copper atoms which gives rise to a blue colouring (Baldrian 2006; Martínez et al. 2005). "Yellow" laccases are classed as those which do not possess a Type I copper atom while the "white" laccases are those which only possess one copper atom (Baldrian 2006). These 'non-true' laccases have been reported to contain different metal ions in place of the four copper ions; normally Zn^{2+}, Fe^{2+} and Mn^{2+} (Lundell et al. 2010). POXA1, from Pleurotus ostreatus, is one example of a "white" laccase which contains one copper ion along with two zinc ions and one iron ion (Baldrian 2006).

Multiple genes encoding the laccase glycoproteins have been reported within fungi, giving rise to the secretion of several isozymes (Isroi et al. 2011; Lundell et al. 2010). This has been reported for most white rots and also for the non-lignin degrading basidiomycetes Coprinopsis cinerea and Laccaria bicolour (Lundell et al. 2010). A study carried out by D'Souza et al. (1999) has reported that the white-rot Ganoderma lucidium secretes at least five isozymes of the enzyme. There are exceptions to this, such as the white-rot Phanerochaete chrysosporium which does not possess specific laccase genes but those encoding the ligninolytic oxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), ferredoxin and several unknown MCOs (Brambl 2009; Isroi et al. 2011; Lundell et al. 2010). Laccases function in the degradation of lignin through catalysing the reduction of molecular oxygen to water molecules alongside the oxidation of phenolic and lower-redox potential compounds to phenoxy radicals (Lundell et al. 2010; Widiastuti 2008).

Fungal laccases are widely used in biotechnological applications and function mainly in lignin depolymerisation and biodegradation through the oxidation of phenolic substrates, comprising approximately 10 % of the lignin polymers, and non-phenolic compounds in the presence of inducers/mediators (Baldrian 2006; Bonnen et al. 1994; D'Souza et al. 1999; Isroi et al. 2011; Lundell et al. 2010; Martínez et al. 2005). The fungal laccases are also known to function alongside peroxidases in the polymerisation of monolignols to create lignin polymers, in the detoxification of hydrolysates through the removal of inhibitors prior to fermentation, and also in the treatment of wastewaters with the modification of toxic components and textile dyes (Campbell and Sederoff 1996; Chandel et al. 2011a; Lundell et al. 2010; Vanholme et al. 2010).

Laccases possess a relatively low redox potential in comparison to the peroxidases involved in lignin degradation but are deemed green biocatalysts due to their use of molecular O_2 instead of H_2O_2 (Cañas and Camarero 2010). The four copper ions are involved in laccase activity where the T1 copper ion is associated with the oxidation of the substrate with concomitant reduction of the ion, followed by the transfer of the electron to the T2/T3 trinuclear copper cluster (Cañas and Camarero 2010; Dwivedi et al. 2011). This mechanism of action involves the oxidation of four substrate molecules and the production of free radicals. The four electrons gained are then donated to molecular O_2 to give H_2O.

Mediator systems are used in conjunction with laccases within industries to increase the overall redox potential of the enzyme. These systems allow for the oxidation of the non-phenolic or aromatic compounds, normally of higher redox potential to the enzymes, through a chain-like mechanism. In this mechanism, the mediator, firstly oxidised by the laccase, diffuses through the matrix and allows for the oxidation of the target compounds (Dwivedi et al. 2011). Laccases are found to work in conjunction with mediators such as ABTS, (2,2'-azinobis (3-ethylbenzathiazoline-6-sulfonic acid)) and syringaldehyde (Baldrian 2006).

5.5.1.2 Lignin Peroxidase

Laccases and peroxidases are known to act in a synergistic manner in the lignification of plants, and the degradation and depolymerisation of lignin polymers (Blanchette 1991; Bonnen et al. 1994; Campbell and Sederoff 1996; Chandel et al. 2011a). These peroxidases (LiP, MnP and VP) are heme-containing hydrogen peroxide-dependent enzymes responsible for the oxidation of the high redox potential components of the lignin polymers (Blanchette 1991; D'Souza et al. 1999). The peroxidases are extracellular, non-specific enzymes produced by most white-rot fungi functioning primarily in the degradation of lignin (Blanchette 1991; Brambl 2009).

Lignin peroxidase (LiP; EC 1.11.1.14) was first discovered in *P. chrysosporium* in 1983 by Tien and Kirk (Chen et al. 2011; Dashtban et al. 2010). LiPs are monomeric proteins possessing a molecular mass of approximately 40 kDa (Isroi et al. 2011). They are similar to classical peroxidases with the iron coordinated to four tetrapyrrole rings and to a histidine residue (Isroi et al. 2011).

LiPs are capable of oxidising a number of different phenolic compounds such as guaiacol, syringic acid and vanillyl alcohol, and are also capable of oxidising non-phenolic aromatic compounds (D'Souza et al. 1999; Dashtban et al. 2010; Piontek et al. 2002). The lignin peroxidases possess an important tryptophan residue on the enzyme surface, trp171 in the isozyme LiPA, which has a role in long range electron transfer from aromatic substrates that are too large to reach the oxidised centre of the enzyme (Isroi et al. 2011).

LiPs can oxidise lignin through this electron transfer mechanism, cleaving various bonds non-catalytically and also through the opening of aromatic rings (Chen et al. 2011). Research by Chen et al. (2011) has indicated the different

binding models and degrading mechanisms of ligninolytic enzymes with lignin. LiPs and laccases directly interact with the lignin structure while MnPs act upon the lignin in an indirect manner.

Hydrogen peroxide is a key component in the degradation of lignin by peroxidases. The presence of H_2O_2 gives rise to an oxidised intermediate of LiP in which the Fe^{3+} ion is converted to Fe^{4+} and a free radical is present on the tetrapyrrole ring (Isroi et al. 2011). The LiP intermediate oxidises a substrate giving rise to a radical cation and a second LiP intermediate in which the free radical is removed but Fe^{4+} is still present (Isroi et al. 2011). This second intermediate oxidises a second substrate molecule giving rise to another radical cation and the original LiP enzyme in its resting state (Isroi et al. 2011). The production of radical cations gives rise to the non-enzymatic reactions such as polymer cleavage allowing for the subsequent degradation of lignin molecules (Blanchette 1991).

5.5.1.3 Manganese Peroxidase

Manganese peroxidases (MnP; EC 1.11.1.13) are defined as H_2O_2–dependent heme-containing peroxidase enzymes capable of degrading lignin substrates. The first MnP was discovered in 1983–1984 in *P. chrysosporium* by Kuwahara et al. (1984) and found to have a molecular mass of 40–50 kDa. This particular peroxidase requires manganese (Mn^{2+}) as a cofactor in order for its oxidative function to occur (Chen et al. 2011; Dashtban et al. 2010; Isroi et al. 2011). MnPs are a more diverse range of enzymes in comparison to the LiPs. The grouping of MnPs is divided according to their length, where the typical long MnPs found in fungi are separated from the short enzyme types which are termed hybrid MnPs (hMnPs) (Lundell et al. 2010).

The presence of Mn^{2+} has a regulatory impact on the production of MnP and LiP by a fungus (Blanchette 1991). If Mn^{2+} is found in low concentrations, LiP is produced predominately, while high concentrations of Mn^{2+} will gives rise to the increased production of MnP (Blanchette 1991). The enzyme structure contains a binding domain for the Mn^{2+} but does not possess the specific tryptophan residue found in LiPs required for long range electron transfer (Isroi et al. 2011). As a result, MnPs do not oxidise the lignin substructures directly but through the use of cofactors and chelators.

Similar to the catalytic cycle of LiP, the presence of H_2O_2 gives rise to an oxidised intermediate of the MnP enzyme (MnP-I) (Isroi et al. 2011). This intermediate acts upon the cofactor Mn^{2+} to give its oxidised form, Mn^{3+}, and a secondary intermediate of MnP (MnP-II). This secondary intermediate, then, acts upon another Mn^{2+} to give Mn^{3+} and H_20. The two Mn^{3+} ions produced by this cycle have the potential as mediators, when bound to chelators typically oxalate, to oxidise the various phenolic substrates including amines, dyes and lignin substructures (Blanchette 1991; Dashtban et al. 2010; Isroi et al. 2011).

5.5.1.4 Versatile Peroxidase

Versatile peroxidases (VP; EC 1.11.1.16), first described in the fungus *Pleurotus eryngii*, possess both functionalities of LiPs and MnPs. They have been discovered in various species of Pleurotus and Bjerkandera but not in *P. chrysosporium*, although genes related to the VP of Pleurotus have been found within its genome (Isroi et al. 2011). The VP enzymes are capable of oxidising the phenolic and non-phenolic aromatic compounds along with the oxidation of Mn^{2+}, similar to MnP. They are structurally similar to the hMnPs and contain the binding site for Mn^{2+} close to the heme group (Lundell et al. 2010). The enzymes also contain the essential tryptophan residue that is required in the electron transfer from aromatic lignin substrates (Isroi et al. 2011). VPL, the versatile peroxidase produced by *P. eryngii*, has been structurally characterised revealing that it possess both MnP and LiP structural motifs. VPL contains the three acidic residues essential for Mn^{2+} binding and a tryptophan residue, trp164, which is structurally related to the trp171 found in LiPA (Isroi et al. 2011).

5.5.1.5 Hydrogen Peroxide-Producing Enzymes

Fungi, white-rot basidiomycetes in particular, require H_2O_2 to allow the extracellular peroxidase enzymes to function in lignin degradation. The H_2O_2 is provided by oxidases that are produced by the fungus and act by reducing molecular O_2 to H_2O_2 alongside the oxidation of a co-substrate (Dashtban et al. 2009; Isroi et al. 2011). Two such oxidases are glyoxal oxidase (GLOX; EC 1.2.3.5) and aryl alcohol oxidase (AAO; EC 1.1.3.7). GLOX is a copper-containing enzyme found in many white-rot fungi, for example *P. chrysosporium*, and can oxidise a variety of co-substrates, typically simple aldehydes (Isroi et al. 2011; Martínez et al. 2005). Some of these substrates are natural substances produced by the metabolism of the fungus, for example, glyoxal and methylglyoxal (Isroi et al. 2011). AAO, a flavoenzyme first discovered in *P. eryngii*, acts upon specific metabolites of the white-rot fungi to give rise to H_2O_2. Chlorinated anisyl alcohols are among the substrates oxidised by this enzyme as well as aromatic aldehydes released during lignin degradation in the presence of aryl alcohol dehydrogenase (AAD; EC 1.1.1.91) (Isroi et al. 2011; Martínez et al. 2005).

5.5.1.6 Phenol Oxidases

Phenol oxidases are classified as a range of copper enzymes which do not exhibit glycosyl hydrolase and peptidase activity. They are capable of oxidising phenolic compounds in the presence of molecular O_2. The phenol oxidases include the LMEs previously described as well as tyrosinases, catechol oxidases and catalase-phenol oxidases.

Tyrosinosis possess two catalytic activities; (1) a cresolase activity with the o-hydroxylation of monophenols and (2) catechol oxidase activity with the subsequent oxidation of o-diphenols to reactive o-quinones (Krebs et al. 2004; Rompel et al. 1999). Tyrosinosis are homo-tetrameric proteins containing four copper atoms with a molecular mass of approximately 60 kDa. They function by catalysing the o-hydroxylation of monophenols to catechol (an o-diphenol) followed by the subsequent oxidation of the o-diphenol to the corresponding o-quinone. Tyrosinosis acts upon substrates which include tyrosine, catechol and L-3, 4-dihydroxyphenylalanine (L-DOPA) (Krebs et al. 2004; Rompel et al. 1999).

Catechol oxidases have been identified among fungal cultures which are separate to Tyrosinosis as they lack cresolase activity (Krebs et al. 2004; Rompel et al. 1999; Sutay Kocabas et al. 2008). These enzymes possess two copper atoms which are coordinated by three histidine residues and have a molecular mass of approximately 60 kDa. This oxidase enzymes catalyses the oxidation of o-diphenols to the corresponding o-quinones and is a key enzyme for melanin synthesis. Catechol oxidase acts on the substrates catechol, chlorogenic acid, catechin and caffeic acid (Sutay Kocabas et al. 2008).

Catalase-phenol oxidases (CATPOs) are bifunctional antioxidant enzymes identified in ascomycetous fungi in recent years (Sutay Kocabas et al. 2008). They possess the ability to decompose H_2O_2, which is a typical catalase activity, as well as the ability to oxidise o-diphenolic compounds in the absence of H_2O_2 (Koclar Avci et al. 2012; Sutay Kocabas et al. 2008). CATPOs are tetrameric heme-containing proteins with a molecular mass of approximately 320 kDa (61-97 kDa per subunit). These enzymes act upon similar substrates of catechol oxidases in addition to L-DOPA (Koclar Avci et al. 2012; Sutay Kocabas et al. 2008).

5.5.1.7 Hemicellulase

The hemicellulosic regions of lignocellulose are considered more accessible to extracellular fungal enzymes in comparison to the cellulosic and lignin components as it does not form crystalline structures or microfibrils. This is due to the heterogeneity of hemicellulose polymers within the plant cell wall, the degree of and the particular side chains and branching along the backbone. As a result, a variety of enzymes with different functionalities are required by microbes to completely hydrolyse the hemicellulose present in lignocellulosic biomass.

Xylanases is the term coined to the group of enzymes that act upon the xylan heteropolymers and comprises of endo-1, 4-β-xylanases (EC 3.2.1.8) which cleave glycosidic bonds in the polymer backbone to release xylo-oligosaccharides and, β-xylosidases (EC 3.2.1.37) which hydrolyse the xylo-oligosaccharides released to xylose (Saha 2003). The majority of xylanases cannot cleave the polymer backbone if substituted xylan units are present. Therefore, accessory enzymes are required to remove the substituted residues. However, some of the accessory enzymes can only cleave the side chain residues from xylo-oligosaccharides (Saha 2003). Some fungi possess complete sets of xylan-degrading enzymes, for

example, Penicillium capsulatum and Talaromyces emersonii and have great potential for use in the biorefinery system (Saha 2003).

Similar to the xylanases, mannanases attack the galactomannan or glucomannan polymers within the plant cell wall with the aid of accessory enzymes capable of removing the substituted residues. Mannanases consist of β-1, 4-mannanase (EC 3.2.1.78) which acts directly upon the backbone and β-mannosidases (EC 3.2.1.25) which acts on the mannan substitutions. Xylanases and mannanases act in a synergistic manner with accessory enzymes to overcome the issue of partial breakdown during hydrolysis. The accessory enzymes involved in hemicellulose hydrolysis also include α-L-arabinofuranosidase (AAF; EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), acetylxylan esterase (EC 3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) (Saha 2003).

Chandel et al. (2011a) provide a detailed review on the bioconversion of pentose sugars produced by the hemicelluloses into ethanol. The demand for microorganisms capable of fermenting pentose sugars and producing high yields of ethanol is great. Currently, the microorganisms are only capable of fermenting a number of hexose or pentose sugars into ethanol and cannot tolerate inhibitors such as furfurals and phenolic (Cañas and Camarero 2010; Chandel et al. 2011a).

5.5.1.8 Cellulase

The degradation of the cellulosic component of lignocellulosic biomasses is carried out by hydrolytic enzymes known as cellulases. Cellulase systems consist of three different enzymes; endoglucanases, cellobiohydrolase (CBHs) and β-glucosidases (Grassick et al. 2004; Mtui 2009). It has been reported that properties of biomass such as lignin composition, accessibility of the cellulose chains to these cellulases, degree of cellulose crystallinity and degree of polymerisation determine the overall extent of biomass saccharification (Agbor et al. 2011). The lignin composition of a particular biomass, for example, can have a negative effect on the hydrolysis of cellulose through adsorption of cellulase enzymes (Alvira et al. 2010). Thygesen et al. (2011) have recently demonstrated that the cellulases penetrate into the porous regions of the cellulose fibrils (at amorphous regions) followed by the depolymerisation of the chains. This initial stage of cellulose saccharification is termed morphogenesis and incorporates the swelling and fragmentation of the cellulose fibres (Arantes and Saddler 2010).

Cellulases are grouped into 11 glycoside hydrolase (GH) families and are classed as modular proteins composed of at least two distinct modules; typically, the catalytic module and the carbohydrate binding module (CBM) (Arantes and Saddler 2010; Brás et al. 2011; Dashtban et al. 2009). A flexible linker joins the carbohydrate binding module (CBM) to the catalytic module allowing for the selective anchoring of the catalytic module to the cellulose fibril (Arantes and Saddler 2010; Dashtban et al. 2009; Turner et al. 2007).

Endoglucanases (EG, EC 3.2.1.4) derived from fungi are usually monomeric proteins possessing little or no glycosylation and are characterised by an open

binding cleft (Dashtban et al. 2009; Grassick et al. 2004). EGs act by cleaving the internal β-1, 4-glycosidic bonds of the cellulose chains and are central to the reduction of the degree of polymerisation within hydrolysis (Alvira et al. 2010; Mtui 2009). A number of isozymes of EGs are produced within fungi, which vary among the different species. This is evident in the ascomycete T. reesei which produces at least five isozymes of EG and, also, in the white rot basidiomycete *P. chrysosporium* which produces three isozymes of EG (Dashtban et al. 2009). It has also been observed that not all cellulases possess a CBM; for example, one of the five EG isozymes of T. reesei, EGIII, does not contain a CBM (Dashtban et al. 2009).

Cellobiohydrolase (CBH, EC 3.2.1.91), exo-acting enzymes, are monomeric proteins with little or no glycosylation (Dashtban et al. 2009). Research into the activity of these particular cellulolytic enzymes has found that CBH acts by surrounding the cellulose chain with its extended loops and creating a tunnel-like site for catalysis (Dashtban et al. 2009; Grassick et al. 2004). With this type of active site, CBH is only capable of attacking the cellulose chains at their terminal ends (Grassick et al. 2004). Different isozymes of CBH have the potential to act from either the reducing or non-reducing end of the cellulose chain (Dashtban et al. 2009). T. reesei can produce two CBH isozymes, one which acts from the reducing end and the other which acts from the non-reducing end of the cellulose chains (Dashtban et al. 2009). The ability of CBHs from T. reesei to degrade from either end of a cellulose chain simultaneously allows for a more efficient hydrolytic process (Dashtban et al. 2009).

In commercial hydrolysis, the addition of β-glucosidases (EC 3.2.1.21) can enhance the hydrolysis reaction by rapidly converting cellobiose to glucose, and thereby reducing the potential of end-point inhibition (Arantes and Saddler 2010). β-glucosidases are the most variable of the three cellulolytic enzymes and are dependent upon structure and localisation. These enzymes can be monomeric proteins with molecular masses of approximately 35 kDa, dimeric proteins or even trimeric proteins with molecular masses greater than 146 kDa (Dashtban et al. 2009).

β-glucosidases have been classified into intracellular, extracellular and cell wall-associated groups and are normally glycosylated to varying degrees. β-glucosidases derived from T. versicolor have been seen to be glycosylated up to 90 % with a molecular weight of approximately 300 kDa (Dashtban et al. 2009). Fungal β-glucosidases have been classified into the GH family 3 based on their amino acid sequences along with other bacterial and plant β-glucosidases (Dashtban et al. 2009).

These three cellulases act in a synergistic manner by providing free chain ends with endoglucanases activity, removing cellobiose molecules from the free ends with cellobiohydrolase activity and hydrolysing the cellobiose to glucose with β-glucosidase activity. Cellulases are considered as the most sought after enzymes throughout a range of industries. In a report by Chandel et al. (2011b), cellulases were considered to comprise 75 % of the demand of total enzymes required by the various industries.

5.5.2 Conclusion

The use of fossil fuels, increase in oil prices and GHG emissions have led to the development of various technologies for biofuel production. The use of first generation biofuels, derived from agricultural crops, has provided the majority of biofuel that is consumed today. Although the percentage of biofuel consumption for transportation worldwide is still minimal, the WEC has stated that it is to increase to 5 % by 2030. There are a number of social implications involved with the production of first generation biofuels. The use of agricultural crops, such as corn and wheat, for biofuel production leads to the displacement of food crops, as well as competitive water use. This feedstock versus food concern has lead into the development of second generation biofuels with sources from renewable lignocellulosic materials and wastes.

Second generation biofuels can be derived from lignocellulosic biomasses including leaf litter, spent compost, bagasse and the straw left after harvesting wheat, barley and oats. The use of these "wastes" as feedstocks reduces the feedstock versus food issues, and therefore eases the rising food prices. The various types of lignocellulosic feed stocks possess varying compositions of the main polymers; cellulose, hemicellulose and lignin, within the plant cell wall and provide varying degrees of recalcitrance to hydrolysis. As a result of this, different biorefinery technologies are in place for the degradation of specific feedstocks.

The biorefinery process can be broken down into four main steps; pre-treatment, hydrolysis, fermentation and separation. Fungal enzymes can be used in the pre-treatment of the lignocellulosic feedstock to disrupt the lignin and cellulose microfibrils and allow for lower energy demands and waste production in comparison to physical and chemical pre-treatment. The use of enzymatic hydrolysis instead of acid hydrolysis leads to more environmentally friendly bioprocessing through the minimal requirements of toxic metal ions and lack of wastewaters. The degraded lignin is removed from the biofuel process and can be used by other industries such as the paper-pulp industry.

Current technologies involved in biofuel production are inefficient and costly where the separate enzymes and chemicals required for each stage of the biorefinery are numerous. The introduction of particular fungi to the biorefinery systems allow for the production of various essential enzymes for lignocellulose breakdown. Although there are still cost concerns with the use of fungal enzymes in the biorefinery and production of biofuels in comparison to fossil fuel ethanol, there is ongoing research into increasing their potential through protein engineering and making the enzymes more readily available.

The addition of fungi capable of secreting lignin-modifying enzymes, hemicelluloses and cellulases is essential for efficient hydrolysis. The white-rot basidiomycetes are the typical fungi used for this role although a number of ascomycetes are emerging with the capability of degrading lignocellulose efficiently. Lignin structures act as barriers within the plant cell walls, disrupting the binding of cellulases and hemicelluloses to their target polymers and decreasing

the overall yield of fermentable sugars. Lignin must be removed efficiently in order to create a cost effective biofuel production facility.

There is a growing interest in the phenol oxidase group of enzymes. This group, containing the lignin-modifying enzymes, tyrosinases, catechol oxidases and catalase-phenol oxidases, can be secreted by fungi and aid in the breakdown and removal of the rigid lignin structures through varying oxidative reactions. There are few publications involving the tyrosinases, catechol oxidases and catalase-phenol oxidases in biofuel production to date. The potential of these phenol oxidases in the breakdown of lignin as well as the decomposition of H_2O_2 by catalase-phenol oxidase could lead to a more environmentally friendly approach to the pretreatment of lignocellulosic materials.

The use of LiPs, MnPs and VPs induced by the fungi, require the presence of H_2O_2 and cofactor, Mn^{2+}, (for MnP and VP) to function correctly. VPs possess great potential within the biotechnological and industrial fields as it has two catalytic functions similar to both LiP and MnP where they contain a tryptophan residue for long range electron transfer and a binding site for Mn^{2+} within their structures. The need for H_2O_2 for the catalytic activity of these enzymes during hydrolysis can be costly and environmentally harmful to add at large scales to the biorefinery system. Certain fungi are capable of producing H_2O_2 -producing oxidases, namely GLOX and AAO to overcome this issue. These enzymes, GLOX and AAO, act on co-substrates such as simple aldehydes and chlorinated anisyl alcohols, respectively, while reducing molecular O_2 to H_2O_2.

Hemicelluloses and cellulases are the major battery of enzymes that are required during biofuel production. They are the key to degrading hemicellulose and cellulose fractions into hexose and pentose sugars, respectively, which can then be fermented into biofuels. The hemicelluloses consists of a large variety of enzymes capable of degrading xylans, mannans and Xyloglucans completely. Accessory enzymes are also important in the removal of substitutions, e.g. acetylation and methylation, along the polymer backbone. Without the accessory enzymes certain hemicelluloses cannot function correctly and can reduce the overall biofuel production yields. The accessibility of cellulases to cellulose can reduce the efficiency of the hydrolysis step as the cellulose is present in both amorphous and crystalline structures that are not completely exposed to the enzymes. The cellulases often require partial hydrolysis of the hemicelluloses, as well as pectins, to be able to adsorb to the amorphous cellulose regions.

Thermostable fungi are advantageous to the biofuel and biorefinery process as they are able to withstand the harsh conditions involved and their addition to the consolidated bioprocessing (CBP) method provide great possibilities for reducing enzymatic hydrolysis costs. Analysis of thermophilic fungi with lignocellulose degradation capabilities is required to verify and optimise the conditions involved for enzyme secretion during the biorefinery process. The removal of lignin for use by other industries, for example the paper and pulp industry, is important for the overall cost scheme of the biorefinery in question.

The efficiency of biofuel production and the costs involved must be reviewed and the system altered to provide competitive fuel prices alongside current fossil

fuels. All components of the lignocellulosic polymers must be consumed or removed and utilised by other industries to obtain an efficient biofuel production facility. Fungal enzymes are essential in the production of biofuels, especially in the pre-treatment and hydrolysis stages of the biorefinery systems and possess great potential in overcoming the issues of recalcitrance, high costs and low biofuel yields.

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Chapter 6
Progress on Enzymatic Saccharification Technologies for Biofuels Production

Pablo Alvira, Mercedes Ballesteros and María José Negro

Abstract A key issue for the biorefineries is the cost-effective conversion of carbohydrates contained in lignocellulosic biomass into fermentable sugars, which will provide a viable route to biofuels and bioproducts. Many different raw materials, conversion methods, and process configurations have been studied for the generation of sugars from lignocellulosic biomass. Most of the schemes for lignocellulosic biomass conversion include a pretreatment step to increase digestibility of the substrates and an enzymatic hydrolysis process, which is a crucial step and determines the overall process efficiency. Due to lignocellulose complex structure, different enzymes are involved in the degradation of the substrates and appropriate combinations of different activities are required for complete hydrolysis. This chapter reviews novel advances in enzymatic hydrolysis technologies for lignocellulose conversion, with special focus on the necessity of optimized enzyme mixtures using accessory activities, and the advantages of operating at high initial substrate concentrations.

6.1 Introduction

Alternative and renewable fuels derived from lignocellulosic biomass offer the potential to mitigate global climate change and reduce the dependence on fossil fuels. Biomass is an abundant source of renewable energy with an estimated production of 200×10^{12} kg worldwide annually (DOE 2003). It is constituted by

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carbon building blocks, which have great potential to be converted into fuels and chemicals (Teter et al. 2010). In this context, it is expected that the present industry based on fossil fuels will gradually be replaced in the near future by biorefineries, which will produce from biomass a diverse array of biobased products and energy in the form of fuels, heat, and electricity. Lignocellulose is composed of up to 75 % carbohydrates and it will become an essential source of fermentable sugars, which form the basis for production of liquid biofuels for the transport sector as well as a large variety of commodity chemicals and biomaterials (Olsson et al. 2004; Himmel and Picataggio 2008).

A key issue for the biorefineries is the cost-effective conversion of carbohydrates contained in lignocellulosic feedstocks into fermentable sugars; and to obtain the so-called sugars platform. The resulting sugars will provide a viable route to primary products such as alcohols, esters, and carboxylic acids. In addition, noncarbohydrate components can be potentially recovered to obtain high added value products, as well as for energy and heat production (Ragauskas et al. 2006).

Breakthrough technologies for converting biomass to fuels and chemicals still need to overcome barriers to reach a cost-effective level. Most of the schemes for lignocellulosic biomass conversion include a pretreatment step followed by enzymatic hydrolysis. The aim of the pretreatment is to increase digestibility of the substrate and boost the yield of fermentable sugars while minimizing the formation and release of toxic byproducts. Subsequent hydrolysis of cellulose and hemicellulose components into monosaccharides requires the action of multienzymatic complexes. The use of enzymes has significant advantages over acid hydrolysis; the reactions are very specific, have low energetic requirements (temperature) and do not produce harmful byproducts. Since lignocellulose is a complex matrix of different polymers, the optimization of both steps, pretreatment and enzymatic hydrolysis in relation to each other, is a major challenge for biomass utilization (Sun and Cheng 2002; Ballesteros 2010; Himmel et al. 2007).

6.2 Why Pretreatment?

Many physicochemical, structural, and compositional factors make the native lignocellulosic biomass recalcitrant and difficult to be hydrolyzed by enzymes. Lignocellulosic biomass is primarily constituted by cellulose, hemicellulose, lignin, and smaller amounts of pectin, protein, extractives, and ashes. These components are present in varying amounts in the different plant tissues and they are intimately associated to form the structural framework of the plant cell wall. Generally, cellulose microfibrils are surrounded by sheaves of hemicellulose that, in turn, are covalently linked to lignin. This matrix of heteropolymers in which cellulose is embedded is responsible for the characteristic biomass recalcitrance (Himmel et al. 2007). To alter structural characteristics of lignocellulose and significantly increase cellulose and hemicellulose accessibility to the enzymes, a pretreatment step is necessary (Mosier et al. 2005). The effectiveness of the pretreatment to improve

the enzymatic hydrolysis has been attributed to a modification in the degree of polymerization and crystallinity of cellulose (Mansfield et al. 1999; Kumar et al. 2009a), to a disruption of the lignin–carbohydrate linkages (Laureano-Perez et al. 2005), to lignin and hemicelluloses removal (Pan et al. 2005), and to an increase of the porosity of the material (Chandra et al. 2007). There are a number of key features for an effective pretreatment of lignocellulose biomass: it should be versatile producing high yields for multiple crops, maturity, and harvesting times, enhance substrate digestibility, should minimize sugar degradation and toxic compounds production, maximize the production of other valuable products (e.g. lignin-derived), minimize energy consumption, not require chemicals, operate at with large particle sizes, low moisture contents, and at high solid concentrations, and be scalable to industrial size (Jørgensen et al. 2007a; Yang and Wyman 2008).

Pretreatment research has been focused on identifying, evaluating, developing, and demonstrating promising approaches that enhance the enzymatic hydrolysis of the pretreated biomass at lower enzyme dosages and shorter conversion times. Over the years, many different pretreatments have been investigated on a wide variety of feedstocks types and have been generally classified into biological, physical, chemical, and physicochemical pretreatments. A combination of physical parameters, such as temperature or pressure, and biological or chemical treatments can be also used in the pretreatment process. Several recent review articles provide a general overview of the field (Carvalheiro et al. 2008; Sánchez and Cardona 2008; Taherzadeh and Karimi 2008; Yang and Wyman 2008; Hendriks and Zeeman 2009; Alvira et al. 2010; Gírio et al. 2010; Tomás-Pejó et al. 2011). Some of the pretreatment methods have been reported to increase efficiently the digestibility of different raw materials; however, their viability at larger scale represents a significant drawback. The mechanism for making the cellulose more accessible to enzymes depends on the pretreatment employed and nature of the raw material. In ozonolysis, CO_2 explosion, and biological pretreatments, lignin is removed; while in steam explosion is only redistributed and in liquid hot water is partially solubilized. Hemicellulose is extensively solubilized during wet oxidation, autohydrolysis steam explosion or acid pretreatment; and mechanical comminution and ammonia fiber explosion have been shown to reduce cellulose crystallinity. Table 6.1 summarizes the advantages and disadvantages, and the main effects of the most common pretreatment technologies developed for lignocellulosic biomass conversion.

The selection of an appropriated pretreatment determines the process configuration requirements for hydrolysis and fermentation since each step has a large impact on all subsequent stages (Yang and Wyman 2008). The chemistry of the pretreatment has a remarkable importance due to its impacts on the global ethanol production process. Furthermore, pretreatment affects also the cost of the following operation steps, i.e., downstream cost by determining fermentation toxicity, enzymatic hydrolysis rates and enzyme loading, as well as fermentation process variables.

Table 6.1 Main effects of the most common pretreatment technologies developed for lignocellulosic biomass conversion (Tomás-Pejó et al. 2011)

	Increases accessible surface area	Cellulose decrystallization	Hemicellulose solubilization	Lignin removal	Lignin structure modification	Production of toxic compounds
Biological	++	0	0	+++	++	0/+
Mechanical comminution	+++	+++	0	0	0	0
Extrusion	+++	+++	0	–	–	–
Acid	+++	0	+++	++	+++	+++
Alkali	+++	+++	++/+++	+++	+++	+
Organosolv	++	–	+++	++/+++	++	++/+
Ozonolysis	++	++	++/+++	+++	++	+
Ionic liquids	++	+++	+++	++/+++	++	++/+
Wet oxidation	+++	–	+++	++	+++	+
Microwave	+++	+++	+	+++	+++	+
LHW	+++	–	+++	+	++	+
AFEX	+++	++	++	+	+++	+
SPORL	+++	++	+++	++	++	+
Supercritical fluids	++/+++	–	++	+++	++	++
Steam explosion	+++	–	+++	++	+++	+++

(+++) high effect; (++) moderate effect; (+) low effect; (0) no effect
LHW: Liquid hot water
AFEX: Ammonia fiber expansion
SPORL: Sulfite pretreatment to overcome recalcitrance of lignocellulose

6.3 Enzymes for Hydrolysis of Lignocellulose

The goal of the enzymatic hydrolysis process is to depolymerise the polysaccharides contained in the pretreated lignocellulosic substrates. Due to the complex structure and composition of lignocellulose, different enzymes are involved and an appropriate combination of different activities is required for complete hydrolysis. Enzyme production and subsequent enzymatic hydrolysis are two of the steps preventing bioconversion processes from becoming cheaper and competitive. In recent years, different programs have funded research at the main biotechnological companies to improve the enzymatic products and reduce their cost. Although significant advances have been accomplished, enzyme mixtures still need to be optimized and adapted for the different raw materials and pretreatments (Himmel et al. 2007; Banerjee et al. 2010).

The enzymes involved in lignocellulose conversion can be classified into cellulases, hemicellulases, ligninases, and nonhydrolytic proteins.

6.3.1 Cellulases

Cellulose is the main component of lignocellulose (generally contains 40–50 % (w/w)) and glucose, the preferred carbon source for many microorganisms. Cellulose is a homopolymer of repeating sugar units of glucose linked by β-1,4-glucosidic bonds. It is highly ordered, water-excluding, insoluble, and presents crystalline regions.

Cellulases are the number of enzymes involved in the hydrolysis of the polymer of cellulose into glucose monomers. Cellulases are produced by different organisms including fungi; aerobic and anaerobic bacteria; termites and some insects (Wilson 2008). The most studied microorganism for cellulase production is the filamentous fungi *Trichoderma reesei (*teleomorph *Hypocrea jecorina)*, which secretes large amounts of extracellular enzymes (Kubicek 1992). *T. reesei* has been extensively subjected to strain development processes to improve cellulase production (Persson et al. 1991). Recently, its genome has been completely sequenced, which provides important information to enable genetic modifications and metabolic engineering (Martinez et al. 2008).

Some microorganisms are capable to produce extracellular multienzymatic complexes or cellulosomes, which can degrade cellulose and hemicellulose. Cellulosomes were first discovered in anaerobic bacteria of genus *Clostridium* (Bayer et al. 2004). *Clostridium* is also capable to ferment sugars; therefore, it has been studied for consolidated bioprocessing (CBP) approaches, which involves cellulase production, cellulose hydrolysis and fermentation in one step (Bayer et al. 2008).

To convert cellulose into glucose, at least three categories of enzymes are involved; endoglucanases hydrolyze internal β-1,4-glucosidic bonds in the

cellulose chain, cellobiohydrolases cleave off cellobiose units from the end of the chain, and β-glucosidase converts cellobiose into glucose. These enzymes are usually constituted by a catalytic domain and a binding carbohydrate domain. They work synergistically to hydrolyze cellulose by creating new accessible sites and relieving product inhibition (Himmel et al. 1996). However, cellulases mechanism is not completely known and a deeper understanding could enhance the efficiency of the process. It has been reported that cellulases show nonlinear kinetics with time and amount of enzyme, and the components act in a processive (sequential) manner (Medve et al. 1998; Kurasin and Valjämäe 2011). It has been also suggested that the rate-limiting step in the hydrolysis of cellulose is not the catalytic cleavage of the beta-1,4-glucosidic bond, but the disruption of a single chain of the substrate from its native crystalline matrix, thereby rendering it accessible to the active site of the enzyme (Bayer et al. 2008). This mechanism was recently reported by real-time visualization (Igarashi et al. 2011). Another study hypothesized that carbohydrate binding module can bind strong and unproductively the cellulose chain, reducing the activity of cellulases (Eriksson et al. 2002).

6.3.2 Hemicellulases

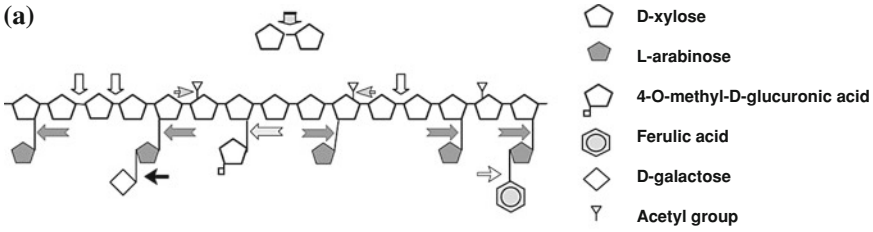
Hemicellulases are the enzymes involved in the degradation of hemicellulose. In contrast to insoluble, highly crystalline, homogenous, and unbranched cellulose polymer, hemicellulose is a branched and substituted heteropolymer composed of a wide variety of subunits, including sugars, sugar acids, and noncarbohydrate moieties. Hemicelluloses represent 15–35 % of plant biomass; and are linked to lignin through ester linkages and to cellulose through interchain hydrogen bonding (Gírio et al. 2010).

The type of hemicellulose and its structural characteristics differ among different plant families or cell types. In addition, the pretreatment produces modifications in the structure and composition of hemicellulose. Acid and hydrothermal pretreatments such as steam explosion or liquid hot water produce extensive hemicellulose solubilization and degradation, yielding hemicellulosic sugars both in monomeric and oligomeric form (Ballesteros et al. 2006; Pérez et al. 2008; Alvira et al. 2010). In contrast, pretreatment with alkali or ammonia fiber/freeze explosion (AFEX) and biological pretreatments generally have little effect on hemicelluloses (Kumar et al. 2009; Wyman et al. 2011). Considering the complexity and variability of hemicelluloses and the modifications produced by the different pretreatments, enzymatic conversion of hemicellulose becomes a challenge. Obtaining an optimal set of enzymes depends on type of biomass, the pretreatment and the desired end products.

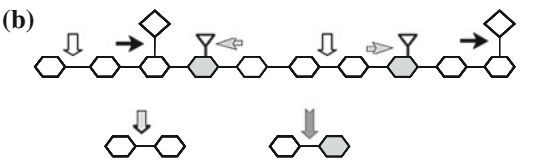
The most relevant hemicelluloses in herbaceous biomass are xylans, especially arabinoxylan. Hardwood contains predominantly glucuronoxylan, while softwood is dominated by glucomannan, galactomannan, and galacto (gluco) mannan

(Dahlman et al. 2003). Other hemicelluloses are also present in plant cell walls, including xyloglucan and β-glucans. This structural and compositional complexity of hemicelluloses extends to the enzymes involved in degradation of these heteropolymers. Hemicellulases are usually classified according to their substrates specificity (xylanases, mannanases, arabinases, galactosidases, etc). Another system, the CAZy database (www.cazy.org), categorizes glucoside hydrolases based on aminoacid sequences and molecular similarities (Henrissat and Davies 1997; Decker et al. 2008).

Hemicellulases can be also divided into depolymerising enzymes, which act on the backbone sugar chains, and debranching enzymes, which catalyze hydrolysis of lateral substitutions (Decker et al. 2008). Debranching enzymes include those acting on glycosidic linkages, such as α-L-arabinofuranosidases or α-D-glucuronosidases; and esterase enzymes, which catalyze ester linkages between sugars and



Symbol	Enzyme	EC	Bound hydrolysed
⇩	Endo-1,4-β-xylanase	3.2.1.8	β-1,4
⇩	β-D-xylosidase	3.2.1.37	β-1,4
⇒	α-L-arabinofuranosidase	3.2.1.55	α-L-1,2; α-L-1,3
⇨	α-D-glucuronosidase	3.2.1.131	α-L-1,2
→	α-D-galactosidase	3.2.1.22	α-L-1,6
⇨	Feruloyl esterase	3.1.1.73	Ester
⇨	Acetyl esterase	3.1.1.72	Ester



Symbol	Enzyme	EC	Bound hydrolysed
⇩	Endomannanase	3.2.1.78	β-1,4
⇩	β-mannosidase	3.2.1.25	β-1,4
⇩	β-glucosidase	3.2.1.21	β-1,4
→	α-D-galactosidase	3.2.1.22	α-D-1,6
⇨	Acetyl mannan esterase	3.1.1.72	Ester

Fig. 6.1 Enzymes involved in arabinoxylan **a** and galactoglucomannan **b** degradation

others components. In Fig. 6.1a, it is shown the typical structure of a cereal arabinoxylan and the enzymes involved in its hydrolysis.

In a biorefinery concept, hemicellulases are valuable tools potentially applicable in many bioconversion processes. In pulp and paper industry, xylanases have been used for biobleaching, in which the enzymes hydrolyze specifically xylan chains and promote partial delignification (Bajpai 2004). Hemicellulases are also used as food additives to poultry and for food industry applications, including coffee processing, fruit and vegetable maceration, bread preparation, and juice clarification (Beg et al. 2001). More recently, hemicellulases have been studied for production of valuable oligosaccharides that can be applicable as food additives (Yang et al. 2005).

Hemicellulases are extensively studied as accessory enzymes to increase the production of fermentable sugars from lignocellulosic raw materials and improve the yields of bioethanol and other biofuels production (Saha 2003). Pretreatments, such as steam explosion, solubilize partially the hemicellulose fraction, which increases accessibility of cellulose chains. In an attempt to reduce the costs and losses associated to pretreatments, the processes should be performed at low severity, which does help the economics of the pretreatment, but often results in less digestible biomass, mostly associated with the decrease of hemicellulose hydrolysis. To maintain the advantages of reduced severity pretreatments, while continuing to increase conversion rates and levels, hemicellulases are becoming much more prominent in the biomass conversion technologies. The action of hemicellulases degrade the hemicellulosic matrix and increase accessibility of cellulose fibers, which improves the enzymatic hydrolysis yields and favors the reduction of cellulase dosages (García-Aparicio et al. 2007; Kumar and Wyman 2009b; Alvira et al. 2011a). In addition, the hydrolysis of hemicellulose fraction increase total amount of fermentable sugars, mainly C-5 sugars. This fact is relevant since pentose-fermenting microorganisms have been developed during last years (Hahn-Hägerdal et al. 2007; Tomás-Pejó et al. 2010).

Many studies report potential uses of hemicellulase enzymes to improve bioconversion processes, being the xylanases the most extensively studied. **Xylanases** include **endoxylanases** and ***β*-xylosidases**. Endoxylanases hydrolyze internal β-1,4-glucosidic bonds in the xylan chain, releasing xylooligomers, while β-xylosidases act on xylobiose and xylooligomers, releasing xylose units. These activities have been widely studied and reported to improve the accessibility of cellulose to cellulases and increase enzymatic hydrolysis yields (Berlin et al. 2005; García-Aparicio et al. 2007; Kumar and Wyman 2009b; Hu et al. 2011). Two mechanisms have been hypothesized to explain this effect: enhanced accessibility of glucan chains due to xylan removal and disruption of xylan linkages to glucan with a subsequent delignification effect (Kumar and Wyman 2008). In addition, xylobiose and xylooligomers have been shown to inhibit enzymatic hydrolysis of glucan and xylan in pretreated lignocellulose (Kumar and Wyman 2009b). Qing et al. (2010) reported a stronger inhibitory effect on cellulases of xylooligomers than xylose or cellobiose. Furthermore, different approaches of enzyme addition strategies and process configurations have also been recently suggested to optimize

the enzymatic hydrolysis and obtain higher fermentation yields (Kumar and Wyman 2009c; Jin et al. 2010; Rémond et al. 2010; Alvira et al. 2011b). These studies hypothesized that the sequence in which the different activities are introduced in the process can improve the overall release of fermentable sugars.

Endomannanases catalyze internal linkages in mannan chains, which constitute galactoglucomannans and glucomannans, the predominant hemicelluloses in softwood (Fig. 6.1b). The main hydrolysis products from these polysaccharides are mannobiose, mannotriose, and various mixed oligosaccharides. These enzymes can have a positive effect on pulping or biofuel production from woody biomass (Agrawal et al. 2011). ***β*-mannosidases** are capable to further degrade mannooligomers to mannose (Tenkanen et al. 1997; Decker et al. 2008).

Xyloglucan is one of the major hemicellulosic polysaccharides in the primary cell wall of various plant species. It is composed of a glucan backbone with xylose substitutions and it is intimately associated to cellulose chains. **Xyloglucanases** degrade xyloglucan by attacking the glucan backbone, even at substituted glucose residues. It has been reported that hydrolysis of xyloglucan enables cellulases to hydrolyze the cellulose polymer more efficiently and improve the enzymatic hydrolysis process from lignocellulosic substrates (Benko et al. 2008).

Debranching enzymes generally remove side groups linked to the main chain of the polysaccharides or to oligomers. The presence of these side chains can restrict the enzymatic hydrolysis of carbohydrates contained in lignocellulosic raw materials. Thus, these enzymes act synergistically with other depolymerising enzymes, which enhance the effectiveness of the enzymatic hydrolysis process.

α-L-arabinofuranosidases cleave arabinose residues from arabinan, arabinoxylan or pectin. In some lignocellulosic materials, this activity favors debranching and degradation of xylan, and also helps to disrupt the lignin–carbohydrate complex since arabinose residues take part in lignin–hemicellulose ether bonds. α-L-arabinofuranosidases are promising tools in various agro-industrial processes, including production of medicinal compounds, improvement of wine flavors, bread quality, pulp treatment, juice clarification, quality of animal feedstock, synthesis of oligosaccharides, and more recently for bioethanol production (Saha 2000; Numan and Bhosle 2006). Moreover, these enzymes work synergistically with other activities, such as endoxylanases (Alvira et al. 2011a), and ferulic acid and acetyl xylan esterases (Poutanen and Puls 1989; Saha, 2000; Raweesri et al. 2008). Supplementation of cellulases with this type of accessory enzymes has the potential to improve the enzymatic hydrolysis process from lignocellulosic feedstocks. Figure 6.2 illustrates the positive and synergistic effect of supplementation with endoxylanases and α-L-Emphasis Type="SmallCaps">L-arabinofuranosidases in the enzymatic hydrolysis of steam explosion pretreated wheat straw.

α-glucuronosidases catalyze the release of glucuronic acid or 4-O-methylglucuronic acid from xylan, showing a synergistic effect with endoxylanases. α-D-galactosidases are involved in degradation of galactomannans and galactoglucomannans, therefore its application in softwood pulping has been considered (Decker et al. 2008).

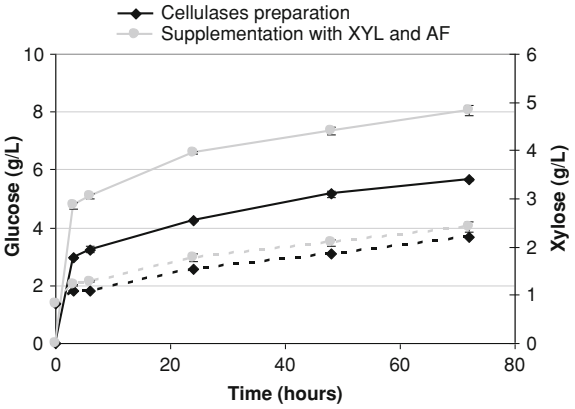


Fig. 6.2 Effect of endoxylanase and α-L-arabinofuranosidase supplementation on glucose (*continuous line*) and xylose (*discontinuous line*) production from steam explosion pretreated wheat straw (Alvira et al. 2011a)

Esterases enzymes can be included in the hemicellulases group since catalyze ester bonds between hemicellulose and other components. Acetyl and hydroxycinnamic acid substituents bound to hemicellulose are removed by **acetyl xylan esterases and ferulic and coumaric esterases**, respectively. Some of these esterases have a broad specificity against different substrates (Crepin et al. 2004). Acetylation occurs on several hemicelluloses, mainly in cereals and hardwoods. Acetyl groups increase solubility and hydration of hemicellulose, therefore deacetylation of xylan and glucogalactomannan decrease the solubility of the polymer. Release of acetyl groups also results in a decrease of the pH and can have an inhibitory or toxic effect to many microorganisms during subsequent fermentation processes (Palmqvist and Hahn-Hägerdal 2000a). As acetyl groups are bound to hemicellulose, they have been reported to inhibit cellulase enzymes and/or limit their access to cellulose chains during enzymatic saccharification processes (Pan et al. 2006). **Acetyl xylan esterases** remove acetyl groups from xylan chain and work synergistically with cellulases and xylanases when using pretreated corn stover, wheat straw, or giant reed (Pan et al. 2006; Selig et al. 2008; Selig et al. 2009; Zhang et al. 2011).

Hydroxycinnamic acids such as p-coumaric and ferulic acid are frequent constituents in hemicelluloses of herbaceous biomasses such as corn stover or wheat straw. These acids take part in the linkages between hemicellulose, frequently arabinose, and galactose side chains, and lignin components (Faulds et al. 2006). The esterase activities contribute to breakdown the lignin–carbohydrate complex having an important role in cell wall development and gut health. **Ferulic and p-coumaric acid esterases** hydrolyze ester bonds between hydroxycinnamic acids and sugars, and release ferulic acid and p-coumaric acid from these polymers. As well as acetic acid, coumaric, and ferulic acid are toxic to many microorganisms and can have a negative effect in bioconversion processes to obtain fermentation products (Palmqvist and Hahn-Hägerdal 2000a). Esterases action can also enhance the accessibility of the cellulose fibers and are potentially used for the production of bioactive chemicals and biofuels (Polizeli et al. 2005). Feruloyl esterase has

been shown to work synergistically with cellulases and xylanases, which can contribute to degrade the lignocellulosic material and reduce the enzyme dosages needed for the process (Vries et al. 2000; Faulds et al. 2006; Tabka et al. 2006; Selig et al. 2008).

6.3.3 Ligninases

Lignin is the second most abundant constituent of plant biomass after cellulose. It is a complex aromatic polymer, highly recalcitrant toward both chemical and biological degradation, where different nonphenolic phenylpropanoid units form a complex three-dimensional network linked by a variety of ether. Although lignin does not contain fermentable sugars, it constitutes a physical barrier that binds unspecifically cellulases and impedes its action; therefore, its removal is a key step in industrial conversion of cellulosic biomass (Ruiz-Dueñas et al. 2009). Some microorganisms, such as the so-called "white-rot" basidiomycetes, are able to depolymerize and mineralize lignin (Martínez et al. 2005). These fungi have developed an extracellular and unspecific enzymatic system of an oxidative nature for lignin degradation. The process involves different enzymes such as laccases, ligninolytic peroxidases, oxidases generating extracellular H_2O_2, reductases, and also low molecular weight compounds that mediate the action of these enzymes (Martínez et al. 2005). The process involves an only enzyme type or various associated enzyme complexes acting synergistically, depending on the species, strains and culture conditions (Martínez et al. 2005).

Laccases have been described for many years in plants, fungi, insects, and bacteria (Mayer and Staples 2002), being their production a characteristic unique of "white-rot" basidiomycetes (Martínez et al. 2005). They are multicopper oxidases that catalyze the one electron oxidation of substituted phenols, anilines, and aromatic thiols to their corresponding radicals with the concomitant reduction of molecular oxygen to water. Their low redox potential only allows the direct oxidation of phenolic lignin units, which only represent a small percentage in lignin (Mayer and Staples 2002). However, in the presence of low molecular weight compounds forming stable radicals that act as redox mediators (Bourbonnais and Paice 1990), laccases also oxidize nonphenolic lignin units. Ligninolytic peroxidases are high redox potential heme peroxidases that require H_2O_2 as co-substrate for the enzyme catalysis (Martínez et al. 2005), and include lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP). The two former were described first in *Phanerochaete chrysosporium* (Martínez 2002). LiP is able to oxidize directly nonphenolic lignin units, whereas MnP generates Mn^{3+} acting preferentially on phenolic units, but also on nonphenolic units via lipid peroxidation reactions (Martínez et al. 2005). VP was described recently in *Pleurotus eryngii* as a new peroxidase which shares catalytic properties with LiP and MnP (Ruiz-Dueñas et al. 1999). Required H_2O_2 by ligninolytic peroxidases is produced by oxidases, such as glyoxal oxidase, a copper

radical enzyme described in *P. chrysosporium* (Kersten 1990), and arylalcohol oxidase described in *P. eryngii* (Guillén et al. 1992). Finally, reductases such as arylalcohol dehydrogenases and quinone reductases are also involved in lignin degradation (Guillén et al. 1997), catalyzing the reduction of phenolic products derived from lignin degradation, and thus avoiding their posterior repolymerization.

The lignin degradation by this nonspecific oxidative system makes "white-rot" fungi useful for a wide range of biotechnological applications of lignocellulosic biomass. Traditionally used in the pulp and paper industry for biopulping or biobleaching (Ruiz-Dueñas et al. 2009), "white-rot" fungi are currently attracting much attention as an alternative or additional method to pretreatments for enhancing enzymatic saccharification of lignocellulosic biomass in ethanol production processes (Kuhar et al. 2008; Dias et al. 2010; Salvachúa et al. 2011). This biological pretreatment consists in a solid state fermentation process in which microbes grow on the lignocellulosic biomass selectively degrading lignin and hemicellulose, while the cellulose is expected to remain intact. Compared to physical and chemical pretreatment methods, biological pretreatment offers some advantages: it is cheaper, safer, less energy consuming, environmentally friendly, and minimizes inhibitors generation (Alvira et al. 2010). However, the rates of this type of pretreatment is still far from industrial requirements, besides of presenting disadvantages, such as long storage times, extended cellulose and hemicellulose consumptions, and low saccharification rate (Alvira et al. 2010). As an alternative, ligninolytic enzymes reduce pretreatment times and present higher lignin specificity, avoiding over carbohydrate consumptions. Among them, laccases are being widely studied on different feedstocks, either individually (Moilanen et al. 2011; Mukhopadhyay et al. 2011) or combined with mediators (Palonen and Viikari 2004).

Ligninolytic enzymes have been also applied in biofuels production to minimize the inhibitory effects of several compounds generated during pretreatments of lignocellulosic biomass. Thermochemical pretreatments, such as steam explosion or dilute acid, generates some soluble inhibitory compounds, derived from a partial sugars and lignin degradation, which can affect enzymatic hydrolysis as well as fermentation steps (Palmqvist and Hahn-Hägerdal 2000a; Panagiotou and Olsson 2007). The nature and concentration of these toxic compounds depend on the severity of pretreatment conditions and the raw material used. They are classified according to their chemical structure and include weak acids, furan derivatives, and phenolic compounds (Palmqvist and Hahn-Hägerdal 2000a). Laccases act selectively on phenolic compounds (Kolb et al. 2012), increasing saccharification yields and enhancing the yeast fermentation performance (Jönsson et al. 1998; Jurado et al. 2009; Kalyani et al. 2012; Moreno et al. 2012). Figure 6.3 shows the positive effect of laccase treatments on ethanol production from steam exploded wheat straw. Compared to chemical and physical detoxification methods, the use of laccases involves fewer inhibitory subproducts, little waste generation and mild reaction conditions (Palmqvist and Hahn-Hägerdal 2000b). Its detoxification mechanism consist in the oxidation of phenolic compounds generating unstable

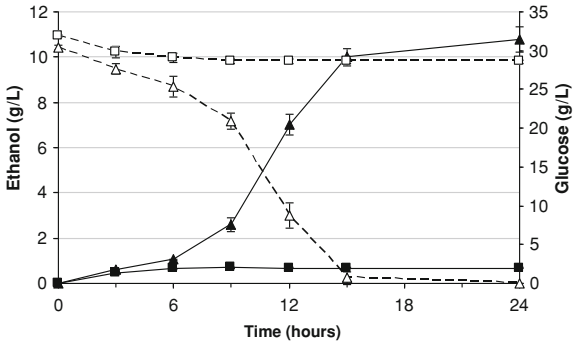


Fig. 6.3 Time course for ethanol production (*filled symbols and continuous lines*) and glucose consumption (*open symbols and discontinuous lines*) during fermentation of steam exploded wheat straw detoxified by laccases (Moreno et al. 2012). Symbols used: *Trametes villosa* laccase (▲, △) and control samples (■, □)

phenoxy radicals that lead to polymerization into less toxic aromatic compounds (Jurado et al. 2009). They have been mainly used to detoxified whole slurries (Jurado et al. 2009; Kalyani et al. 2012; Moreno et al. 2012), prehydrolysates (effluents from pretreated slurries filtration step) (Jönsson et al. 1998), and enzymatic hydrolysates (Jurado et al. 2009; Moreno et al. 2012). Moreover, yeast strains resistant to the phenols of prehydrolysates by laccase expression have been developed (Larsson et al. 2001). Finally, although in a lesser extent, LiP has been also successfully studied as detoxification method (Jönsson et al. 1998).

6.3.4 Noncatalytic Proteins

Non-catalytic proteins such as swollenins and expansins have attracted some attention. These proteins do not catalyze hydrolysis of cellulose or hemicellulose but have been shown to disrupt the crystalline structure of cellulose, thus making it more accessible to enzymes (Jørgensen et al. 2007a). Expansins are plants proteins that enable and regulate the extension of plant cell walls (Cosgrove 2000). Their cell-wall loosening action has been shown to weaken the lignocellulose structure and enhance the hydrolysis of cellulose by cellulases (Baker et al. 2000).

Swollenins are proteins with sequence similarity to the plant expansins isolated from *Trichoderma reesei*. Similarly to the expansins this protein with no catalytic activity appears to disrupt the structure of cellulose microfibers, possibly by breaking hydrogen bonds (Saloheimo et al. 2002). Other non-catalytic proteins of fungal origin belonging to glycosyl hydrolase family 61, the so-called GH61, have been shown to stimulate the activity of cellulases in synergism assays. This positive effect can significantly reduce the total protein loading required to hydrolyze lignocellulosic biomass (Merino and Cherry 2007; Harris et al. 2010).

Several GH61 genes were transformed into *T. reesei* resulting in strains expressing GH61, and obtaining fermentation broths with enhanced hydrolytic capability (Merino and Cherry 2007).

6.4 Factors Affecting Enzymatic Hydrolysis and Challenges for Bioconversion Processes

Enzymatic hydrolysis of lignocellulosic biomass is hindered by different obstacles that limit the enzymes action. Although a significant breakthrough in reducing the cost to produce enzymes has been accomplished by companies such as Novozymes, Genencor, Verenium, or DSM, enzymatic hydrolysis still requires high enzyme loadings and it is still an economic barrier for biofuels production. Therefore, the efficiency of enzymatic hydrolysis to produce sugars from lignocellulosic biomass should be further improved. Main factors affecting enzymatic hydrolysis can be divided into enzyme-related and substrate-related factors. Enzyme-related factors include end-product inhibition, thermal inactivation, enzymes synergism, and irreversible adsorption of the enzymes to lignin. The main substrate-related factors that limit enzymatic hydrolysis comprise degree of polymerization and crystallinity of cellulose, accessible surface area, and lignin and hemicellulose content (Mansfield et al. 1999; Esteghlalian et al. 2001).

For implementation of biofuels production at industrial scale, two main challenges can be identified to improve the process: **operating at high solid content** in the enzymatic hydrolysis process and **optimizing the enzyme complexes performance** to minimize enzyme loadings.

6.4.1 Optimizing Enzyme Complex

Optimizing the multienzyme mixtures involves different strategies, which include improvements in the enzyme production process, screening of novel enzyme-producing microorganisms, metagenomic approaches, random mutagenesis, genetic engineering of cellulolytic microorganisms and/or specific enzymes, addition of surfactants and enzyme recycling.

The cost of **enzymes production** is considered a major bottleneck for industrial scale development of bioconversion processes. Many organisms are capable to produce cellulases; however, the filamentous fungus *Trichoderma reesei* has been the most extensively studied. *T. reesei* produces mainly cellulases but also other activities at lower level such as xylanases or swollenins (Foreman et al. 2003; Martinez et al. 2008), which can have a positive effect on the saccharification process. One possibility to reduce costs is on-site enzyme production in a biorefinery plant. In an on-site production process, part of the lignocellulosic material

already available can be used as a cheap carbon source in the enzyme production. This system can provide appropriate enzymatic mixtures and reduce costs since downstream processes can be minimized (Jørgensen and Olsson 2006). Moreover, production of enzymes using as carbon source the lignocellulosic substrate that will be subjected to enzymatic hydrolysis has shown significant advantages. It has been reported that the composition of the carbon source can have an effect on the production of the enzymes because certain polysaccharides or monosaccharides can induce the expression of specific enzymes (Juhász et al. 2005; Kovacs et al. 2009; Sipos et al. 2010). Therefore, the cultivation of the fungus on one type of lignocellulosic material would induce different enzymes and can result in an enzyme mixture with a composition especially suitable for the hydrolysis of this particular material (Juhász et al. 2005).

It has been detailed in previously that many different enzymatic activities can be required for complete hydrolysis of lignocellulosic substrates. Thus, the **composition of the enzymatic mixtures** needs to be adapted to the characteristics of the substrate employed, that is the composition of the raw material and the modifications occurred during the pretreatment. In summary, enzyme mixtures will need to be tailored for each raw material and pretreatment, e.g., through addition of substrate-specific accessory enzymes, such as xylanases, mannanases, esterases, etc. A number or articles in recent years have evidenced the importance of accessory enzymes (Berlin et al. 2005; Berlin et al. 2006; Alvira et al. 2011a). The optimization of the enzyme cocktails for each type of pretreated substrate would favor the reduction of enzyme loadings and also the severity of the pretreatments.

Recover and recycle the enzymes from reaction suspension and bound to the residual substrate (mainly onto lignin) at the end of the enzymatic hydrolysis represents an interesting strategy to reduce the cost of the process. Different methods have been suggested to recover the enzymes, such as adsorption by contact with fresh substrate and ultrafiltration techniques. Readsorption of free cellulases onto fresh lignocellulosic substrates has been shown as an effective method for free enzyme recovery. Cellulases remain active after hydrolysis of lignocellulosic substrates and therefore can be recycled in the process (Tu et al. 2007). Ultrafiltration can also result an effective method for enzyme recovery and for continuously removing the enzymatic hydrolysis products. Qi et al. (2012) studied a two-step process using a combination of ultrafiltration for recycling the enzymes and nanofiltration for concentrating glucose, which could improve the fermentation efficiency of lignocellulosic hydrolyzates and lower the separation and purification cost of fermentative products. In a different article, it was studied the cellulase adsorption and recycling from both the liquid and the solid phase after enzymatic hydrolysis of pretreated wheat straw. It was observed that alkali treated wheat straw showed better recycling efficiency when compared to acid treated, which indicates that the efficiency of enzyme recycling is affected by the content and distribution of lignin. It was shown that ultrafiltration method had the benefit to retain β-glucosidases, compared to the absorption recycling method (Qi et al. 2011).

Another possibility to avoid unproductive adsorption of enzyme and improve the enzymatic hydrolysis performance is the addition of **additives or surfactants** to the substrate. These compounds occupy the binding sites on lignin and reduce binding potential of cellulases. This strategy can enhance the cellulose conversion and lower enzyme loading requirements. Different compounds have been evaluated as additives in the enzymatic hydrolysis. Tween 20 and Tween 80 are non-ionic surfactants that have shown a positive effect on enzymatic hydrolysis. Addition of these compounds has been reported to decrease adsorption of cellulase proteins and protect them from deactivation, having the potential to reduce total enzyme loadings (Tu and Saddler 2010; Yang et al. 2011). Polyethynelglycol (PEG) also showed a positive effect on the enzymatic hydrolysis of several pretreated lignocellulosic materials and it is a low-cost commodity product. Addition of PEG prevented unproductive binding of cellulases onto lignin and increased free cellulase activity (Sipos et al. 2011).

6.4.2 Operating at High Solid Content

Operating at high solid content in the enzymatic hydrolysis process is crucial for large-scale development of bioproduct and biofuel production processes. The aim of utilizing high solid content is to reach high sugar concentrations and subsequently high concentrations of fermentation products, such as ethanol (Jørgensen et al. 2007a; Hodge et al. 2009). Furthermore, maintaining high substrate concentrations throughout the conversion process is important for the energy balance and economic viability of biofuels production. Obtaining high concentration of fermentation product reduces global production cost since downstream processing and water consumption can be lowered. In case of ethanol production, distillation increases significantly the energy demand of the process, especially when ethanol concentrations are below 4 % (Öhgren et al. 2006).

In general, higher substrate loadings results in higher concentration of sugars. However, it has been shown that enzyme performance gradually decreases as substrate concentration increased. This can be attributed to enzyme inhibition by end products or toxics, presence of high concentrations of lignin and mass transfer limitations (Jørgensen et al. 2007a; Kristensen et al. 2009). In addition, some recent studies have reported a decrease in the adsorption capacity of cellulase enzymes to cellulose at high substrate loadings due to the effect of hydrolysis products (Kristensen et al. 2009; Wang et al. 2011). To overcome these barriers, different process configurations and strategies have been suggested to increase solids concentration in bioconversion processes.

Operating at high initial solids content (above 10–15 % (w/w)) involves technical barriers. Viscosity of the pretreated materials is usually very high, which implies mass transfer limitations and mixing difficulties. Operating fed-batch processes by adding fresh substrate when viscosity decreases has been shown as an effective strategy to increase substrate concentrations in fermentations processes

(Ballesteros et al. 2002; Varga et al. 2004). Another possibility is carrying out a prehydrolysis prior to initiate the simultaneous saccharification and fermentation (SSF) process. Using this strategy, the enzymes act at optimum temperature and reduce viscosity, which can result in higher substrates loadings (Rosgaard et al. 2007; Manzanares et al. 2011). A recent advance for operating at high consistency is the development of novel bioreactors with improved mixing capacity and low energy consumption (Jørgensen et al. 2007b; Zhang et al. 2009).

Another problem when operating at high substrate concentration is product inhibition. Cellobiose, glucose, and hemicellulose-derived sugars have been shown to inhibit the enzymes action (Xiao et al. 2004). In SSF processes, sugars released by the action of the enzymes are converted directly to ethanol by the fermenting microorganism, which reduces end-product inhibition (Ballesteros et al. 1994; Olsson et al. 2006). Constant removal of glucose during the process has been also proposed (Andric et al. 2010).

Degradation compounds originated from carbohydrates and lignin during the pretreatment affect the enzymatic hydrolysis (Tengborg et al. 2001; García-Aparicio et al. 2006) and the fermenting microorganisms (Palmqvist and Hahn-Hägerdal 2000a; Oliva et al. 2003; Oliva et al. 2004). At high substrate loadings, the concentration of these compounds increases, therefore their influence in the bioconversion process can become more significant. Washing the pretreated material has been typically employed to eliminate toxic compounds and increase enzymatic hydrolysis and fermentation yields. To avoid washing and use the whole slurries, detoxification strategies such as laccase treatments have been studied to reduce the concentration of phenolic compounds and increase substrate concentrations in fermentation (Moreno et al. 2012).

Different articles reported the utilization of high substrate concentrations for ethanol production. Using wet oxidized and steam exploded corn stover, a substrate consistency of 15 % and 10–30 % dry matter (DM) in fermentation experiments, respectively, was studied (Varga et al. 2004; Lu et al. 2010). Using corn stover pretreated by combination of stream explosion and alkaline hydrogen peroxide, it was reached a solids loading of 30 % (Yang et al. 2010). With hydrothermal pretreated and steam pretreated wheat straw, it was possible to carry out hydrolysis and SSF at high substrate concentrations up to 20–30 % (Jørgensen 2009; Ballesteros et al. 2011), and with steam pretreated spruce it could be reached a consistency of 14 % (Hoyer et al. 2010).

6.5 Conclusion

Efficient utilization of lignocellulosic materials in a biorefinery depends on the advances in pretreatment technologies, enzyme saccharification, and fermentation of sugars to fuels and chemicals. Optimization of pretreatment and enzymatic hydrolysis processes is crucial to make bioconversion processes from lignocellulosic biomass viable and cost-effective. The aim of the pretreatment is increasing

the digestibility of carbohydrates while minimizing degradation of sugars and generation of toxic compounds. The pretreatment has to be adapted to the different raw materials and should be validated at large scale. The cost and efficiency of enzyme products still represents a major bottleneck to improve the economy of industrial biorefineries. To reduce costs of enzymatic hydrolysis processes, it is required the optimization of enzymatic mixtures in order to increase sugars production yields, reduce pretreatment severity, and decrease enzyme dosages. Complexity of lignocellulosic substrates involves that enzyme cocktails should be adapted for each raw material and type of pretreatment. In addition, operating at high solid content should be considered as a key issue for biofuels production. Finally, the integration of all the process steps has a remarkable importance to increase overall process efficiency and promote large-scale development. The type of biomass and pretreatment determines the process configuration requirements for hydrolysis and fermentation as each step has a large impact on all subsequent stages.

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Chapter 7
Microbial Glycoside Hydrolases for Biomass Utilization in Biofuels Applications

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Abstract Renewable biomass is predicted to have the potential to meet at least a quarter of the world demand for transportation fuel, but to do so both terrestrial lignocellulosic as well as marine algal resources need to be efficiently utilized. In the processes where these biomasses are converted into different types of energy carriers (such as fuel-alcohols e.g. ethanol or butanol) microbial glycoside hydrolases (GHs) have a role in the saccharification process. During saccharification polymeric carbohydrate resources (e.g. starch, cellulose or hemicellulose) are hydrolyzed into mono and oligosaccharides that can be utilized by the organism selected to ferment these carbohydrates into the desired energy-carrier. This chapter aims to shed light on different processing alternatives for the conversion of lignocellulose or algal starch into mono or oligosaccharides, and what roles the microbial GHs have as processing aids in these conversions.

7.1 Introduction to Biofuels

With the depletion of crude oil, attention has gone toward use of natural recoverable resources for production of biofuels. Public and scientific attention is also driven by factors such as the price, concern over greenhouse gas emissions, as well as support from government subsidies. In 2010, worldwide biofuel production reached 105 billion liters and provided 2.7 % of the fuels for road transport (Shrank and Farahmand 2011). Moreover, it is predicted that biofuels have the

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potential to meet more than a quarter of world demand for transportation fuels by 2050 (Platts 2011).

A biofuel is by definition a fuel, whose energy is derived from biological carbon fixation. This includes fuels derived directly from solid biomass or fuels obtained by conversion of biomass into energy-carrying compounds such as fuel-alcohols (e.g. methanol, ethanol, and butanol), biodiesel, hydrogen, or biogas (Chandra et al. 2012). These biofuels are liquid or gaseous, and with this they meet the requirements of (a) being portable, (b) being easy to handle (they can be pumped), and (c) to burn cleanly. Bioethanol is the biofuel that today is produced in largest amounts. Ethanol production accounted for more than 80 % of the biofuel production volume (86 billion liters in 2010, of which almost 90 % were produced in the US and Brazil) (Shrank and Farahmand 2011).

Biofuels are defined as first, second or third generation, based on the type of biomass and technology used for its production (Fig. 7.1). First generation biofuels are made by sugar, starch, and vegetable oil by established technologies, and include mainly ethanol, biogas, and biodiesel. The use of first generation technology has, however, been the subject of considerable media attention and political debate to draw attention to the environmental and social impacts of producing biofuels from food crops (European Biofuels Technology Platform 2009). Second generation biofuels, include the ethanol and biogas as above, but in this case the fuels are produced from cellulosic materials (lignocellulosic feedstocks) which are more challenging to degrade into fermentable sugars for further conversion. Second generation biofuels also include other types of fuels e.g. hydrogen, other bio-alcohols, and mixed compounds (Fig. 7.1).

The lignocellulosic biomasses are not food crops or are the nonedible parts of the food crops, and demand technology developments for efficient processing. Moreover, the feedstocks should be defined as sustainable, and sustainability is for instance judged based on the availability of the feedstock, the impact on green house gas (GHG) emissions and the impact on biodiversity and land use (European Biofuels Technology Platform 2009). Recently, a third generation of biofuels has also been suggested implying the use of (macro and micro) algae as biomass (An et al. 2011). Algae can be cultured on sea or wastewater and do not require the same use of land area. Algae has to date mainly been considered for biodiesel, hydrogen, and biogas production (Demirba 2011; Aitken and Antizar-Ladislao 2012), but an emerging interest has also risen for their use in ethanol production (Demirbas 2010; Aitken and Antizar-Ladislao 2012; Harun et al. 2010). In this chapter, our focus is on the second and third generation biomass resources, and what possibilities the microbial glycoside hydrolases (GHs) give us to access and degrade the polymeric carbohydrate fibers into shorter oligo and monosaccharides fermentable by microorganisms for conversion into metabolites, which are the energy carriers of biofuels. The oil fractions used for biodiesel production have been reviewed elsewhere (see for example Stuart et al. 2010) and are not considered in this chapter.

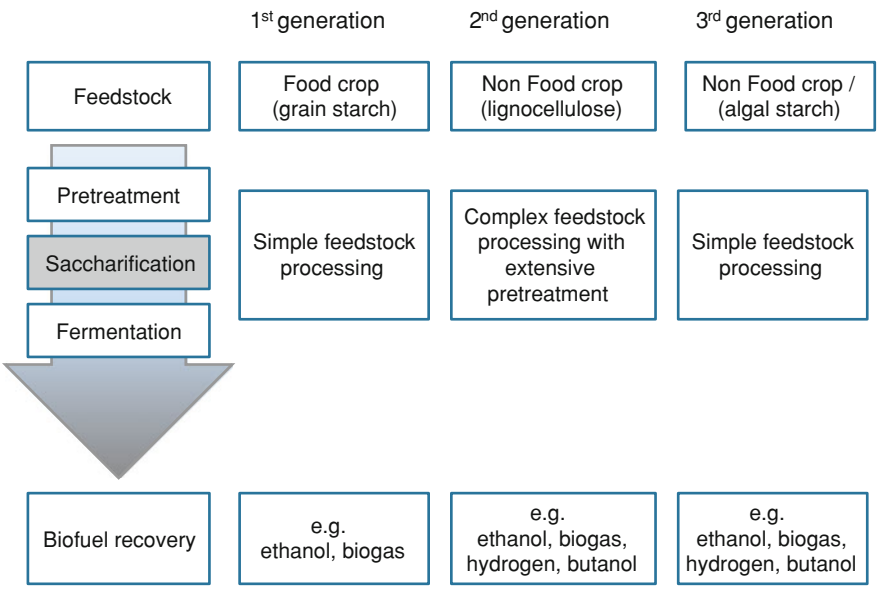

Fig. 7.1 A simple division of 1st, 2nd and 3rd generation biofuels from carbohydrates, based on feedstock and processing. GHs have a role in the saccharification step, which is the degradation of polymeric carbohydrates into smaller oligo or monosaccharides allowing fermentation into the desired biofuel. Biodiesel production is excluded from the scheme as it is based on oil and has a different overall processing scheme

7.2 Biomass for Second and Third Generation Biofuels

7.2.1 Lignocellulosic Biomass—Raw Material for Second Generation Biofuel

Lignocellulosic feedstocks consist of mainly cellulose, hemicellulose, and lignin and can be found in the cell walls of almost all plant-derived materials, such as wood and grass, agricultural residues, and municipal solid wastes. The relative composition of the lignocellulosic material, however, varies greatly, depending on source (Chandel and Singh 2011; Garrote et al. 1999; Mosier et al. 2005) and for an overview, the weight percentage of dry biomass of representative lignocellulosic materials are listed (Table 7.1).

Cellulose (β-1-4-glucan), a linear polymer of glucose units, is the major component of the lignocellulose (accounting up to 50 % of the total plant dry weight), the most abundant form of biologically fixed carbon in the biosphere, and a primary target for biofuels that are metabolites from microbial conversions (as in bioethanol production). It is hence a material of high interest to utilize well, but also a very recalcitrant material, making its utilization difficult. A major challenge is still to manage to convert lignocellulose in high yields to fermentable sugars

Table 7.1 Percent dry weight composition of some lignocellulosic feed stocks and paper wastes

Feedstock	Cellulose	Hemicellulose	Lignin
Corn stover	37.5	22.4	17.6
Corn fiber	14.3	16.8	8.4
Pine wood	46.4	8.8	29.4
Poplar	49.9	17.4	18.1
Wheat straw	38.2	21.2	23.4
Switch grass	31.0	20.4	17.6
Office paper	68.6	12.4	11.3
Newspaper	61	16	21

Extracted from Mosier et al. (2005); Chandel and Singh (2011)

(see also Sect. 7.3. Lignocellulosics requires pretreatment for degradation) and to follow this with an efficient process that reduces the oxygenated carbohydrates to fuel molecules (Chundawat et al. 2011). In the process to obtain fermentable sugars, microbial GHs are used as catalysts to obtain saccharification (hydrolysis) of different polysaccharides in the biomass (explained more in the sections below). The microbial GHs are catalysts designed to degrade complex carbohydrate polymers into mono or oligosaccharides, that allow uptake and metabolism by the microorganism selected as cell-factory for the conversion into the desired biofuel, even if the microorganism on its own is not capable to degrade the polymeric carbohydrate forms.

It has been predicted that based on available land, the energy potential of lignocellulosics worldwide allows an energy outtake of approximately 100 EJ/annum (1 EJ = 1×10^{18} J, covering woody biomass, straw, and energy crops) (Parikka 2004), which is to be compared to the global energy demand (425 EJ in 2001) (Lewis and Nocera 2006) showing that approximately one-quarter of the current demand can be obtained, and thus additional resources are needed to cover a shift from fossil to renewable resources. A means to increase the possible overall energy outtake is to also turn to biomasses from marine environments.

7.2.2 Algal Biomass—Marine Resources as Rawmaterial for Third Generation Biofuels

Algae have the possibility to provide a high-yield source of biofuels without compromising food supplies, forests, or arable land (Subhadra and Edwards 2010; John et al. 2011; An et al. 2011), thereby being an interesting complement to the lignocellulosic second generation agricultural feedstocks. Marine environments are predicted to supply approximately 50 % of global biomass (Carlsson et al. 2007; John et al. 2011) thereby significantly increasing the potential of biomass as a source of transportation fuel. Algae represent a large number of different photosynthetic species (both heterotrophic and autotrophic). The autotrophic species

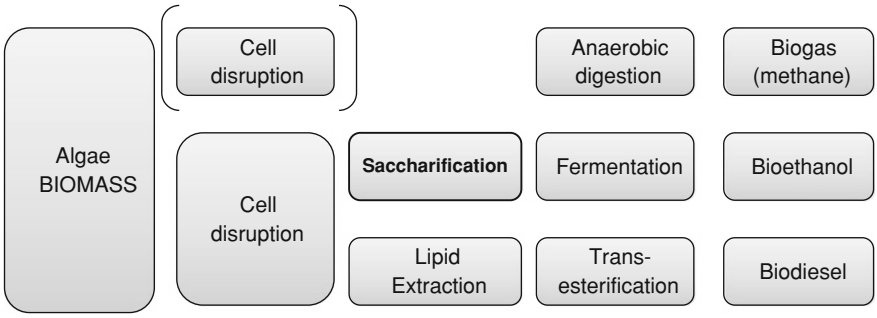

Fig. 7.2 Schematic process steps for the three fuel types that are currently considered as most suitable for energy production from algal biomass (adapted from Aitken and Antizar-Ladislao 2012). Enzymatic processing using GH is primarily predicted in the saccharification (or polysaccharide hydrolysis step, in bold) to boost fermentable sugars in the bioethanol production process

can fix inorganic carbon from CO_2 which is assimilated into for example carbohydrates (John et al. 2011), which can be converted into fermentable sugars for further conversion into selected energy carriers (Fig. 7.2). The heterotrophic species take up organic molecules and convert into mainly lipids and protein, of which the lipid fraction is of interest for biodiesel production (Fig. 7.2). Some species, called mixotrophic algae, can utilize both processes (John et al. 2011). Through these processes, carbohydrates, lipids, and proteins can be produced in a very short time, allowing as frequent harvests as in 1–10 days for some microalgae (Harun et al. 2010).

Based on size and morphology, algae are roughly grouped as macro- or microalgae. As the name implies, microalgae are microscopic frequently unicellular organisms. Macroalgae are multicellular, and are composed of structures resembling higher plants, with the difference that they are buoyant and do not need the lignin-containing structural polymer complexes that are necessary for terrestrial plants. This makes their polysaccharides easier to degrade into fermentable sugars (John et al. 2011; Chen et al. 2009). The types of polysaccharides available in the algae differ dependent on the species, but there are species shown to have both high cellulose and high starch content (Table 7.2). Use of organisms with high starch content, would allow the same type of processing of the starch as for first generation biofuels, involving use of starch degrading GHs.

7.3 Lignocellulose Requires Pretreatment for Degradation

In plant biomass, cell wall models predict cellulose microfibrils (polymers of β-1, 4-linked glucose packed by hydrogen bonds) surrounded by a matrix of hemicellulose (e.g. xylans, mannans, glucans, and xyloglucans), pectin (polymers of

Table 7.2 Carbohydrate, protein, lipid and starch content in some selected algae

Algal source	Protein (%)	Lipid (%)	Carbohydrate (%)	Starch (% of biomass after oil extraction)
Chlamydomonas C. reinhardtii	48	21	17	53 (strain UTEX90) 45 (strain UTEX2247)
Chlorella	51–58	14–22	12–17	12–37
C. vulgaris	57	2	26	*n.d.
C. pyrenidosa				
Dunaliella D. salina	57	6	32	n.d.
Scenedesmus S. obliquus	50–56	12–14	10–17	23 (strain TISTR85446)
Spirulina	n.d.	n.d.	n.d.	37–56
S. fusiforma	60–71	6–7	13–16	n.d.
S. maxima	46–63	4–9	8–14	n.d.
S. platensis				

*n.d. = not determined

From Aitken and Antizar-Ladislao (2012); John et al. (2011); Rodjaroen et al. (2007)

mainly galacturonic residues, common in the middle lamella of the cell wall), and lignin (phenyl-propanoid polymers). Unbranched hemicelluloses form hydrogen bonds with the surface of cellulose microfibrils, while branched hemicellulose form bonds (mainly ester linkages) with the phenolic acids in lignin (Chundawat et al. 2011; Sjostrom 1993). This association of cellulose, hemicellulose, pectin and lignin results in bundles, or macrofibrils formation.

Due to this physicochemical, structural, and compositional complexity cellulose is resistant to microbial as well as enzymatic digestion. Many microorganisms also lack the enzyme systems necessary for efficient degradation of the lignocellulosic material, and this is especially evident in cases, such as the conventional way of ethanol production, where a single noncellulolytic microorganism (typically *Saccharomyces cerevisiae*) is used for the conversion from carbohydrate to biofuel. Thus, it is necessary to start with a pretreatment step which reduces the crystallinity of the cellulose, removes lignin and hemicellulose, and improves the porosity of the biomass. This enhances the accessibility of cellulose to enzymes, which in turn leads to a more efficient conversion of cellulose to fermentable sugars. Over the years, an impressive number of pretreatment methods have been developed that breaks down the intertwined interaction, among others, between lignin, cellulose and hemicelluloses, and a summary of the common pretreatment methods is given in Table 7.3. The efficiency of the treatment methods varies from method to method, and depends on the type and source of the biomass treated.

For a given biomass, among the available methods, a suitable pretreatment can be selected based on: (a) process cost, (b) susceptibility of the treatment product to enzymatic hydrolysis, (c) effect of pretreatment on hemicelluloses and cellulose, (d) presence or absence of by products that inhibit enzyme activity and fermentation processes, and (e) amount and type of chemical consumption.

Table 7.3 Summary of lignocellulosic pretreatment methods

Pretreatment	Process	Principles	Notable remarks	References
Physical	Milling, chipping, grinding	Easier handling and increased surface area to volume ratio which facilitates reactivity. Decreased degree of lignocellulose crystallinity	First step in multiple pretreatment processes. High power consumption	Tassinari and Macy 1977; Cadoche and Lopez 1989; Galbe and Zacchi 2007
	Irradiation	The energy breaks the hydrogen bonds of the cellulose crystalline structure and makes it prone to enzymatic digestion	Efficient in the presence of lignin. Expensive and not convenient for large scale application	Kumakura and Kaetsu 1983; Kumakura et al. 1982
	Hydro-thermal	Uses water at high temperature and pressure that dissolves most of the lignin and hemicelluloses which in turn facilitates the hydrolysis of the cellulosic fraction	Generates acetic and other organic acids	Mosier et al. 2005; Negro et al. 2003
	Pyrolysis	Use of high temperature to disrupt the lignocelluloses	Efficient when carried out in the presence of oxygen	Shafizadeh and Bradbury 1979
Physio-chemical	Explosion e.g. steam explosion, ammonia fiber explosion, CO_2 explosion	Alters the structure of cellulosic biomass to make it more accessible. Exposes biomass to high temperature and pressure followed by a sudden pressure fall to make an explosive decomposition	Steam explosion is the most commonly used method for the pretreatment of lignocellulosic biomass	Grous et al. 1986; Brownell et al. 1986; Emmel et al. 2003; Kumar et al. 2009
Chemical	Acid	Increases the biomass porosity by removing the hemicelluloses and altering the lignin structure, and this facilitates enzymatic digestibility	Accompanied with aldehyde formation. Consumes significant energy for pretreatment and product recovery	Mosier et al. 2005; Kumar et al. 2009
	Alkali	Removes lignin, acetyl, and various uronic acid substitutions from the biomass by saponification that breaks the intermolecular ester bonds. This improves enzymatic digestibility of the biomass	Utilize lower temperatures and pressures compared to other pretreatment methods	Kassim and El-Shahed 1986; Fox et al. 1989; MacDonald et al. 1983
	Wet oxidation	Treatment of biomass in the presence oxygen/air and water at high temperature and pressure opens the crystalline structure of cellulose	All biomass fractions are affected. Hemicelluloses degrade substantially	Palonen et al. 2004; Varga et al. 2004; Martin et al. 2007
	Ozonolysis	Targets lignin degradation by attacking and cleavage of aromatic rings structures	The cellulose and hemicellulose fractions remain intact	Neely 1984; Euphrosine-Moy et al. 1991
	Solvent extraction	Use solvents to remove lignin and some hemicelluloses to facilitate enzymatic hydrolysis of lignocellulosic biomass. Often accomplished at moderately high temperature	Requires removal of the solvent from the treated biomass	Pan et al. 2005; Pan et al. 2006; Araque et al. 2007
Biological	Microbiological	Micoorganisms (often fungi) degrade lignin and hemicelluloses	Long process but low in energy consumption and requirement	Kurakake et al. 2007

7.4 Enzymatic Hydrolysis of Lignocellulose

7.4.1 Hydrolysis of Cellulose to Fermentable Sugar

Cellulose is one of the most important resources for production of the biofuel ethanol. However, the common organisms used in the production of bioethanol, *S. cerevisiae* and *Zymomonas mobilis* cannot utilize cellulose. Therefore, cellulose has to be depolymerized to a fermentable sugar (glucose) that can be utilized by these organisms to produce bioethanol. Although there are alternative chemical methods of cellulose depolymerization, the enzymatic hydrolysis is a preferred process as it results in high-quality hydrolysate (no side products) and uses mild reagents (enzymes), which is beneficial from a sustainability perspective.

The major cellulose degrading enzymes belong to the GHs. These cellulose degrading enzymes are subcategorized as (a) endo-glucanases (E.C. 3.2.1.4) enzymes that randomly attack the β-1, 4-linkages within the polymer chain and release oligosaccharides, (b) exo-glucanases or cellobiohydrolases that cleave off cellobiose either from the reducing (E.C. 3.2.1.176) or nonreducing ends (E.C. 3.2.1.91) of the chains, and (c) β-glucosidases (E.C. 3.2.1.21) which degrade smaller chain oligosaccharides releasing the terminal, nonreducing β-D-glucosyl residue (Fig. 7.3). All the cell wall degrading enzymes are classified under multiple glycoside hydrolase families (which are based on similarities in sequence and structure), showing examples of convergent evolution. Endo-glucanases are for example classified under many different GH-families, with different folds and with both retaining (GH5, 7, 12, 44, 51) and inverting (GH6, 8, 9, 45, 48, 74, 124) reaction mechanisms (see: http://www.cazy.org). The cellobiohydrolases are structurally related to endo-glucanases and the enzymes acting from the reducing end are mainly classified under GH7 and 48, while those acting from the nonreducing end are predominantly found in the inverting GH6 and 9. The β-glucosidases are classified under GH1, 3, 9, 30 and 116, of which the inverting GH9 also harbor structurally related endo-glucanases and cellobiohydrolases.

The architecture of the plant cell wall degrading enzymes varies and many of the microbial enzymes are composed of a number of modules (Mba Medie et al. 2012). The ancillary modules are often carbohydrate binding modules (CBMs), which are believed to target the enzyme (catalytic module) toward certain parts of the cell wall.

The use of endo-glucanases, exo-glucanases, and β-glucosidases in combination exhibits a synergistic effect on depolymerization of both crystalline and amorphous cellulose to the fermentable sugar glucose (van Dyk and Pletschke 2012). The commercially available GHs which are currently in use by the sector are in principle originated from fungi and cellulases account about 20 % of the total enzyme market which is estimated to be 6 billion dollar in 2012 (Mathew et al. 2008). However, a vast number of alternative enzymes exist, and many efforts have for example been put in developing methods utilizing thermostable enzymes (Turner et al. 2007), allowing processing at higher temperatures. The cost

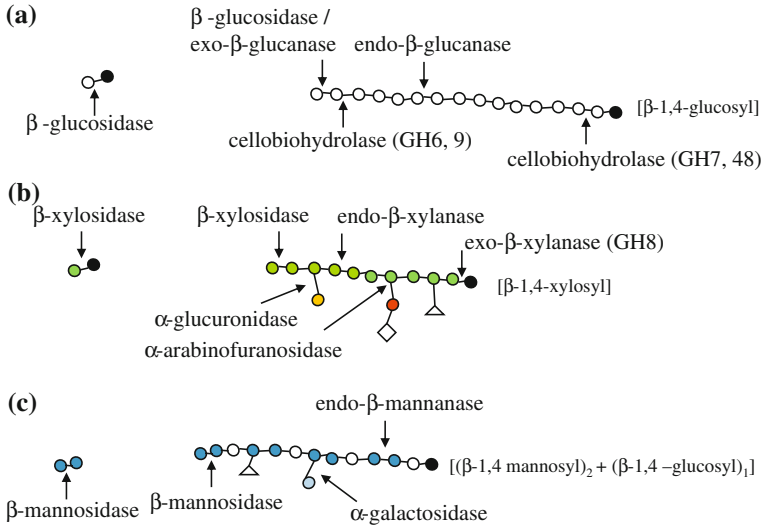

Fig. 7.3 Simplified structures and sites of enzymatic attack on polymers from lignocellulose. A cellulose chain fragment is shown (**a**), along with hypothetical fragments of the hemicelluloses xylan (**b**), and glucomannan (**c**). Sites of attack of some of the major enzymes acting on the respective material are indicated by *arrows*. The glycosidic bond type of the main chain is indicated in *brackets* to the right of each polymer fragment. Carbohydrates are indicated as *circles*, and the reducing end of each main chain is shown as a *black* circle. White = glucose, green = xylose, yellow = glucuronic acid, red = arabinose, dark blue = mannose, light blue = galactose, Acetate groups are shown as *triangles*, phenolic groups as *diagonals*, and methyl groups as *rombs*. Adapted from Turner et al. (2007)

of cellulases is still high (Cheng and Timilsina 2011) and it needs a concerted effort to bring it down to a comfortable price floor.

In addition to GHs, it is recently shown that oxidative enzymes such as cellobiose dehydrogenase and polysaccharide monooxygenases also cleave the glycosidic bonds in cellulose and play a role in its degradation (Phillips et al. 2011, Mba Medie et al. 2012). Some of these enzymes, such as the chitin-binding protein (Cbp21) or the oxidative enzymes classified under GH61 (Vaaje-Kolstad et al. 2010; Harris et al. 2010), have been shown to open up structures in crystalline polysaccharides (like cellulose and chitin) that are inaccessible by other GHs.

7.4.2 Hydrolysis of Hemicelluloses

The thin profit margin of ethanol production from lignocellulose is partly due to the utilization of only the cellulose fraction of the biomass (Gowen and Fong 2010). Thus, the utilization of hemicelluloses is expected to increase the profitability of the process, and this has initiated a remarkable degree of research

activity. As for cellulose, the utilization of hemicelluloses requires hydrolysis of the polymers into oligomeric and monomeric units. However, unlike cellulose, hemicelluloses are structurally and chemically heterogenous and in general vary from source to source (Beg et al. 2001). Both chemical and enzymatic hydrolysis processes exist that can depolymerize hemicelluloses; however, from a sustainability perspective, the enzymatic hydrolysis is preferable over the chemical route. Xylans are the most common type of hemicellulose in plants, and are heteropolysaccharides with homopolymeric backbone chains of 1, 4-linked β-D- xylopyranose units (Saha 2003; Garrote et al. 1999; Koukiekolo et al. 2005).

Xylanases that degrade this fraction of the biomass are often used in the hydrolysis of lignocellulosic biomass. Like the cellulolytic enzymes, xylanases can be divided into endo-acting xylanases (E.C. 3.2.1.8, available in GH5, 8, 10, 11, 43), exo-acting xylanase (E.C. 3.2.1.156, found in GH8) acting from the reducing end, and complemented with xylosidases (E.C. 3.2.1.37, for example in GH1, 3, 39, 43, 52, 54, 116, 120) acting from the nonreducing end (Shallom and Shoham 2003). The enzymes acting on hemicellulose are like the cellulolytic enzymes also frequently modular, composed of catalytic as well as ancillary domains (Shallom and Shoham 2003). It can also be noted that a single GH-family often includes enzymes of many different specificities. Mutagenesis studies have shown that exchange of only a few residues in the glycone binding site will change the binding preference of one monosaccharide for another (Corbett et al. 2001) and in GH-family 1 and 3 many of the glycosidases can for example hydrolyze gluco- as well as xylo-oligosaccharides (Yernool et al. 2000; Zhou et al. 2012).

Use of other hemicellulose degrading enzymes such as mannanases (EC 3.2.1.78, acting on the different mannan containing hemicelluloses, mainly classified under GH5, 26 and 113), mannosidases (EC 3.2.1.25, GH1, 2 and 5), galactosidases (EC 3.2.1.23, GH1, 2, 3, 35, 42), and arabinofuranosidases (EC 3.2.1.55, GH3, 43, 51, 54, 62) (Fig. 7.3) can together with xylanases (dependent on the biomass used) further increase the monosaccharide yield of certain materials, to obtain better conversion of hemicelluloses to their monomeric units.

Most of this work has been implemented to improve the production of ethanol, and in this field a sizable portion of the research has also been related to engineering fermentative organisms to make them utilize the monomeric pentoses and produce ethanol (Hahn-Hägerdal et al. 2007). The fermentation of pentoses to ethanol undoubtedly improves the overall production of ethanol from lignocellulosic biomass. However, the inability of naturally existing commercial ethanol producer strains has hindered its implementation. (*S. cerevisiae*, does not naturally utilize pentoses). Today, there are many metabolically engineered yeast strains that successfully produce ethanol from xylose (Matushika et al. 2008, 2009; Kuhad et al. 2011) and this will in the future improve the ethanol yield per unit mass of the lignocelluloses used and is predicted to improve the profit margin for companies involved in production of ethanol. Another field of engineering involves introduction of cellulolytic enzymes to enable cellulose degradation or use of cellulolytic microorganisms followed by modification of their ethanol production pathway, described more in the following section.

7.5 Exogenous or Endogenous Enzymes for Saccharification of Lignocellulose

The production of ethanol from biomass can be accomplished in any of three known processes. In the conventional process, the hydrolysate which is obtained from a separate hydrolysis of pre-treated biomass, is used to formulate fermentation media for production of ethanol often using *S. cerevisiae* or *Z. mobilis* (Fig. 7.4). This method is the most common process. However, it is believed that the separate processing steps made the process relatively expensive, and hence alternative methods have been developed. The process known as simultaneous saccharification and fermentation (SSF) or simultaneous saccharification and co-fermentation (SSCF) is one of the alternative approaches. In this process, the hydrolysis of the pre-treated biomass (with addition of exogenously produced cellulose degrading enzymes) and the fermentation process for production of ethanol performed simultaneously in the same reactor. However, this process requires biomass feedstock which is extensively pre-treated (Carere et al. 2008). Although extensive pretreatment is necessary to ensure easy handling and efficient enzymatic degradation of the cellulose, it is expensive. The third alternative process of ethanol production from lignocellulosic biomass is consolidated bio-processing (CBP), a system in which cellulase production, substrate hydrolysis, and fermentation is accomplished in a single process step by cellulolytic micro-organisms (with endogenous cellulose degrading enzymes) (Carere et al. 2008;

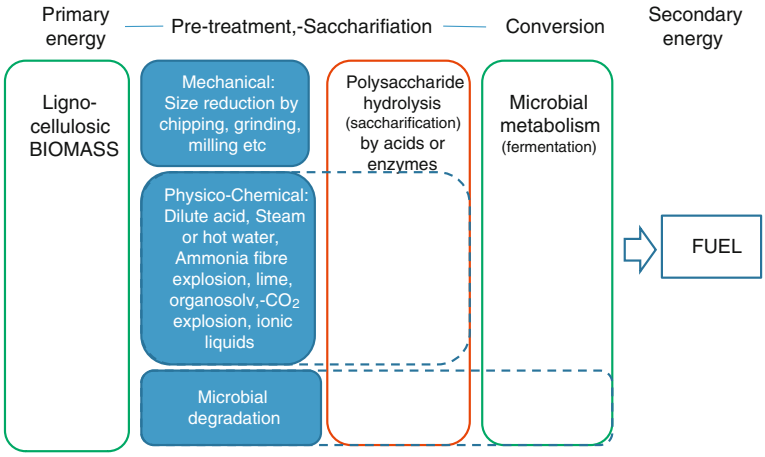

Fig. 7.4 For the conversion of recalcitrant lignocellulosic biomass the pretreatment is a necessity to gain enough efficiency in the following conversion. The different methods range from low to high pH, and novel methods are still under development. The possibilities of microorganisms to take up polymeric sugars are often limited, and hence the final pretreatment step is frequently a hydrolysis step, allowing uptake of mono and oligosaccharides in the organism (s) selected for the conversion into the energy carrier. In this step, microbial GHs have a potential

Hasunuma and Kondo 2012). Combining these three processes in a single step is expected to substantially cut down the ethanol production cost. However, despite this great promise, so far, there is not even a single microorganism that fulfills all the required traits of substrate hydrolysis and ethanol production at commercial level. This has led to the option of metabolically engineering selected microorganisms that can potentially be used for production of ethanol from biomass through (CBP) system (Hasunuma and Kondo 2012).

There are two possible approaches to engineer organisms for the CBP system, i.e., to recombinantly express the necessary cellulose degrading enzyme in a commercial ethanol producer strain such as *S. cerevisiae* (van Zyl et al. 2007; van Wyk et al. 2010) or enhance the ethanol producing capabilities of known cellulolytic microbes such as *Geobacillus*, *Clostridium* or *Fusarium*. For example *Fusarium oxysporum* is known to produce several cellulose and hemicellulose degrading enzymes and ferment both hexose (glucose) and pentose (xylose) into ethanol with reasonably good yield (1.8 mol ethanol/mole of glucose and 1 mol ethanol/mole of xylose) (Panagiotou and Christakopoulos 2004; Xiros and Christakopoulos 2009). Thus, if the ethanol yield is increased through metabolic engineering, this organism is attractive for CBP of ethanol production. To date, attempts in this direction have been made in the cellulolytic moderate thermophile *Geobacillus thermoglucosidasius*, by elimination of the lactate dehydrogenase and pyruvate formate lyase pathways together with upregulation of the expression of pyruvate dehydrogenase (Cripps et al. 2009) resulting in improved ethanol production. Unlike aerobic microorganisms which generally produce noncomplexed extracellular cellulases, some anaerobic cellulolytic microorganisms such as *Clostridium* spp. are also of interest, as these organisms degrade cellulose using a large extracellular complex of enzymes that act in a consortium known as the cellulosome (Carere et al. 2008). Cellulosomes often contain GHs, polysaccharide lyases and carboxyl esterases which are arranged around the noncatalytic protein scaffolding which consists of cohesins linked to enzymes and carbohydrate binding modules using dockerins (Ding et al. 2008; Gilbert 2007; Bayer et al. 2007; Fontes and Gilbert 2010). This complex consortium degrades not only cellulose, but also other plant biomass fractions such as hemicelluloses and pectin which makes it attractive for bioethanol production.

7.6 Use of Algal Starch for Production of Ethanol

A wide range of research aiming to utilize algae for production of energy is ongoing. Like plant biomass, the algal biomass contains carbohydrates that can be used for production of ethanol through fermentation (Goh and Lee 2010; Brennan and Owende 2010). Starch has been the most ideal substrate for production of ethanol. However, the starch used in conventional fermentation comes from grains and this created a competing demand between food and energy production and hence not favored from social, economical, and political stands. The use of starch from

nonfood sources alleviates the problem emerged from the fierce competition between food and energy production. Different algal genera such as *Chlorella, Glacilaria*, *Spirulina*, *Prymnesium*, *Ulva* etc. are known to accumulate starch (Zemke-White and Clements 1999) (Table 7.2), which can be used for bioethanol production the same way grain starch is being used. For hydrolysis of the starch, different enzymes from the α-amylase superfamily (Fig. 7.5) are utilized. The family consists of sequence-related retaining enzymes (classified under GH13, 70 and 77)

If the algal starch is considered for ethanol production, it needs to be gelatinized, and enzymatically liquefied and saccharified as in the conventional grain starch processing for ethanol production (Turner et al. 2007). In liquefaction, thermostable α-amylase (EC 3.2.1.1) is used to obtain oligosaccharides, followed by saccharification using *β*-amylase (EC 3.2.1.2) to obtain maltose or glucoamylase (EC 3.2.1.3) to obtain glucose. The efficiency of the saccharification can also be increased by adding debranching enzyme (or pullulanase, EC 3.2.1.41). Recently, it has also been shown that presence of cyclodextrins [produced by cyclodextrin glycosyltransferase (CGTases EC 2.4.1.19)] can increase the ethanol tolerance of e.g. *S. cerevisiae* in the following fermentation step (Liang et al. 2011).

However, gelatinization of starch is an energy intensive process and there has been a growing interest to decrease the energy consumption of starch processing.

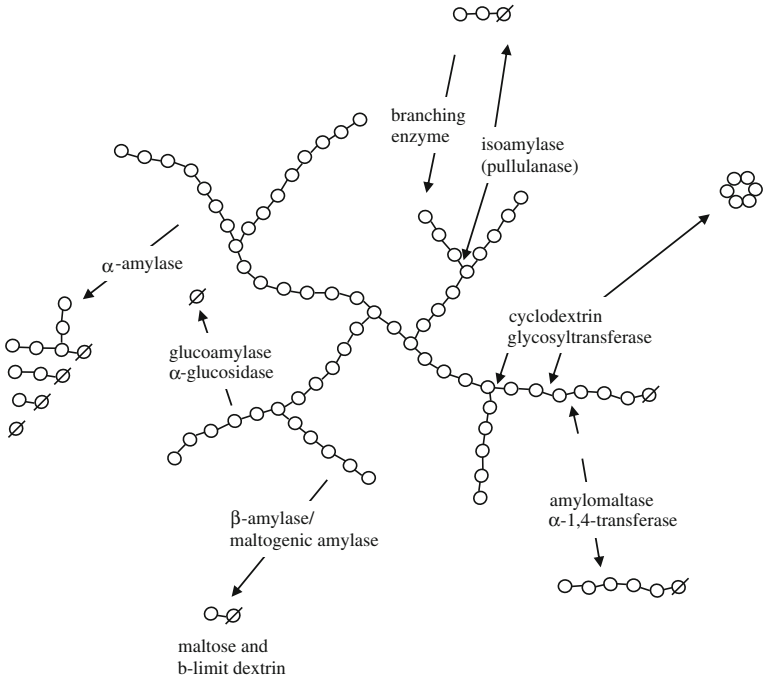

Fig. 7.5 Enzymatic attack on part of a hypothetical amylopectin molecule. Circles are glucose molecules and circles with a line through represents a reducing glucose molecule. Adapted from Turner et al. (2007)

The high temperature-cooking of starch (at 140–180 °C) which is necessary to disrupt the starch granules increases the ethanol production cost. Direct grain raw starch saccharification is one possible alternative to achieve a reduction in the energy consumption of the process (Robertson et al. 2006). Indeed, the use of low temperature-cooking fermentation systems has been tried and it succeeded in reducing the energy consumption significantly (Matsumoto et al. 1982, Shigechi et al. 2000). However, raw starch is known to be resistant to enzymatic hydrolysis and this has limited its application. Interestingly, algal raw starch is known to degrade efficiently compared to raw starch coming from food grade cultivars (Meeuse and Smith 1962).

7.7 Conclusion

Biofuel production from renewables requires use of lignocellulosic biomass as well as algal biomass to allow large production volumes. This requires hydrolysis of lignocellulose as well as algal starch. For starch processing, enzymatic technologies are developed and in addition a number of enzyme mixes are available. Lignocellulose hydrolysis is more complex, but the understanding is progressing and current research shows that hydrolysis is stimulated by interplay with hydrolyzing enzymes (like the microbial GHs) and oxidizing enzymes. A question for the future is if use of exogenous enzymes is economically feasible, or if endogenous enzymes in engineered organisms for high production are more desired for future biofuel production systems. The advantage using exogenous enzymes is that saccharification is general and can in a following step be used for different fermentation processes (using different microorganisms, and for production of different types of energy carriers). Endogenous enzymes can, however, be a promising alternative when developing efficient organisms for a single process. In the future, it is likely that both strategies will be in use for different purposes.

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Chapter 8
Developing Cellulolytic Organisms for Consolidated Bioprocessing of Lignocellulosics

Willem H. van Zyl, Riaan den Haan and Daniel C. la Grange

Abstract Lignocellulosic biomass represents an abundant, renewable feedstock for the production of biofuels and chemicals in a potentially sustainable manner. The main reason that it is not widely used at present is the technological barrier that there is no low-cost technology, either biological or thermochemical, to overcome the recalcitrance of lignocellulose. An organism that hydrolyzes the polysaccharides in biomass and simultaneously produces a commodity product such as ethanol at a high rate and titer could significantly reduce the costs of biomass conversion through the biological route. This would allow a combination of steps that are currently accomplished in different reactors into a consolidated bioprocess (CBP). While no ideal wild-type organism has been identified that can be used in CBP, several candidates are in various stages of development. This chapter assesses the *status quo* for CBP organismal development either by enabling noncellulolytic organisms to grow on cellulosic substrates, by improving product forming abilities of native cellulose utilizing organisms, or by engineering organisms with improved cellulolytic and product forming abilities. Furthermore, the state of the art of feedstock, pretreatment, and process integration options are briefly assessed.

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8.1 Introduction

The increasing premium many governments place on greater energy security and environmental concerns juxtaposed with the increasing demand for and cost of oil has led to the development of an active biofuels industry (Van Zyl et al. 2011). First generation biofuels such as ethanol from corn starch or sugarcane already contribute considerable amounts of liquid fuels in several countries. However, these technologies suffer from a shortage in the availability of feedstock in order to displace a more significant amount of petroleum-based fuels. Lignocellulosic biomass represents the most abundant source of carbon in nature and is the only source that could possibly provide sufficient feedstock to satisfy the world's energy and chemicals needs in a sustainable and renewable manner (Hill et al. 2006; Van Zyl et al. 2011). Second generation biofuels, such as ethanol from cellulosic biomass, therefore seek to overcome the problem of feedstock supply shortage by utilizing the energy contained in total plant biomass. Current technologies for conversion of biomass into ethanol via a biological route commences with a pretreatment step during which physical and/or chemical processes are used to make the polymeric fractions more accessible to enzymatic hydrolyses (Stephanopoulos 2007). Feedstock origin will determine the optimal type of pretreatment required, which in turn defines the optimal enzyme mixture required in subsequent hydrolysis steps and the composition of the hydrolysis products.

Four biologically mediated events occur after pretreatment during the conversion of lignocellulose into ethanol namely: (1) production of depolymerizing enzymes, (2) hydrolysis of the polysaccharide components of pretreated biomass, (3) fermentation of the hexose (*C*6) sugars present, and (4) fermentation of pentose (*C*5) sugars present (Lynd et al. 2002). Improvements in biomass conversion technology generally involve the consolidation of two or more of these steps (Fig. 8.1). Hydrolysis and fermentation steps can be combined in simultaneous saccharification and fermentation (SSF) of hexoses or simultaneous saccharification and co-fermentation (SSCF) of both hexoses and pentoses. These processes avoid the feedback inhibition effect that released sugars have on the enzymes but are done at the cost of having to lower the temperature to a level that is suboptimal for enzymatic activity if a mesophilic process organism is used. The ultimate objective is a one-step consolidated bioprocessing (CBP) of lignocellulose to bioethanol, where all four steps occur in a single reactor and a single microorganism or microbial consortium converts pretreated biomass into a commodity product such as ethanol without the need for added enzymes. CBP would represent a breakthrough for low-cost biomass processing, due to the economic benefits of process integration (Galbe et al. 2005; Hahn-Hägerdal et al. 2007; Hamelinck et al. 2005; Robinson 2006) and avoiding the high costs of enzymes that make the biological conversion route unattractive (Anex et al. 2010; Kazi et al. 2010).

Lignocellulosic plant biomass consists of 40–55 % cellulose, 25–50 % hemicellulose, and 10–40 % lignin, depending on whether the source is hardwood, softwood, or grasses (Sun and Cheng 2002). The main polysaccharide present is

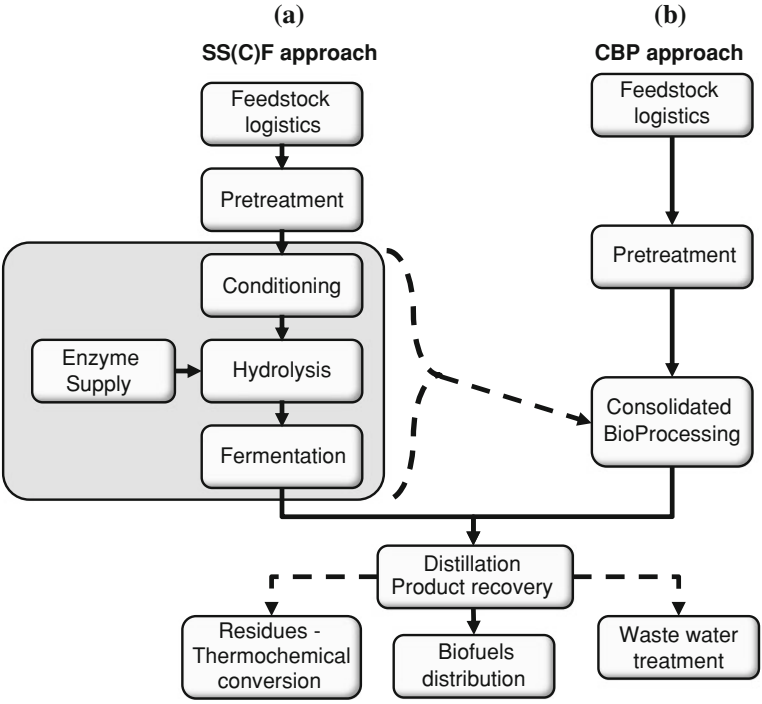

Fig. 8.1 **a** Scheme for simultaneous saccharification and (co)-fermentation (*SS(C)F*) process is illustrated in the shaded box. **b** If a recombinant organism that produces cellulases is employed in a consolidated bioprocessing (*CBP*) process, savings in process energy required by running the process at moderate temperatures and the cost of exogenous enzymes, as well as the reduction in operating units can have substantial economic benefits

water-insoluble crystalline cellulose which also represents the major fraction of fermentable sugars. Full enzymatic hydrolysis of crystalline cellulose requires the synergistic action of three major types of enzymatic activity, namely (1) endoglucanases that act in amorphous regions of cellulose and release cellodextrins as well as provide free chain ends, (2) exoglucanases, including cellodextrinases and cellobiohydrolases that act on the crystalline part of cellulose in a processive manner from free chain ends and release mainly cellobiose, and (3) β-glucosidases that hydrolyze cellobiose and small cello-oligosaccharides to glucose (Zhang and Lynd 2004). Hemicellulose refers to a number of heterogeneous polysaccharide structures, such as (arabino)xylan, galacto(gluco)mannan, and xyloglucan (Sun and Cheng 2002). These chemically diverse polymers are linked together through covalent and hydrogen bonds and can be chemically bound to the lignin fraction. Different pretreatment protocols may remove variable amounts of hemicelluloses, but it remains imperative from an economic perspective that sugars contained in the hemicellulose fraction of lignocellulose are also converted into ethanol (Hahn-Hägerdal et al. 2001). The compositions of hemicelluloses present in

lignocellulosic feedstocks and the large variety of enzymes required to hydrolyze them are reviewed elsewhere (Girio et al. 2010; Van Zyl et al. 2007). Fungi and several bacteria release the entire complement of cellulases and hemicellulases produced into their surrounding growth media. These organisms have so-called free enzymes systems and these form the basis of most commercial enzyme preparations. Free enzymes systems may contain a large diversity of enzymes whose expression may be variably induced depending on the growth substrate (Herpoël-Gimbert et al. 2008). In contrast, some bacteria such as *Clostridium cellulolyticum* and *Clostridium thermocellum* have been shown to produce lignocellulase complexes, named cellulosomes, on their cell walls (Bayer et al. 2008). The cellulosomal architecture involves multiple catalytic components assembled on a scaffoldin subunit through strong noncovalent protein–protein interactions between cohesin modules on the scaffoldin and dockerin modules on the individual enzymes (Himmel et al. 2010). This highly ordered structure of multiple enzymes in close proximity to the substrate results in a high level of enzyme–substrate–microbe synergy (Fierobe et al. 1999; Lu et al. 2006). Cellulosome producing organisms display a wide variety of catalytic subunits on the cell surface via scaffoldin, allowing the organisms to modulate cellulosome activity according to the substrate (Raman et al. 2009).

8.2 Feedstocks

The rapid increase in the demand for biofuels necessitates sustainable feedstock resources and conversion technologies (Sastri et al. 2008). In recent years, significant progress has been made toward the development of feedstocks and technologies for the production of biofuels from lignocellulosic biomass (Fortman et al. 2008). Potential cellulosic feedstocks are numerous and widespread and include woody biomass, perennial grasses, and agricultural and forest residues (Table 8.1). These feedstocks have gained popularity because they provide high biomass yield and in some cases multiple harvests per year are possible. Some of these have recently been genetically modified to provide additional beneficial properties, including reduced or altered lignin (Masarin et al. 2011; Shadle et al. 2007; Voelker et al. 2011; Wadenback et al. 2008; Wang et al. 2012), improved digestibility (Guillaumie et al. 2008; Harris et al. 2009; Shadle et al. 2007), and increased biomass production (Eriksson et al. 2000).

Eucalyptus species are among the most planted hardwoods in the world. The lumber and paper industries have been harvesting *Eucalyptus* and other woody biomass sustainably for many years (Somerville et al. 2010). Continued sustainable growth of these industries is aided by increased use of electronic media as well as paper recycling (Counsell and Allwood 2007). Harvesting is however likely to increase as legislation in many countries changes in favor of renewable energy. The potential energy available in woody biomass is enormous. It is estimated that biomass harvested in the Northern Hemisphere from wood products has

Table 8.1 Summary of biofuel feedstocks

Crop	Growth cycle (months)	Water needs (mm/season)	Average productivity (dry t/ha/year)	Ethanol yield (L/ha)
Wood				
Poplar	36	900	8	2,000
Willow	36	800	11	2,750
Eucalyptus	36	800	12	3,000
Perennial grass				
Switchgrass	12	700	15	5,000
Miscanthus	12	750	25	7,500
Napier grass	3	1,500	40	12,500
Agricultural crop				
Sugarcane	15	2,000	21	10,000
Maize	4	750	10	3,800
Drought resistant crop				
Agave	60	400	20	7,500

Adapted from Somerville et al. (2010). Ethanol yield refers to the total amount produced from a feedstock, including grain and stover or sugar and bagasse

an energy content equal to 107 % of the liquid fuel consumption of the United States (Goodale et al. 2002). Since the genome sequences of poplar (Brunner et al. 2004), willow, and eucalyptus (Paiva et al. 2011) became available, research has focused on genetically manipulating these species in order to develop feedstocks more suitable to the bioethanol industry. Much of the research is aim at reducing the cost of pretreatment by altering the lignin content (Gonzalez-Garcia et al. 2012; Porzio et al. 2012; Somerville et al. 2010).

Perennial plants such as switchgrass, *Miscanthus*, and Napier grass have high photosynthetic capacity, as well as water and nitrogen use efficiency (Ansah et al. 2010; Somerville et al. 2010). They are fast growing and have efficient root systems allowing them to reach deep into the soil for water. The root produces a network of stems and roots that holds onto soil to prevent erosion. A stand of switchgrass can be harvested annually for 10 years, while *Miscanthus* will remain productive for 10–15 years. These and other perennial grasses are capable of averaging around 30 metric tons of dry matter per hectare per year, however, perennial grasses are not being grown on a meaningful scale as yet. This is mainly because there is currently no commodity market to guarantee the price. Recently, genetically engineered switchgrass lines were produced with reduced lignin content (Fu et al. 2011). These transgenic switchgrass lines are phenotypically normal, but have reduced thermochemical, enzymatic, and microbial recalcitrance. They therefore require 300–400 % lower cellulase dosages and less severe pretreatment.

Maize is the largest crop in the world in terms of grain production at around 820 million metric tons per annum (Somerville et al. 2010). A more or less equal amount of stems and stripped cobs (stover) is potentially available for the production of biofuel. Since crop residue help to prevent erosion and guard against the loss of nutrients, excess removal would also increase the amount of fertilizer

needed to maintain good crop yields. The amount of residue that can be removed without compromising soil quality depends on soil type, topography, climatic conditions, and management practices (Hood et al. 2007). Irrespective of the amount of residue left on the land for soil maintenance, a significant amount of this cheap feedstock is still available for the production of cellulosic ethanol. Maize has been genetically modified to express hydrolytic enzymes to aid in stover hydrolysis (Hood et al. 2007) while altering the regulation of lignin biosynthesis simplifies pretreatment (Sticklen 2007).

Ethanol from sugarcane constitutes one of the largest sources of biofuels in the world (Somerville et al. 2010). Currently, 4.6 million hectares of sugarcane are used for bioethanol production in Brazil; however, the Brazilian government stated that this would be increased substantially to a maximum of 63.5 million hectares (Decree No.6.961 2009). Approximately 60 million hectares of this allocated land would be available for biofuels production. Estimates, based on the expected increase in sugarcane crops and the additional amount of ethanol produced as cellulosic fuel, are that Brazil could produce 14 % of the current world transportation fuel demand of 4,900 Gl by the year 2030 (Somerville et al. 2010). South Africa produces about 20 million tons of cane (about 50 % of Africa's production) on 325,000 hectares of land. If the full potential of the estimated 6 million hectares of land suitable for sugarcane production in Angola, Malawi, Mozambique, Tanzania, Zambia, and Zimbabwe are also realized, about 400 million tons of cane can be produced, which could yield 49 Gl, about 20 % of Africa's current total petroleum consumption (Fortman et al. 2008; Somerville et al. 2010). By selecting sugarcane lines with decreased lignin Masarin et al. (2011) could show increased conversion of the glucan component into glucose using commercial enzymes. Similar studies might in future provide sugarcane lines that would allow cost-effective production of ethanol from bagasse (Fortman et al. 2008).

Almost one-fifth of the terrestrial surface on the Earth is semiarid and prone to droughts with rainfall of between 200 and 800 mm per year (Davis et al. 2011). If this is combined with agricultural land that has fallen out of production, the amount of land available for the production of biomass using drought resistant species such as *Agave* is vast. *Agave* spp. thrives under these conditions and produces up to 34 dry tons of biomass per hectare per year. Obtaining biomass in sufficient quantities to merit the construction of commercial scale facilities will be a major concern in the future; fortunately, there are a number of different crops suitable for different environmental conditions that could enable sustainable production of sufficient quantities of biomass.

8.3 Conversion Technologies

Currently, the two primary methods for the conversion of biomass into biofuel are biochemical and thermochemical (Hess et al. 2007; Miller 2010). A detailed discussion on the thermochemical conversion of lignocellulosic feedstocks falls

outside the scope of this chapter, but is reviewed elsewhere (Bhaskar et al. 2011; Demirbas 2001). The cost and biofuel yield for each of these vary significantly depending on the implemented strategies as well as the feedstock used. Similarities to current technology used for the production of ethanol from maize have led to biochemical conversion attracting the most attention (Summarized in Fig. 8.1). Feedstock logistics and biofuel distribution is shared by both conversion technologies.

One of the major challenges of biomass harvesting and delivery to conversion facilities remains yield and density, which determines the volume of the biomass. The density of grassy feedstock to woody feedstocks can vary from $\sim$70 to $\sim$300 kg/m^3. For a 200–1,000 million liter per annum cellulosic ethanol plant, 0.8–4.0 million tons of dry biomass are required, which would require 50–250 trucks per day to deliver the biomass (Verma et al. 2011). Innovative ways of harvesting and delivering biomass to conversion facilities have to be developed to ensure cost-effective production of cellulosic ethanol at significant quantities. These could include dedicated production of biomass (e.g., as found in the sugar and paper-and-pulp industries) or the development of biomass commodity markets, parallel to agricultural commodity markets, such as grain and livestock. Defining of strict specifications for biomass delivered will be crucial to ensure a uniform feedstock for take-off by biomass conversion industries.

The aim of pretreatment is to open up the structure of lignocellulosic material by decreasing the crystallinity of cellulose, removing the hemicellulose fraction, and breaking down lignin (Verma et al. 2011). This increases the surface area making the cellulose more accessible to hydrolytic enzymes. A number of pretreatment processes have been reported in the literature (Table 8.2). Each has advantages and disadvantages. It is therefore important to optimize the pretreatment process for a particular feedstock keeping in mind the subsequent fermentation process to be used. Optimization of pretreatment should ideally be done together with enzymatic hydrolysis, this would ensure development of a process that would yield the maximum amount of fermentable sugars. The sugar stream produced through enzymatic hydrolysis is then fermented to ethanol.

8.4 CBP Organismal Development

While several wild-type microorganisms possess the ability to produce a broad range of enzymes required to hydrolyze all the polysaccharides found in lignocellulose, none of them display the ability to directly convert these into a desired product such as ethanol at economically feasible rates and titers (Hahn-Hägerdal et al. 2006; Lynd et al. 2005). In contrast, microorganisms with favorable product producing qualities often cannot utilize all the sugars available in biomass, lack cellulolytic ability, and may exhibit sensitivity to the inhibitors present in pretreated lignocellulosic biomass. Often microorganisms may also produce product mixtures where desirable products are produced along with undesirables. Some

Table 8.2 A summary of pretreatment processes reported in the literature

Category	Pretreatment method	Feedstocks used for	Advantage	Disadvantage	Reference
Physical pretreatment	Steam explosion	Highly crystalline feedstocks with complex lignin—straw	Increases enzyme digestibility Cost effective Suitable for large scale	Low hemicellulose sugar yield High temperatures required	Excoffier et al. (1991); Heitz et al. (1991); Kristensen et al. (2008); Verma et al. (2011)
	Liquid hot water	Corn fiber Wheat straw	Good cellulose digestibility High pentose recovery (up to 80 %) Low inhibitor concentrations Suitable for large scale	High temperature and pressure used	Allen et al. (2001); Dien et al. (2006); van Walsum et al. (1996)
	Mechanical size reduction (milling, crushing, grinding)	Spruce and aspen	High rate and extent of enzymatic saccharification of cellulose	Expensive Time-consuming Energy intensive No lignin removal	Ryu and Lee (1983)
	Radiation	Wheat straw	Decreased crystallinity Partial depolymerization of lignin	Slow Energy intensive Expensive	Imai et al. (2004); Yang et al. (2008)

(continued)

Table 8.2 (continued)

Category	Pretreatment method	Feedstocks used for	Advantage	Disadvantage	Reference
Chemical pretreatment	Catalyzed steam explosion	Softwood	One of the most cost-effective pretreatment methods	SO_2 is highly toxic Generates carbohydrate derived inhibitors	De Bari et al. (2007); Stenberg et al. (1998)
			Less inhibitory compounds than steam explosion	Incomplete destruction of lignin	
	Dilute acid	Most biomass sources including, hardwood, softwood, agricultural residues, waste paper, and municipal waste	High xylose yield	Sulfuric acid highly corrosive Fermentation inhibitors are produced Prehydrolyzate must be neutralized	Dien et al. (2006); Sun and Cheng (2005); Torget and Teh-An (1994)
	Alkaline pretreatment	Hardwood, agricultural residues	High degree of delignification Good solubilization of hemicellulose	Long treatment times required	Kim et al. (2003); Prior and Day (2008); Vancov and McIntosh (2011)
			Low temperature process	Conversion of alkali into irrecoverable salts	
	Ammonia fiber/freeze explosion (*AFEX*)	Most biomass sources	High degree of delignification	Low hemicellulose recovery	Bals et al. (2010); Holtzapple et al. (1992); Murnen et al. (2007)
		Agricultural residues	Negligible inhibitor production	Ammonia recovery essential for cost-efficient pretreatment	
		Not suited for high lignin feedstock	No particle size reduction needed		

(continued)

Table 8.2 (continued)

Category	Pretreatment method	Feedstocks used for	Advantage	Disadvantage	Reference
	Organosolv	–	High degree of delignification Recovery of pure low molecular weight lignin	Expensive Require high-pressure equipment Solvent recovery essential to reduce cost	Pan et al. (2008)
	pH controlled liquid hot water	Corn stover	Yield high degree of soluble oligosaccharides and mono saccharides Less that 1 % carbohydrates lost to degradation products	Currently not economically feasible	Kim et al. (2009); Mosier et al. (2005); Weil et al. (1998)
	Ionic liquids	Sugarcane bagasse, wheat straw	Effective at mild reaction conditions Possible to recover almost 100 % of ionic liquid Low toxicity biodegradable and non-flammable	High cost of ionic liquids	Feng and Chen (2008); Fukaya et al. (2008); Kuo and Lee (2009); Zhu (2008)
Biological pretreatment	–	Softwood, corn stover, bamboo	No chemicals needed Low energy requirements Mild reaction conditions Environmentally safe	Very slow process Large-space requirements	Hwang et al. (2008); Kurakake et al. (2007); Singh et al. (2008)

Verma et al. (2011), Hendriks and Zeeman (2009), Zheng et al. (2009), Wyman et al. (2005)

characteristics of an ideal CBP organism are given in Table 8.3. It may also be possible to perform CBP with a mixture of organisms with the desired properties of cellulolytic ability and product formation. The development of symbiotic consortia for lignocellulosic biofuel production was recently reviewed (Zuroff and Curtis 2012). Due to the variety of lignocellulosic feedstocks likely to be available, the diversity in pretreatment methods and the difference in desired products produced, there is scope for development of CBP organisms with a range of different traits (La Grange et al. 2010). Three different approaches have been followed to develop such an organism: (1) engineering product forming ability into organisms that are efficient biomass degraders, (2) engineering cellulolytic ability into organisms that have attractive product producing attributes, and (3) engineering cellulolytic ability and product formation into organisms with other particular positive attributes.

8.4.1 Engineering Product Forming Ability into Biomass Degraders

Several species of cellulolytic fungi, such as *Trichoderma reesei*, naturally produce a large repertoire of saccharolytic enzymes to digest lignocellulose efficiently, assimilate all lignocellulosic sugars, and convert these sugars into ethanol, showing that they naturally possess all pathways for conversion of lignocellulose into bioethanol (Chambergo et al. 2002; Lynd et al. 2002). It has been shown that a biorefinery consuming thousands of tons of biomass per day will require many tons of cellulase preparation to operate assuming that enzymes with far greater specific activity are not identified (Xu et al. 2009). Currently, only fungi naturally produce the required amounts of cellulase and some strains of *T. reesei* produce more than 100 g/L cellulase (Cherry and Fidantsef 2003). Thus, advantages of *T. reesei* as a CBP organism are: (1) the production of sufficient quantities of

Table 8.3 Characteristics of an ideal CBP organism

1. Ability to ferment all hexoses and pentoses present in lignocellulose
2. High product yield, titer, and productivity
3. High product and inhibitor tolerance
4. General robustness for industrial processes, cellulase production in toxic environment
5. Tolerance to low pH and high temperature
6. Amenability to DNA manipulation
7. High levels of heterologous protein production and secretion (if cellulolytic ability must be engineered)
8. Concurrent fermentation of sugars (hexose and pentose co-utilization)
9. GRAS status
10. Recyclability in successive processes
11. Minimum nutrient supplementation requirement

Van Zyl et al. (2007)

cellulases at reasonable cost, (2) several strains are established commercially, and (3) it can utilize all lignocellulose sugars for production of ethanol. Challenges to overcome before *T. reesei* can be considered as a CBP organism include the observations that ethanol yield, rate of production and tolerance are low, and that mixing during fermentation may require more energy owing to the filamentous cell morphology. Preliminary studies showed that *T. reesei* could produce cellulases when grown aerobically on cellulose that continued to degrade cellulose to sugars and ferment these sugars to ethanol when cultures were rendered anaerobic (Xu et al. 2009). However, acetic acid was produced as a major byproduct. The major limitation for efficient ethanol production by *T. reesei* does not lie in the absence of the relevant genes and pathways but are more likely related to the low expression of these genes or the activity of the enzymes encoded. Approaches to solving these problems are to enhance the expression of the relevant genes at the transcriptional level and/or to introduce heterologous genes that encode enzymes with higher activities and to knockout genes responsible for the production of byproducts. Recently, the laccase gene *lacA* from *Trametes* sp. AH28-2 was heterologously expressed in *T. reesei* under control of a constitutive promoter (Zhang et al. 2012). Transformants were identified that were able to secrete the recombinant laccase. Reducing sugar yields obtained from saccharification of corn residue by crude enzyme extracts prepared from the transformants increased by 31.3–71.6 %, respectively, compared to the host strain.

Another filamentous fungus, *Fusarium oxysporum*, also produces the enzymes required to break down cellulose and hemicellulose while simultaneously fermenting the released sugars to ethanol albeit at relatively low yields (Anasontzis et al. 2011; Panagiotou et al. 2005). In SSF of a cellulosic substrate a *F. oxysporum* wild-type strain was able to grow in aerobic conditions and produced ethanol with a yield of 0.35 g/g cellulose under anaerobic conditions. The strain was also shown to effectively produce a complete system of hydrolytic enzymes when grown on various agro-industrial lignocellulose by-products, such as dry citrus peels, corn cob, and brewer's spent grain with concomitant ethanol production (Anasontzis et al. 2011; Xiros et al. 2008). It was hypothesized that homologous overexpression of cellulases and hemicellulases under constitutive control, could provide a higher breakdown rate of the biomass and thus increase the supply of sugars to the ethanol production pathway. To this end, the endoxylanase two of *F. oxysporum*, was overproduced under control of the constitutive *Aspergillus nidulans gpdA* promoter (Anasontzis et al. 2011). The fermentative performance of the transformants were evaluated and compared to that of the wild type in simple CBP systems using corn cob or wheat bran as sole carbon sources. Transformants produced approximately 60 % more ethanol compared to the wild type on corn cob and wheat bran likely due to the ~2–2.5-fold higher extracellular xylanase activities in the fermentation broths of the transformants.

High-temperature conversion process conditions potentially provide a significant energy saving since reactors would not have to be cooled to mesophilic conditions before inoculation and then reheated for distillation (Xu et al. 2010). Furthermore, it has been shown that a 10 °C increase in temperature approximately doubles

enzymatic reaction rates, decreasing the amount of enzyme required (Ibrahim and El-diwany 2007). In addition, the use of reaction and fermentation temperatures in excess of 60 °C minimizes the risk of contamination. Since cellulose hydrolysis and sugar release is in most cases the rate-limiting step in a typical CBP process, high-temperature hydrolysis will be therefore be advantageous. Thermophilic bacteria capable of cellulose hydrolysis and ethanol production show great potential as CBP organisms (Xu et al. 2010). The cellulosome producing thermophilic Gram-positive anaerobic bacterium *C. thermocellum* is regarded as a potential CBP organism as it is very efficient at hydrolyzing crystalline cellulose (Lynd et al. 2002). While growth of wild-type strains is inhibited in the presence of ethanol concentrations above 2 % (v/v) strains have been evolved that remained viable at ethanol concentrations of up to 8 % (v/v) (Xu et al. 2010). This group also investigated the effect of some of these inhibitors on cellulosome activity of *C. thermocellum*. It was shown that that some organic acids actually promoted cellulolytic activity and that the *C. thermocellum* cellulosome could tolerate certain concentrations of furfural (up to 5 mM), *p*-hydroxybenzoic acid (up to 50 mM) and catechol (up to 1 mM). The *C. thermocellum* cellulosomes were also able to tolerate higher ethanol concentrations and temperatures than the *T. reesei* enzymes used commercially.

8.4.2 Engineering Cellulolytic Ability into Process Organisms

The yeast *Saccharomyces cerevisiae* has long been employed for the industrial production of ethanol (Kuyper et al. 2005; Van Dijken et al. 2000). Attributes that make it suitable for industrial ethanol production include a high rate of ethanol production from glucose (3.3 g/L/h), high ethanol tolerance, and its GRAS status. However, this yeast species has a number of shortcomings in terms of a CBP processing organism such as its inability to hydrolyze cellulose and hemicellulose or utilize pentose sugars available in lignocellulosic biomass. A number of research groups have been working on improving the substrate range of *S. cerevisiae* through genetic engineering to include the monomeric forms of sugars contained in plant biomass including xylose (Hahn-Hägerdal et al. 2007; Kuyper et al. 2005), arabinose (Karhumaa et al. 2006) and cellobiose (van Rooyen et al. 2005). There have been many reports detailing the expression of one or more cellulase or hemicellulose encoding gene(s) in *S. cerevisiae* (Van Zyl et al. 2007). Strains of *S. cerevisiae* were created that could grow on and ferment cellobiose, the main product of the action of cellobiohydrolases, at roughly the same rate as on glucose in anaerobic conditions (van Rooyen et al. 2005). Recently the high affinity cellodextrin transport system of the model cellulolytic fungus *Neurospora crassa* was reconstituted into *S. cerevisiae* (Galazka et al. 2010) leading to growth of a recombinant strain also producing an intracellular β-glucosidase on cellodextrins up to cellotetraose. Subsequently, strains of *S. cerevisiae* were engineered to co-ferment mixtures of xylose and cellobiose

(Ha et al. 2011). A xylose fermenting strain was engineered to also produce a high-affinity cellodextrin transporter and an intracellular β-glucosidase to hydrolyze cellobiose. It was shown that intracellular hydrolysis of cellobiose minimized glucose repression of xylose fermentation allowing co-consumption of cellobiose and xylose that improved ethanol yields. This was partly due to circumventing the competition between xylose and glucose for transport into the cell. Sadie et al. (2011) recently showed that expression of the gene encoding lactose permease of *Kluyveromyces lactis* (*lac*12) also facilitated transport of cellobiose into a recombinant *S. cerevisiae* strain. This report further showed the successful expression of a *Clostridium stercorarium* cellobiose phosphorylase (*cepA*) and that strains co-producing the heterologous CepA and Lac12 were able to grow on cellobiose as sole carbohydrate source.

There have also been reports showing production of cellulases in *S. cerevisiae* specifically with the aim of enabling the organism to grow on a polymeric substrate. Cho et al. (1999) showed that for SSF experiments with a strain co-producing a β-glucosidase and an exo/endocellulase activity, loadings of externally added cellulase could be reduced. Fujita et al. (2002, 2004) reported co-expression and surface display of cellulases in *S. cerevisiae* and high cell density suspensions of a strain displaying the *T. reesei* endoglucanase II, cellobiohydrolase II, and the *Aspergillus aculeatus* β-glucosidase were able to directly convert 10 g/L phosphoric acid swollen cellulose (PASC) into approximately 3 g/L ethanol. An *S. cerevisiae* strain co-expressing the *T. reesei* endoglucanase 1 (*cel*7*B*) and the *S. fibuligera* β-glucosidase 1 (*cel*3*A*) was able to grow on and convert 10 g/L PASC into ethanol up to 1.0 g/L (Den Haan et al. 2007). Jeon et al. (2009) constructed a similar strain that produced significantly more endoglucanase activity than the strain reported by Den Haan et al. (2007) and notably improved conversion of PASC into ethanol was achieved. When the processive endoglucanase Cel9A of the moderately thermophilic actinomycete *Thermobifida fusca* was functionally produced in *S. cerevisiae* growth of the strain expressing only this one cellulase encoding gene could be demonstrated on media containing PASC due to a sufficient amount of glucose cleaved from the cellulose chain (van Wyk et al. 2010). It was shown that the enzyme released cellobiose and glucose from cellulosic substrates in a ratio of approximately 2.5:1. In an effort to construct an engineered yeast with efficient cellulose degradation, Yamada et al. (2010) developed a method, cocktail delta(δ)-integration to optimize cellulase expression levels. Different cellulase expression cassettes encoding β-glucosidase, endoglucanase, or cellobiohydrolase, were integrated into yeast chromosomes in one step, and strains expressing an optimum ratio of these cellulases were selected for by growth on media containing PASC as carbon source. Although the total integrated gene copy numbers of an efficient cocktail δ-integrant strain was about half that of a conventional δ-integrant strain, the PASC degradation activity (64.9 mU/g-wet cell) was higher than that of a conventional strain (57.6 mU/g-wet cell) suggesting that optimization of the cellulase expression ratio improved PASC degradation activity more than overexpression. Matano et al. (2012) enhanced cellulase activities on a recombinant *S. cerevisiae* yeast cell surface displaying *T. reesei*

EG2 and CBH2 and *A. aculeatus* BGL1 by additionally integrating *eg*2 and *cbh*2 genes into the recombinant strain. As a result, a high ethanol titer (43.1 g/L) was produced from high-solid (200 g-dry weight/L) pretreated rice straw by performing a 2-h liquefaction and subsequent 72-h fermentation in the presence of 10 FPU/g-biomass added cellulase. Ethanol yield from the cellulosic material by the recombinant strain reached 89 % of the theoretical yield, which was 1.4-fold higher than the strain without additional gene copies.

As exoglucanase activity is required for the successful hydrolysis of crystalline cellulose, the addition of successful, high level expression of a cellobiohydrolases to these strains should enable conversion of crystalline cellulose into ethanol. While there have been reports of successful expression of cellobiohydrolase encoding genes in *S. cerevisiae* the titers achieved were generally low (Ilmen et al. 2011). Recently the expression of relatively high levels of exoglucanases in *S. cerevisiae* was reported for the first time (Ilmen et al. 2011; Mcbride et al. 2012). Ilmen et al. (2011) reported a large increase in the maximum titer achieved for two critical exocellulases: Cel6A (CBH1) and Cel7A (CBH2). The cellulase expression levels achieved in this study meets the calculated levels for growth on cellulose at rates required for an industrial process (Olson et al. 2012). Using these exoglucanases, a yeast strain was constructed that was able to convert most of the glucan available in paper sludge into ethanol (Mcbride et al. 2012). The strain was also able to displace 60 % of the enzymes required to convert the sugars available in pretreated hardwood into ethanol in an SSF configuration. A similar strain expressing three alternative cellulases produced ethanol in one step from pretreated corn stover without the addition of exogenously produced enzymes fermenting 63 % of the cellulose in 96 h to 2.6 % (v/v) ethanol (Khramtsov et al. 2011). These results demonstrate that cellulolytic *S. cerevisiae* strains can be used as a platform for developing an economical advanced biofuel process.

As it has been shown that the close proximity of multiple enzymes on the cell surface enables synergistic hydrolysis of lignocellulosic materials, several groups have attempted to reconstruct a minicellulosome on the *S. cerevisiae* cell surface (Ito et al. 2009; Lilly et al. 2009; Tsai et al. 2009; Wen et al. 2010). Ito et al. (2009) constructed a chimeric scaffoldin to allow cell surface display of both *T. reesei* EG2 and *A. aculeatus* BGL1, yielding yeast strains capable of hydrolyzing *β*-glucan. *S. cerevisiae* strains were also engineered to display a trifunctional minicellulosome consisting of a mini-scaffoldin containing a cellulose binding domain and three cohesin modules, anchored to the cell surface and three types of cellulases, EG2 and CBH2 originating from *T. reesei*, and BGL1 from *A. aculeatus*, each bearing a C-terminal dockerin (Wen et al. 2010). This strain was able to break down and ferment PASC to ethanol with a titer of 1.8 g/L. Tsai et al. (2010) engineered yeast strains capable of displaying a trifunctional scaffoldin carrying three divergent cohesin domains originating from *C. thermocellum*, *C. cellulolyticum* and *Ruminococcus flavefaciens*. In addition, strains were constructed that secreted one of the three corresponding dockerin-tagged cellulases namely an EG from *C. thermocellum*, an exoglucanase from *C. cellulolyticum*, or a BGL from *R. flavefaciens*. Using a yeast consortium composed of one strain displaying the

mini-scaffoldin and three strains secreting the dockerin-tagged cellulases, the secreted cellulases were docked onto the displayed mini-scaffoldin in a predictably organized manner. By adjusting the ratio of different populations in the consortium, cellulose hydrolysis and ethanol production was successfully fine-tuned and ~30 % of 10 g/L PASC was solubilized in 73 h. Displaying cellulosomal components on the yeast cell surface was also recently employed to create strains that could convert xylan into ethanol (Sun et al. 2012). These strains displayed mini-hemicellulosomes that consisted of a mini-scaffoldin originating from *C. thermocellum* tethered to the cell surface through the *S. cerevisiae* α-agglutinin adhesion receptor and up to three enzymes. Up to three types of hemicellulases, an endoxylanase (*T. reesei* Xyn2), an arabinofuranosidase (*Aspergillus niger* AbfB), and a *β*-xylosidase (*A. niger* XlnD), each with a C-terminal dockerin, were assembled onto the mini-scaffoldin via cohesin-dockerin interactions. The resulting quaternary trifunctional complexes exhibited an enhanced hydrolysis rate of arabinoxylan over the other configurations. Furthermore, in strains with an integrated xylose utilizing pathway, the recombinant yeast displaying a mini-hemicellulosome containing the xylanase and xylosidase could simultaneously hydrolyze and ferment birchwood xylan to ethanol although less than 1 g/L ethanol was produced.

Zymomonas mobilis is a well-known Gram-negative fermenting bacterium that produces ethanol at very high rates and is used to produce some traditional alcoholic beverages (Zhang et al. 1997). However, *Z. mobilis* cannot ferment or utilize the pentose sugar xylose and it cannot hydrolyze polysaccharides. Zhang et al. (1997) engineered a *Z. mobilis* strain capable of fermenting the major pentose sugars present in plant material, namely xylose and arabinose. Co-fermentation of 100 g/L sugar (glucose:xylose:arabinose—40:40:20) yielded an ethanol concentration of 42 g/L in 48 h. Brestic-Goachet et al. (1989) expressed the *Erwinia chrysanthemi cel5Z* in *Z. mobilis*. The maximum endoglucanase activity obtained was 1,000 IU/L with 89 % of the enzyme secreted to the extracellular medium. Expression of the *Ruminococcus albus β*-glucosidase enabled *Z. mobilis* to ferment cellobiose to ethanol very efficiently in 2 days and most of the recombinant enzyme was secreted (Yanase et al. 2005). Recently, numerous strains of *Z. mobilis* were shown to possess native extracellular activities against carboxymethyl cellulose (Linger et al. 2010). Furthermore, two cellulolytic enzymes, *E*1 and GH12 from *Acidothermus cellulolyticus*, were produced heterologously as active, soluble enzymes in *Z. mobilis*. While the *E*1 enzyme was less abundant, the GH12 enzyme comprised as much as 4.6 % of the total cell protein. Additionally, fusing predicted secretion signals native to *Z. mobilis* to the N-termini of these enzymes was shown to direct secretion of significant levels of active *E*1 and GH12 enzymes, though a significant portion of both still resided in the periplasmic space.

8.4.3 Engineering Cellulolytic Ability and Product Formation into Organisms with Favorable Attributes

Several yeast species other than *S. cerevisiae* have innate properties that make them attractive as possible CBP organisms (Lynd et al. 2005). The promise of thermotolerant yeast species as CBP organisms and recent data on ethanol production from cellulosic and hemicellulosic materials with thermotolerant yeast strains in SSF and CBP was recently reviewed by Hasunuma and Kondo (2012). The *A. aculeatus* β-glucosidase was introduced to the multistress tolerant yeast *Pichia kudriavzevii* (*Issatchenkia orientalis*) and the transformant could convert cellobiose into ethanol under acidic conditions and at temperatures exceeding 40 °C (Kitagawa et al. 2010). Strains of the yeast *Kluyveromyces marxianus* can grow at temperatures as high as 52 °C and can convert a wide range of substrates, including xylose, into ethanol (Fonseca et al. 2007). Successful SSF with a variety of feedstocks at elevated temperatures has been demonstrated with *K. marxianus* (Fonseca et al. 2007; Pessani et al. 2011). Recently, SSF conversion of pretreated switchgrass to ethanol with *K. marxianus* IMB3 was shown at 45 °C with a yield of 86 % of the theoretical maximum achieved in 168 h (Pessani et al. 2011). Genes encoding thermotolerant varieties of cellobiohydrolase, endoglucanase, and β-glucosidase were expressed in combination in a strain of *K. marxianus* (Hong et al. 2007). The resulting strain was able to grow in synthetic media containing cellobiose or carboxymethylcellulose as sole carbon source but the hydrolysis of crystalline cellulose was not shown. A *K. marxianus* strain was also engineered to display *T. reesei* endoglucanase II and *A. aculeatus* β-glucosidase on the cell surface was shown to convert 10 g/L cellulosic β-glucan into 4.24 g/L ethanol at 48 °C within 12 h (Yanase et al. 2010).

Strains of the methylotrophic yeast *Hansenula polymorpha* are also able to grow at elevated temperatures up to 48 °C and ferment glucose, cellobiose, and xylose to ethanol (Ryabova et al. 2003). Additionally, attributes such as process hardiness and a high capacity for heterologous protein production make this yeast an attractive candidate for CBP. The promise of *H. polymorpha* as a CBP organism was recently emphasized when strains were constructed that could ferment starch and xylan (Voronovsky et al., 2009). *Scheffersomyces stipitis* (formerly *Pichia stipitis*) has a substrate range including all the monomeric sugars present in lignocellulose (Jeffries and Shi 1999). Some *S. stipitis* strains produce low quantities of various cellulases and hemicellulases to break down wood into monomeric sugars although it cannot utilize polymeric cellulose as carbon source (Jeffries et al. 2007). Among the enzymes naturally produced are a β-glucosidase that allows the yeast to ferment cellobiose. Endoglucanases were successfully produced in *H. polymorpha* (Papendieck et al. 2002) and *S. stipitis* (Piotek et al. 1998). As these yeasts are capable of growth on cellobiose the recombinant strains should have the ability to hydrolyze amorphous cellulose although this aspect was not tested. The xylanolytic ability of *S. stipitis* was enhanced by the co-expression of xylanase and xylosidase encoding genes resulting in strains that displayed improved biomass

production on medium with birchwood glucuronoxylan as sole carbohydrate source (Den Haan and Van Zyl, 2003). Although mutant strains of *S. stipitis* with increased ethanol tolerance were recently isolated, *S. stipitis* remains a relatively poor fermenter (Watanabe et al. 2011). However, its ability to consume acetic acid and reduce the furan ring in furfural and hydroxymethylfurfural (HMF) creates an opportunity for this yeast to remove some of the toxins produced during cellulosic biomass conversion (Agbogbo & Coward-Kelly, 2008). This could be very beneficial in wastewater treatment. While these yeast strains all have promising attributes in terms of CBP organisms, the ethanol rates, titers, productivities, and tolerance of all of these candidates have to be improved.

Although the mesophilic Gram-negative bacterium *Escherichia coli* cannot hydrolyze cellulose or produce ethanol at appreciable quantities it has been shown to catabolize all major sugars present in plant biomass, producing organic acids and ethanol (Alterthum and Ingram 1989). Furthermore, *E. coli* has unparalleled genetic and metabolic tractability and is therefore an excellent candidate for metabolic engineering. *E. coli* has been engineered to biosynthesize the most chemically diverse range of chemicals of any organism, including hydrogen, higher alcohols, fatty acids, and terpenes (Bokinsky et al. 2011). Bräu and Sahm (1986) successfully modified *E. coli* metabolism by expressing the *Z. mobilis* pyruvate decarboxylase at high levels yielding a strain that produced ethanol at levels comparable with *Z. mobilis*. Subsequent work has focused on improving ethanol yields and tolerance, growth rate, and strain stability (Chen et al. 2009; Da Silva et al. 2005; Ingram et al. 1987, 1991; Ohta et al. 1991b; Yomano et al. 1998). Wild-type *E. coli* strains are incapable of rapid growth on cellobiose (Moniruzzaman et al. 1997); however, *Klebsiella oxytoca* contains a phosphoenol-dependent phosphotransferase system (PTS) enabling it to utilize cellobiose. The *K. oxytoca casAB* operon was expressed in the ethanol producing strain of *E. coli*. While expression was initially poor, a mutant was generated which produced 45 g/L ethanol—a yield of 94 % of the theoretical maximum. *E. coli* was also engineered to assimilate cellobiose through a phosphorolytic mechanism (Sekar et al. 2012). Cytoplasmic expression of the *Saccharophagus* cellobiose phosphorylase was shown to enable *E. coli* to use cellobiose and it was shown that the endogenous LacY permease was responsible for the transport of cellobiose. Conversion of cellobiose into ethanol in a KO11 strain background was achieved. Several endoglucanases have been expressed in *E. coli* allowing it to hydrolyze amorphous and soluble cellulose to shorter cello-oligosaccahrides (Da Silva et al. 2005; Seon et al. 2007; Srivastava et al. 1995; Wood et al. 1997; Yoo et al. 2004; Zhou et al. 2001). Zhou et al. (2001) successfully reconstructed the predominant secretion system type in Gram-negative bacteria, the type II secretion system, encoded by the *out* genes from *E. chrysanthemi*, in *E. coli*. This enabled *E. coli* to secrete more than 50 % recombinant *E. chrysanthemi Cel5Z* it produced. Recently, Shin et al. (2010) demonstrated a co-cultivation or binary strategy for CBP of xylan. Two *E. coli* strains were engineered to function cooperatively to transform xylan into ethanol. The first strain was engineered to co-express *axeA*, the acetylxylan esterase encoding gene from *Streptomyces violaceoruber* and *xyl*11*A* encoding a

xylanase gene from *Bacillus halodurans*. The recombinant enzymes were secreted into the growth medium by a method of *lpp* deletion with over 90 % efficiency. Secreted enzymes hydrolyzed xylan into xylo-oligosaccharides, which could be utilized by the second strain, designed to convert xylo-oligosaccharides into ethanol. The second strain was based on the KO11 strain optimized for ethanol production. The *KxynB* gene encoding *β*-xylosidase from *Klebsiella pneumonia* and *KxynT* encoding xyloside permease from *K. pneumoniae* were introduced into KO11. Co-cultivation of the two strains converted xylan into ethanol with a yield of about 55 % of the theoretical value. Bokinsky et al. (2011) engineered strains of *E. coli* that expressed either an endoglucanase and a *β*-glucosidase or a xylanase, and xylobiosidase under control of native promoters. Secretion was achieved through fusion of the proteins to OsmY, previously shown to enable secretion of fused proteins from *E. coli*. Growth of these strains could be demonstrated on model cellulosic and hemicellulosic substrates. Furthermore, the strains grew using either the cellulose or hemicellulose components of ionic liquid-pretreated biomass or on both components when combined as a co-culture. Strains were further engineered with biosynthesis pathways for the production of fatty-acid ethyl esters, butanol and pinene to demonstrate the production of fuel substitutes or precursors suitable for gasoline, diesel, and jet engines. Although titers and conversion rates were very low, direct conversion of ionic liquid-treated switchgrass into these biofuel components could be demonstrated without externally supplied hydrolase enzymes.

K. oxytoca is a hardy prototrophic Gram-negative bacterium with the ability to transport and catabolize cellobiose, cellotriose, xylobiose, xylotriose, sucrose, and all monomeric sugars present in lignocellulosic biomass (Zhou and Ingram 1999). Four different fermentation pathways are present in *K. oxytoca* producing lactic acid, succinate, formate, acetate, ethanol, and butanediol (Ohta et al. 1991b). Through metabolic engineering and expression of the *Z. mobilis pdc* and *adhB* genes, it was possible to generate a recombinant *K. oxytoca* strain to produce ethanol from soluble sugars at 95 % of the maximum theoretical yield (Wood and Ingram 1992). Interestingly, *K. oxytoca* has the ability to ferment xylose and glucose at equivalent rates which shortens the time required to ferment the mixtures of glucose and xylose typically present in lignocellulosic hydrolysates (Ohta et al. 1991a). Zhou and Ingram (1999) constructed a *K. oxytoca* strain expressing the *E. chrysanthemi cel*8*Y* and *cel*5*Z* endoglucanase encoding genes and the genes that encode the type II secretion system. They showed that both *Cel*8*Y* and *Cel*5*Z* were secreted effectively by *K. oxytoca* and that the strain was capable of fermenting amorphous cellulose and producing a small amount of ethanol without the addition of cellulases.

The thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum* is also under development as a CBP organism for biomass conversion. *T. saccharolyticum* grows in a temperature and pH range of 45–65 °C and pH 4.0–6.5 and is able to ferment a wide range of sugars present in cellulosic biomass including cellobiose, glucose, mannose, galactose, xylose, and arabinose (Shaw et al. 2008a). Unlike most organisms *T. saccharolyticum* metabolizes xylose and glucose at almost

the same rate (Shaw et al. 2008a, 2008b) but produces organic acids in addition to ethanol. Knockout mutants were created that produced almost exclusively ethanol from xylose. Furthermore, a strain with *hfs* and *ldh* deletions exhibited an increased ethanol yield from consumed sugars (Shaw et al. 2009). *T. saccharolyticum* naturally produces both a xylanase and a β-xylosidase enabling it to ferment xylan directly to ethanol (Lee et al. 1993). Furthermore, *T. saccharolyticum* was able to produce as much ethanol from Avicel with four filter paper units (FPU) of externally added enzyme in SSF as *S. cerevisiae* was with 10 FPU, the result of improved enzyme efficiency at higher temperatures (Shaw et al. 2008b). This shows the potential of this thermophile as CBP organism if a cellulolytic system can be established.

Another group of bacteria with great CBP potential is from the genus *Geobacillus*. These are thermophilic bacilli with certain species being able to ferment sugars like glucose, xylose, and arabinose at temperatures of between 55 and 70 °C, producing a mixture of lactate, formate, acetate, and ethanol (Barnard et al. 2010). Certain species like *Geobacillus* R7 also have the ability to produce lignocellulose-degrading enzymes including cellulases, xylanases, and lignases. All the above-mentioned attributes make *Geobacillus* a very good candidate for CBP; however, the production of lactate and formate is not desirable. Therefore, genetic engineering of these strains has been carried out at a British company, TMO Renewables Ltd., to improve ethanol production by *Geobacillus*. *Geobacillus thermoglucosidasius*, capable of oligosaccharide fermentation, was engineered by deletion of the *ldh* and *pfl* genes and upregulation of *pdh* to produce relatively high yields of 0.42–0.47 g ethanol/g hexose sugar, although the yield was somewhat less for pure pentose sugars (Cripps et al. 2009).

Bacillus subtilis, the best-characterized Gram-positive microorganism, is a major industrial microorganism and its potential as a CBP organism is increasingly apparent (Zhang and Zhang 2010). Among other advantages, *B. subtilis* has GRAS status, a very high protein-secreting capability, rapid growth rate with low nutrient requirement utilizing soluble pentose and hexose sugars, and native hemicellulase production. Furthermore, it tolerates high concentrations of salts and solvents, has an available genomic DNA sequence, and well-developed recombinant DNA techniques and fermentation technologies. Although it natively produces several polysaccharide-degrading enzymes, *B. subtilis* is noncellulolytic. Recently however, six cellulase genes sourced from *C. thermocellum* were cloned and expressed in *B. subtilis* and were efficiently secreted into the culture broth, showing synergistic activity on PASC and Avicel (Liu et al. 2012). It was also shown that a recombinant *B. subtilis* could grow on amorphous cellulose or pretreated biomass in chemically defined minimal *M*9 media through overexpression and secretion of its intrinsic glycoside hydrolase family five endoglucanase (Zhang et al. 2011). This stain was further optimized for direct conversion of cellulose to lactate. Finally, a mini-cellulosome was assembled on the surface of *B. subtilis*, displaying a mini-scaffoldin that could bind with three cellulases—an endoglucanase (Cel5), a processive endoglucanase (Cel9), and a cellobiohydrolase (Cel48) all originating from *C. thermocellum* (You et al. 2012). The cell-bound cellulosome exhibited

4.5- and 2.3-fold higher hydrolysis ability than cell-free cellulosome on Avicel and PASC, respectively, degrading up to 24–63 % of the substrates in 72 h.

8.5 Integrating Consolidated Bioprocessing with Existing Bio-Based Industries

The cost of second generation biofuels remains high despite significant advances in recent years. The high capital investment and energy demands of second generation cellulosic ethanol technologies necessitate their integration with first generation bio-based and thermochemical processes to minimize costs and improve energy efficiency and overall economics (Van Zyl et al. 2011). Different biological and thermochemical processes will be discussed and their potential integration in a few bio-based industries highlighted.

Three thermochemical options are available for the conversion of biomass: combustion, pyrolysis, and gasification. The simplest option is combustion (burning) of biomass in the presence of air, which generates hot gases at temperatures of around 800–1,000 °C and energy that can be harvested as heat. Pyrolysis entails heating biomass in the absence of air to about 500 °C converting the biomass into liquid (bio-oil), solid (char), and gaseous fractions. Bio-oils represent biomass in liquid form with a higher density and can be upgraded to transport fuels. Char can be used to improve soil fertility and as replacement to activated charcoal. In contrast, gasification is the conversion of biomass by partial oxidation at higher temperatures (in the range of 800–900 °C) to generate syngas that can be used for synthesis of different synthetic fuels (using the Fischer–Tropsch process) or burned for heat production. Both bio-oils and char also can be gasified as renewable feedstock for synthetic fuels production (Aden and Foust 2009; Bridgwater 2012; McKendry 2002).

During the biological processes for lignocellulose hydrolysis–fermentation nonfermentable lignin-rich residues remain, which contain large amounts of energy. These residues can provide all the heat and electricity needed for cellulosic ethanol production through high-efficiency processes, such as a high pressure boiler coupled with a multistage steam turbine (Aden and Foust 2009; Piccolo and Bezzo 2009) (Fig. 8.1). It can also provide surplus electricity production for sale into local electricity grids (Cardona and Sanchez 2007; Leibbrant 2010; Reith et al. 2002). Heat integration within biological and thermochemical routes for second generation biofuels production have the potential to increase overall energy efficiency by as much as 15 % and can reduce capital and operational costs substantially (Aden and Foust 2009; Galbe et al. 2005; Kazi et al. 2010; Leibbrant 2010). The organic loads in wastewater can be lowered through anaerobic digestion, simultaneously producing biogas that can be captured and used to generate electricity and/or process heating (Banerjee et al. 2009). Waste streams can also be combined with waste streams from local municipalities, integrating industrial and municipal wastes to improve water qualities in densely populated urban areas.

In existing industries, such as the sugar and pulp-and-paper industries, only part of the biomass is used, resulting in substantial quantities of residues remaining. Many of these residues are suitable for the production of cellulosic ethanol. By integrating such existing industries with cellulosic ethanol production, significant saving in the cost of the transport and handling of the feedstock and integration of energy consumption can be achieved (Aden and Foust 2009; Anex et al. 2010; Gnansounou et al. 2005; Hahn-Hägerdal et al. 2006; Kazi et al. 2010; Piccolo and Bezzo 2009). Improvement in better waste streams treatment can also be obtained (Goh et al. 2010; Hahn-Hägerdal et al. 2006; Soccol et al. 2010). Swedish researchers suggested that such integration could reduce the cost of cellulosic ethanol production with up to 20 % in Sweden (Hahn-Hägerdal et al. 2006; von Sivers and Zacchi 1995).

The integration of second generation cellulosic ethanol production with first generation production from sugars or starches can have multiple benefits, including providing economies of scale, reduction of capital costs and investor risk, and increased economic attractiveness and environmental acceptance (Gnansounou et al. 2005). Such integration can provide joint feedstock supply, fermentation, water and nutrient recycle, distillation, and further opportunities for energy integration (Easterly 2002; Galbe et al. 2007). This is particularly true for sugar-rich crops, such as sugarcane, sweet sorghum, and sugar beet, where integration with cellulosic ethanol processes ensure optimum use of the feedstock and its logistics (Gnansounou et al. 2005; Sims et al. 2008). These crops also allow flexibility of switching between the production of crystallized sugar and ethanol, as is currently done in Brazilian sugar mills (Gnansounou et al. 2005). Similar integration possibilities also exist for starches (corn, grain sorghum, triticale, etc.), where ethanol could be produced from the fiber-rich residues (Cardona and Sanchez 2007; Linde et al. 2010).

8.6 Conclusions

The consideration of cellulosic feedstocks for the production of biofuels could help address the demand for biofuels, but could also realize sustainability and environmental benefits. The options and quantities of feedstocks have substantially broadened and the choice of feedstocks will vary between geographical areas in terms of climate and the availability of suitable land. To date no ideal organism has been developed for CBP conversion of biomass. Yeasts are usually sufficiently robust for industrial conversion of lignocellulosics, however, lack the necessary substrate range, particularly the ability to degrade complex polysaccharides such as cellulose. The *S. cerevisiae* strain developed by the Mascoma Corporation represents the best CBP organism engineered thus far as this strain could convert several cellulosic substrates to ethanol with addition of minimal exogenous enzymes in an SSF configuration (Ilmen et al. 2011; Mcbride et al. 2012). While the advantages of using the yeasts *P. stipitis*, *K. marxianus*, and *H. polymorpha* are

well appreciated, they are not as robust as *S. cerevisiae* strains and the engineering of cellulolytic ability into these strains are currently rudimentary. None of the strains are as yet capable of utilizing crystalline cellulose and the high-level production of an exocellulase remains a requirement. New information about secretion pathways, chaperones, and metabolic engineering should help alleviate this problem in the future. Filamentous fungi on the other hand have a wide substrate range, but grow relatively slowly and do not produce enough of a desirable commodity product at titers required for commercial processes.

Bacteria generally have higher productivity rates (higher growth rates) but often lack process robustness. Compared to *S. cerevisiae*, all of the bacterial species discussed above are relatively sensitive to inhibitors associated with lignocellulosic hydrolysates (Yomano et al. 1998; Zhou and Ingram 1999). Engineering enhanced protein secretion allowed sufficient secretion of endoglucanases in *E. coli* (Zhou and Ingram 1999) and *K. oxytoca* (Ji et al. 2009). *E. coli* and *K. oxytoca* strains capable of breaking down cellulose could also be modified to produce other commodity products such as lactic acid, succinic acid, acetic acid, or 2, 3-butanediol (Ji et al. 2009). The *Geobacillus* strain used by TMO Renewables Ltd., is capable of producing ethanol at appreciable titers from pretreated lignocellulosic feedstock and represents a very promising organism for CBP.

Different candidate CBP organisms are in various stages of development for cellulose conversion into commodity products, notably ethanol. However, it has been suggested that the chance to develop one CBP organism for the conversion of all cellulosic feedstocks is rather slim. It is more likely that more than one organism may eventually be used in various biomass conversion processes and the choice may depend on the sugar composition of the feedstock, the pretreatment method used, and the end product(s) required. The high-cost disadvantage of current second generation biofuels configurations may be partially addressed through innovative methods of process integration with existing bio-based industries, in order to minimize capital investment, optimize energy efficiency, and improve overall economics. Integration between first and second generation technology processes may be the most effective way to bring second generation biofuels to market.

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Part IV
Biofuel Resources

Chapter 9
Potential Bioresources as Future Sources of Biofuels Production: An Overview

Veeranjaneya Reddy Lebaka (L)

Abstract In recent years, biofuels are receiving increasing public and scientific attention, because of the crude oil reserves of the world that are predicted to deplete in about 40 years and other factors such as uncertainties related to oil price, greenhouse gas emission, and the need for increased energy security and diversity. Biofuels are a wide range of fuels that are in some way derived from renewable bioresources. It is reported that fossil fuels—oil, coal, and natural gas—dominated the world energy economy, covering more than 80 % of the total primary energy supply. Renewable energy sources accounted for 9.8 % of the world's total primary energy supply in 2007. The wonderful development of the biofuel industry was heralded in the past few years, from the late 1970s as the renewable energy source to worldwide shortages of fossil fuels. Biofuels production is the process of preparing raw materials—starch- or sugar-containing for fermentation by microorganisms, which is currently the only microorganism used for converting sugar into alcohol and the heart of the fermentation process is the yeast cell. Demand for biofuels is increasing at a rate that will require serious consideration of alternatives to the primarily glucose-/starch-based feedstock over the next decade. Various lignocellulosic biomass sources such as agricultural residues, oils, oilseeds, wood and forest wastes, municipal solid wastes, wastes from the pulp and paper industry, and algae have the potential to serve as low-cost and abundant feedstock for biofuels production. Next generation biofuel production from high hydrocarbon (Latex producing) yielding plants and oligogeneous microorganisms are attracting the interest of many investigators in the area of novel and advanced fuels. Advanced genetic engineering tools offer the possibility of improved biodegradative capabilities of cellulases (cellulosomes) by

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reconstituting cellulosomes and with potent enzymes from different microbial species. Fast-growing grass species, halophytes specifically grown on marginal land and aquatic macrophytes, algae and other oil accumulating microorganisms could provide biofuel feedstock for biorefineries in the future. This chapter focuses the current status and future prospectus of research on 'liquid biofuel production from different potential substrates'. The first section discusses the introduction of biofuels, the second section gives a detailed presentation on the status and concerns of biofuels, and the third section discusses the types of biofuels and the production of especially liquid biofuels from different bioresources that are currently in use and also points to potential bioresources for future use.

9.1 Introduction

Consistent access to power, fuel, and water is important for continued growth and maintenance of human civilization. With world energy consumption predicted to increase to 54 % between 2001 and 2025, considerable focus is being directed toward the development of sustainable and carbon neutral energy sources to meet the future needs. Biofuels are an attractive alternative to current petroleum-based fuels as they can be utilized as transportation fuels with little change to current technologies. Liquid (ethanol, biodiesel) or gaseous (methane or hydrogen) biofuels are derived from organic materials such as starch, oilseeds, and animal fats, or cellulose (Fig. 9.1). Biofuels are receiving increasing public and scientific attention in their use as an alternative to petroleum-based fuels in transportation driven by four factors. First, there are increasing concerns about the world's dwindling petroleum supply amid continuing growth in demand and price volatility. Global demand for petroleum is projected to increase roughly from 50 % by 2030 to 118 million barrels per day (mbd), with the United States, Europe, and China projected to lead consumption at 28, 16, and 15 mbd, respectively. Since 1990, India's oil production has grown from about 650 thousand barrels per day to about 1 mbd in 2008.

Meanwhile, consumption has increased from 1.2 mbd from 1990 to around 3 mbd in 2008. Second, many nations are increasingly concerned about energy security. As global crude oil supply steadily declines, the remaining petroleum reserves grow increasingly concentrated, i.e., come under the control of a steadily declining number of producers. One consequence is an increasing dependence of petroleum importing regions on an ever-decreasing number of suppliers. For example, India imports the majority of its oil from about 10 countries. The gap between consumption and production has been expanding rapidly in the past few years due to growth. Consequently, India's oil imports have risen from US$ 6 billion in 1990 to $15 billion in 2000 and to US$77 billion in 2008. Various nations have taken steps explicitly aimed at increasing energy security by investing in domestic resources or considering strategies for developing alternative

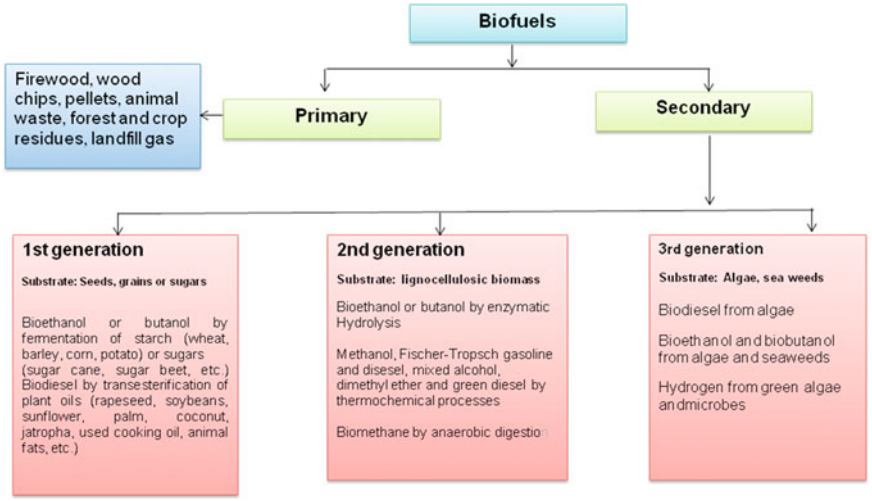

Fig. 9.1 Classification of biofuels depending on substrates used

energy sources. Third, addressing climate change will require a shift to nonpetroleum fuels, which presently account for one-fifth of the world's fossil carbon dioxide emissions and are rising at a rate of roughly 2.5 % per year. Several analyses conclude that biofuels could play a major role in strategies aimed at reducing carbon emissions and, when combined with carbon capture and sequestration technologies capable of extracting carbon dioxide from the atmosphere, minimizing atmospheric concentrations of greenhouse gases (GHGs). The fourth motivation for promoting biofuels derives from its potential to support development in rural areas of both industrialized nations, where governments are under increasing pressure to eliminate subsidies to the agricultural sector, and developing nations, where creating economic alternatives for rural communities is an abiding challenge (Azar et al. 2006; Sagar and Kartha 2007).

9.2 Concerns of Biofuel Production

The various biomass feedstock used for producing biofuels can be grouped into two basic categories: 'first generation' feedstock, which is harvested for sugar, starch, and oil content and can be converted into biofuels using conventional technologies, and 'second generation feedstock', which is harvested for total biomass and can only be converted into biofuels by advanced technical processes. Second generation feedstock can be acquired from various woody plants (e.g., hybrid poplar, eucalyptus), grassy crops (e.g., miscanthus, switchgrass, sweet sorghum), agricultural residues (e.g., bagasse, straw), and municipal solid waste (e.g., waste paper and yard waste). Second generation feedstock is considered the

least expensive and most abundant form of biomass and in energy content is comparable to first generation feedstock. The limiting factor for the use of second generation feedstock for biofuel production is the nonavailability of low-cost production and processing technologies that efficiently convert biomass into liquid fuel (Fig. 9.2). Consequently, current production economics are more favorable for conversion of first generation feedstock into biofuels. Biomass processing costs in ascending order are as follows: lignocellulose, starches, vegetable oils, latex (terpenes), algal lipids. Vegetable oils, starches, and sugarcane currently have lower conversion costs than other feedstock. Terpenes and algal lipids are currently too expensive to be used as liquid biofuel feedstock. Hence, many countries that foster biofuel development generally promote the use of agricultural and oil crops that are already produced on a large scale for human and animal consumption. For example, all of Brazil's ethanol production is derived from sugarcane, currently the highest volume ethanol feedstock worldwide. In the US, more that 90 % of the ethanol comes from corn, the world's second largest fuel crop and one of the most important agricultural crops globally. In Europe, about 70 % of the biodiesel is made from rapeseed, the world's second largest source of plant oils, with most of the remainder coming from sunflower seeds. Moreover, nearly all of the biodiesel produced in the US comes from soybeans, the world's largest source of plant oil for food and fuel. Interestingly, the two crops with the largest planted area worldwide, wheat and rice, are not significant sources of biofuel due to high

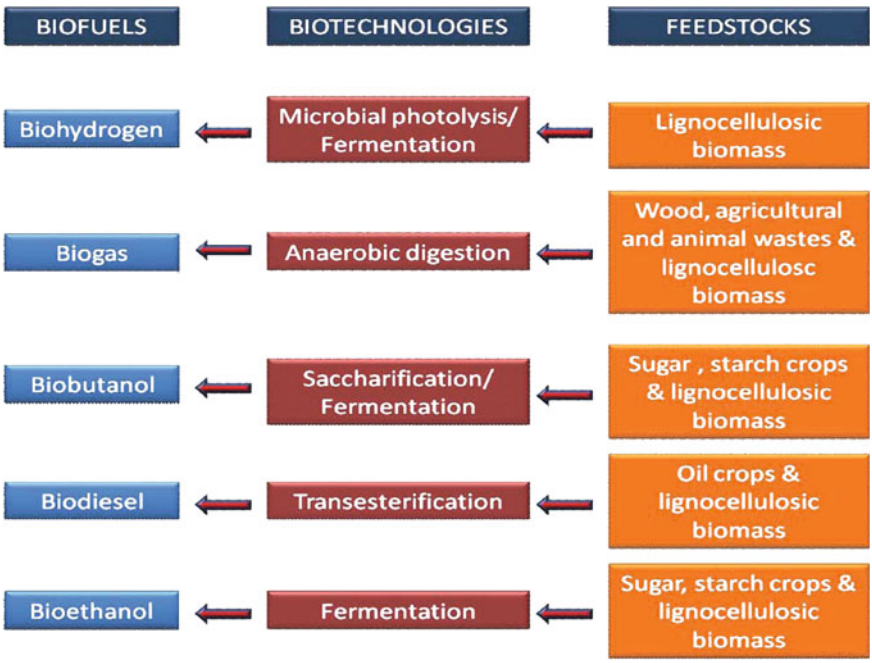

Fig. 9.2 Technologies used in production of various biofuels

demand as food. The use of agricultural crops as first generation feedstock for biofuel production raises ethical and moral questions due to the rising cost of food as well as direct competition with agriculture for arable land and food processing facilities.

Roughly 29 % (51×10^9 ha) of the Earth's surface is covered by land. Of this, about 10 % (4.9×10^9 ha) has been put into productive use. Productive use lands can be classified under three categories: (1) pastures and rangeland, (2) crops, and (3) settled land. Roughly 67 % of productive use land is devoted to pastures and rangeland, 29 % is devoted to crops, and human settlements cover about 4 % (about 0.2×10^9 ha). Agricultural food production doubled between 1961 and 1996 with only a 10 % increase in the land under cultivation, although irrigated cropland has increased by about 70 % over the past four decades (Foley et al. 2005). Agriculture accounts for an estimated 70–80 % of global water use, although for many countries the percentage is even higher (Gui et al. 2008). As world population and affluence increase, agriculture will face increased pressure to produce food more efficiently and to maintain the ecological sustainability of arable lands. In addition, the intensive use of fertilizers and pesticides on crops has also caused significant environmental problems. Rainforests in Brazil and South East Asia are currently being cleared at an unprecedented rate for soybean and oil palm plantations for the production of biofuels. Environmentalists are now debating the negative impact of biofuels production by deforestation activities and destruction of ecosystems (FAO 2006; Gui et al. 2008).

Approximately, 8 % of plant-based oil production is used as biodiesel, and this has contributed to price increases of oil crops such as rapeseed, palm, and soybean over the last few years. Increasing demand for corn for ethanol production in the United States has escalated the price of corn in Mexico, almost tripling between 2006 and 2007, and has led to a tortilla crisis in that country. This is an issue beyond just culinary or cultural overtones because poor Mexicans get more than 40 % of their protein from tortillas. At the same time, chicken feed costs in the United States increased by 40 % between the summer of 2006 and early 2007 because of rising corn prices. In addition, prices of sugar beets, wheat, maize, sugarcane, oilseeds, and cassava were 10, 16, 23, 43, and 54 % higher, respectively, than their baseline 2020 prices (Johanson and Azar 2007).

The Food and Agriculture Organization estimates that there were 963 million chronically undernourished people in developing countries in 2008 with attendant enormous human suffering and social and economic costs. About three quarters of these people are extremely poor rural inhabitants, mainly landless small farmers living in impoverished regions, underemployed agricultural laborers, and other artisans and traders whose livelihood depends on these groups (Manzoye 2001). The impoverishment of these groups has increased in many instances as the prices of agricultural commodities have shown not only a decline over the long term but also short-term volatility. Moreover, a decline in food prices often does not benefit this group because they are not purchasers of food. In such instances, a rise in food prices due to biofuels benefits countries and households that are net producers of

food, including the rural poor whose livelihoods are closely tied to the agricultural economy (Runge and Senauer 2007).

The world's arable land should remain for food crop cultivation in the light of environmental, economic, ethical, and moral issues. Furthermore, most of the second generation feedstock plants can be grown on marginal lands, thus avoiding competition with agriculture and fostering price stability in food commodities. Field et al. (2008) estimated abandoned lands at approximately 450 Mha worldwide. Additionally, a great deal of degraded land faces abandonment in the future if overuse continues. Estimates of moderately degraded lands (those lands with significant decreases in productivity) are up to 910 Mha worldwide, and the combined abandoned and degraded land area of 1,300–1,400 Mha is substantial when compared with the 5,700 Mha used for agriculture and animal production (Daily 1995; FAO 2000). Farming of biofuel crops on marginal or abandoned lands allows wasteland utilization, combats desertification, and also has potential economic benefits for small farmers.

9.3 Liquid Biofuels

The two primary pathways involved for producing liquid biofuels from biomass are biochemical conversion and thermochemical conversion. The most commonly known liquid biofuels such as ethanol, biodiesel, and butanol are produced through biochemical conversion of plant products (juice, grains, and whole biomass) that are derived from photosynthesis (Fig. 9.3). There are two main thermochemical pathways for converting biomass into liquid fuel, i.e., pyrolysis and thermochemical gasification. Pyrolysis uses high temperatures in the absence of oxygen to convert the biomass into liquid 'bio-oil', solid charcoal, and light gases (Kheshgi et al. 2000). Bio-oil is best suited for use as a fuel for stationary electric power or thermal energy applications, rather than as a transportation fuel (Ma and Hanna 1999). Thermochemical gasification, which entails partial combustion of a feedstock, involves decomposition of biomass into a gas consisting primarily of hydrogen, carbon monoxide, water vapor, nitrogen (unless gasified in oxygen rather than air), and small quantities of methane and higher hydrocarbons. This synthesis gas (or "syngas") can be then be cleaned and used as chemical feedstock in a manner very similar to petrochemical feedstock. Thermochemical gasification is an attractive alternative for producing biofuels because it can be used on many feedstock in addition to the starch, sugar, or oil food crops that are the basis of ethanol and biodiesel today. It can make use of the cellulosic fraction of biomass, as well as the lignin fraction, which typically comprises 20–30 % of woody biomass. Thermochemical gasification makes potential energy sources of waste streams, agricultural residues, and dedicated energy crops that can be grown on less-valuable land than annual food crops. Four biofuel options undergoing

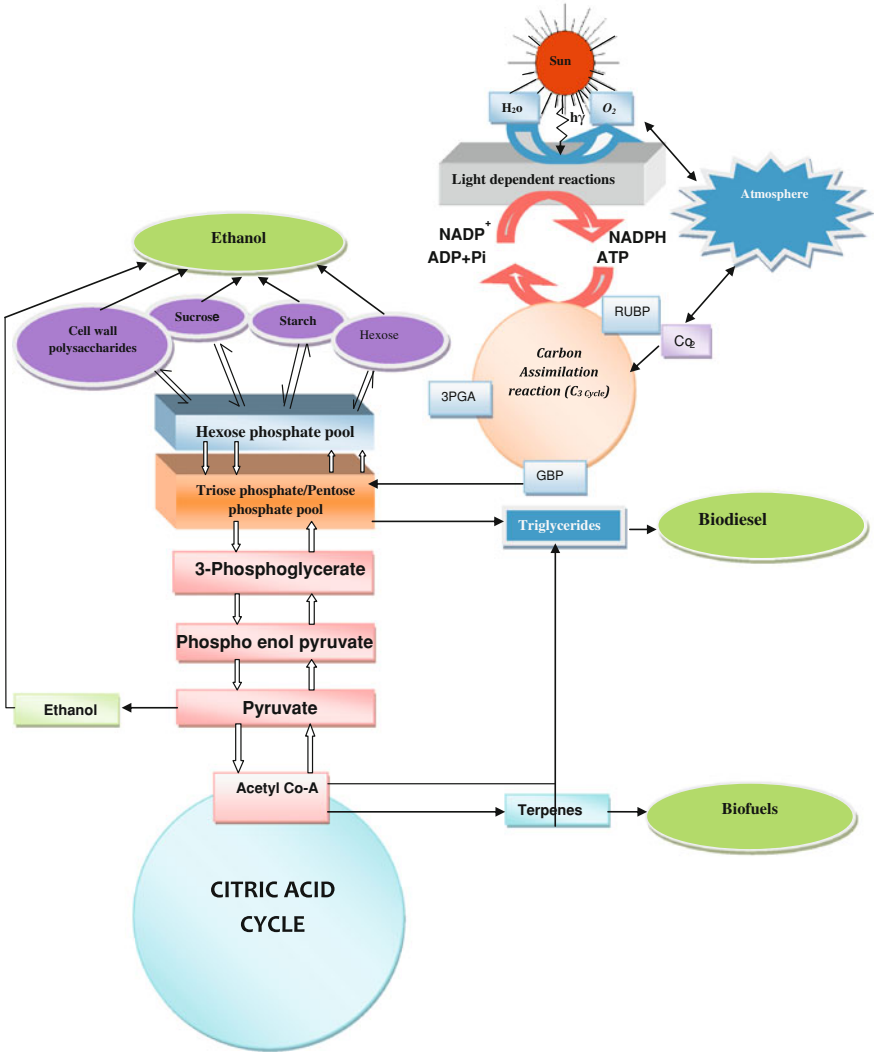

Fig. 9.3 Photosynthesis as the source of all biofuels

development that are produced via the thermochemical gasification route are methanol, hydrogen, Fischer–Topsch liquids, and dimethyl ether (Hamelinck and Faaji 2006). The importance of other alternative fuels such as biobutanol, biojet fuel, biohydrogen, biotolune, and numerous other hydrocarbon compounds have not received significant attention as compared to ethanol and biodiesel for various reasons.

9.3.1 Bioethanol

Ethanol if produced using a renewable substrates is named as bioethanol. Ethanol, both renewable and environmentally friendly, is believed to be one of the best alternatives, leading to a dramatic increase in its production capacity. China and India contributed 11 % to global ethanol production in 2006, and production levels were much lower in other countries (Johnston 2008; Naik et al. 2010). Ethanol can be produced from a variety of substrates including sugar-laden crops (e.g., sugarcane, sugar beet), starch-laden crops (e.g., corn and cassava), and lignocellulosic biomass. The substrate selection depends on availability depending upon countries' agricultural practices (Table 9.1). Cellulosic ethanol derives its appeal from the fact that it can be acquired from various choices of second generation feedstock. Production of ethanol from sugar-laden crops is the simplest route; the main steps are milling, pressing, fermentation, and distillation. Ethanol production from starch-laden crops requires the additional steps of liquefaction and saccharification (conversion of sugar). Presently, roughly 60 % of ethanol production is sugar based and 40 % is starch based. A key characteristic of cellulosic biomass is that it

Table 9.1 Fuel ethanol programs and feedstock used in some countries

Country	Feedstock	Percentage of ethanol in gasoline blends, % (v/v)	Remarks
Brazil	Sugarcane	24	ProAlcool program; hydrous ethanol is also used as fuel instead of gasoline
USA	Corn	10	Oxygenation of gasoline is mandatory in dirtiest cities; taxincentives; some states have banned MTBE; 5 % blends are also available
Canada	Corn, wheat, barley	7.5–10	Tax incentives; provincial programs aimed to meet Kyoto protocol
Colombia	Sugarcane	10	Began in November 2005; total tax exemption
Spain	Wheat, barley	–	Ethanol is used for ETBE production; direct gasoline blending is possible
France	Sugar beet, wheat, corn	–	Ethanol is used for ETBE production; direct gasoline blending is possible
Sweden	Wheat	5–85	Blends are also available; there is no ETBE production
China	Corn, wheat		Trial use of fuel ethanol in central and northeastern regions
India	Sugarcane	5–10	Ethanol blends are mandatory in all states
Thailand	Cassava, sugarcane, rice	10	All gasoline stations in Bangkok must sell ethanol blends; ethanol blends will be mandatory from 2007

is naturally resistant to decomposition into sugar molecules. Following pretreatment with steam and/or acid, cellulosic biomass produces a liquid slurry consisting mostly of hemicelluloses and lignin. More efficient and less-costly enzymes can be used to decompose cellulose and hemicelluloses into fermentable sugars, followed by treatment with specialized bacteria or yeasts that convert sugars into ethanol. The use of lignocellulosic biomass in biofuel production and future research needs have been recently summarized by Carroll and Somerville (2009).

The world's top ethanol fuel producers in 2008 were the United States with 9.0 bg (billion US liquid gallons) and Brazil (6.47 bg), accounting for 89 % of world production. Strong incentives, coupled with other initiatives, are giving rise to fledging ethanol industries in countries such as Canada, China, Thailand, Colombia, India, Australia, and some Central American countries. Nevertheless, ethanol is yet to make a significant impact on world oil consumption. Currently, India produces about 0.66 bg of ethanol, which is primarily used for potable and industrial purposes. Despite a 2004 mandate requiring 5 % ethanol blending with fuel, currently only a very small amount of ethanol is blended with petrol (Licht 2008).

9.3.1.1 Substrates for Ethanol Production

Well-studied and commercial technologies for ethanol production are crop based, utilizing substrates such as sugarcane juice, beet juice, molasses, and corn starch. Since the cost of raw materials can be as high as 40 % of the bioethanol cost (von Sivers et al. 1994; Wyman 1999), researchers have concentrated on utilizing lignocelluloses since the later 1990s. This natural and potentially cheap and abundant polymer is found as agricultural waste (wheat and rice straw, corn stalks, soybean residues, sugarcane bagasse), industrial waste (pulp and paper industry), forestry residues, municipal solid waste, etc. (Wiselogel et al. 1996). It has been estimated that lignocelluloses accounts for about 50 % of the biomass in the world and 10–50 billion tons.

9.3.1.2 Ethanol from Sugars

In general, the sugarcane juice and molasses (by-products of sugar mills) are the main feedstock for ethanol production. In Brazil, about 79 % of ethanol is produced from fresh sugarcane juice and the remaining percentage from cane molasses (Wilkie et al. 2000). Sugarcane molasses is the main feedstock for ethanol production in India; cane juice is not presently used for this purpose (Ghosh and Ghose 2003). Sugar beet juice and molasses are other sources of fermentable sugars for ethanol fermentation (production) especially in Europe. In the industrial production of ethanol, yeast, *Saccharomyces cerevisiae* is the most employed microorganism due to its capability to hydrolyze cane sucrose into glucose and fructose, two easily assimilable hexoses/sugars. Aeration is an

important factor for growth and ethanol production by *S. cerevisiae*. Although this microorganism has the ability to grow under anaerobic conditions, small amounts of oxygen are needed for the synthesis of substances like fatty acids and sterols. The oxygen may be supplied through the addition to the medium of some chemicals like urea hydrogen peroxide (carbamide peroxide), which also contributes to the reduction of bacterial contaminants as claimed in the patent of Narendranath et al. (2000). Other yeasts, as *Schizosaccharomyces pombe*, present the additional advantage of tolerating high osmotic pressures (high amounts of salts) and high solids content (Bullock 2002). Among bacteria, the most promising microorganism is *Zymomonas mobilis*, which has a low energy efficiency resulting in a higher ethanol yield (up to 97 % of theoretical maximum). However, its range of fermentable substrates is too narrow (glucose, fructose, and sucrose). Another disadvantage of the use of this bacterium during the fermentation of sugarcane syrup and other sucrose-based media is the formation of the polysaccharide levan (made up of fructose units), which increases the viscosity of fermentation broth, and of sorbitol, a product of fructose reduction that decreases the efficiency of the conversion of sucrose into ethanol (Lee and Huang 2000).

The high osmolality of the media based on cane molasses is negative for ethanolic fermentation. This osmolality is related to the concentration of sugars and salts in the medium. Different studies have been carried out in order to obtain *S. cerevisiae* strains with greater salt and temperature tolerance. For example, Morimura et al. (1997) developed by protoplast fusion and manipulating culture conditions, flocculating strains capable of growing at 35 °C and at molasses concentration of 22 % (w/v). Under these conditions and using repeated-batch cultures at laboratory scale, ethanol concentration of 91 g/l and productivities of 2.7 g/(l h) were obtained. However, the principal approach for avoiding the negative influence of salts and other compounds on the fermentation is through the conditioning of molasses by the addition of different compounds neutralizing the inhibitory effects of the medium components. In addition, molasses should be supplemented with nutritional factors promoting the yeast growth.

With the prevailing interest in high gravity fermentation both from quality and economic considerations, research and development efforts are being made to understand the process of alcohol production, namely excretion and tolerance by fermenting yeast strain. Earlier, several researchers investigated the increased production of ethanol by adding certain growth factors such as ergosterol, oleic acid, vegetable oils, fatty acids, soy flour, skimmed milk powder, chitin, polysaccharides, and fungal mycelium (Andersen and Stier 1953; Casey et al. 1984; Deepak and Visvanathan 1984; Viegas et al. 1984; Damoano and Wang 1985; Patil and Patil 1989). Similar studies were made by Reddy and Reddy (2005, 2006) using malted horse gram flour and finger millet flour.

9.3.1.3 Ethanol from Starch

Starch is a one of the best and most high yielding feedstock for ethanol production, but yeast *S. cereviciae* cannot utilize it directly. Hydrolysis is required to produce ethanol from starch by fermentation. Starch was traditionally hydrolyzed by acids, but the specificity of the enzymes, their inherent mild reaction conditions, and the absence of secondary reactions have made the amylases to be the catalysts generally used for this process. There are two steps present in hydrolysis of starch using amylases. First, these starch suspensions should be brought to high temperatures (90–110 °C) for the breakdown of starch kernels. The product of this first step, called liquefaction, is a starch solution containing dextrines and small amounts of glucose. In second step, the liquefied starch is subject to saccharification at lower temperatures (60–70 °C) through glucoamylase obtained generally from *Aspergillus niger* or *Rhizopus* species (Pandey et al. 2000; Shigechi et al. 2004). Apar and Ö zbek (2004) provide information about the effects of operating conditions on the enzymatic hydrolysis of corn starch using commercial a-amylase. In previous years, the possibility of hydrolyzing starch at low temperatures for achieving energy savings is being investigated (Robertson et al. 2006).

Potential Starchy Substrates for Ethanol Production

Corn:

Ethanol is produced almost exclusively from corn in the USA. Corn is milled for extracting starch, which is enzymatically treated for obtaining glucose syrup. Then, this syrup is fermented into ethanol. There are two types of corn milling in the industry: wet and dry. During the wet-milling process, corn grain is separated into its components. Starch is converted into ethanol and the remaining components are sold as co-products. During dry-milling, grains are not fractionated and all their nutrients enter the process and are concentrated into a distillation co-product utilized for animal feed called Dried Distiller's Grains with solubles (DDGS) (Gaulati et al. 1996).

Wheat:

Generally in Europe, ethanol is mostly produced from beet molasses; in some countries like France it is also produced from wheat by a process similar to that of corn. Some efforts have been made for optimizing fermentation conditions. For example, Wang et al. (1999) have determined the optimal fermentation temperature and specific gravity of the wheat mash. Soni et al. (2003) have optimized the conditions for starch hydrolysis using α-amylase and glucoamylase obtained by solid-state fermentation of wheat bran.

Cassava:

Cassava is an important alternative source of starch for ethanol production and for production of glucose syrups. Cassava is the tuber that has gained most interest due to its availability in tropical countries being one of the top ten more important tropical crops. Ethanol production from cassava can be accomplished using either

the whole cassava tuber or the starch extracted from it. Starch extraction can be carried out through a high-yield large-volume industrialized process as the Alfa Laval extraction method (FAO 2004), or by a traditional process for small- and mid-scale plants. This process can be considered as the equivalent of the wet-milling process for ethanol production from corn. The production of cassava with high starch content (85–90 % dry matter) and less protein and minerals content is relatively simple.

Others:

Besides corn and wheat, ethanol can be produced from rye, barley, triticale (Wang et al. 1997), and sorghum (Prasad et al. 2007). For these cereals, some pretreatments have proved to be useful. Abd-Aziz (2002) suggested the utilization of sago palm for ethanol production in the case of Malaysia. The ethanol production from bananas and banana wastes using commercial a-amylase and glucoamylase has been studied by Hammond et al. (1996). In their work, an ethanol yield of 0.5 L EtOH/kg dry matter of ripe bananas was obtained. The processing of starch-containing food wastes by adding malt to the pulverized feedstock has been patented (Chung and Nam 2002). One of the most promising crops for fuel ethanol production is sweet sorghum, which produces grains with high starch content, stalks with high sucrose content and leaves, and bagasse with high lignocellulosic content. In addition, this crop can be cultivated in both temperate and tropical countries requiring only one-third of the water needed for cane cropping and half of the water required by corn. Moreover, it is tolerant to the drought, flooding, and saline alkalinity (Winner Network 2002). Grassi (1999) reports that from some varieties of sweet sorghum, the following productivities can be obtained: 5 ton/Ha grains, 8 ton/Ha sugar, and 17 ton dry matter/Ha lignocellulosics. The estimated price for fuel ethanol production from this feedstock is US$200–300/$m^3$, whereas the corresponding one for sugarcane ethanol is 260, for corn ethanol is 300–420, and for lignocellulosic ethanol is 450.

9.3.1.4 Ethanol from Lignocellulosic Biomass

Lignocellulosic complex is the most abundant biopolymer on the Earth. It is considered that lignocellulosic biomass comprises about 50 % of world biomass and its annual production was estimated at 10–50 billion tons. Many lignocellulosic materials have been tested for bioethanol production as observed in Table 9.5. In general, prospective lignocellulosic materials for fuel ethanol production can be divided into six main groups: crop residues (cane bagasse, corn stover, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp), hardwood (aspen, poplar), softwood (pine, spruce), cellulose wastes (newsprint, waste office paper, recycled paper sludge), herbaceous biomass (alfalfa hay, switchgrass, reed canary grass, coastal Bermudagrass, timothy grass), and municipal solid wastes (MSW). The composition of most of these materials can be found elsewhere (e.g. Sun and Cheng 2002). Numerous studies for developing large-scale production of ethanol from lignocellulosics have been

carried out in the world. However, the main limiting factor is the higher degree of complexity inherent to the processing of this feedstock. This is related to the nature and composition of lignocellulosic biomass. Two of the main polymers of the biomass should be broken down into fermentable sugars in order to be converted into ethanol or other valuable products. But this degradation process is complicated, energy-consuming, and incompletely developed.

Pretreatment of Lignocellulosic Biomass

Feedstock pretreatment is the main processing challenge in the ethanol production from lignocellulosic biomass. The lignocellulosic complex is made up of a matrix of cellulose and lignin bound by hemicellulose chains. During the pretreatment, this matrix should be broken in order to reduce the crystallinity degree of the cellulose and increase the fraction of amorphous cellulose, the most suitable form for enzymatic attack. Additionally, the main part of hemicellulose should be hydrolyzed and lignin should be released or even degraded. The fact that the cellulose hydrolysis is affected by the porosity (accessible surface area) of lignocellulosic materials should be also considered. The yield of cellulose hydrolysis is less than 20 % of the theoretical when pretreatment is not carried out, whereas the yield after pretreatment often exceeds 90 % of theoretical (Lynd 1996). Therefore, the aim of the pretreatment is the removal of lignin and hemicellulose, the reduction of crystalline cellulose, and the increase in the porosity of the materials. Additionally, the pretreatment should improve the formation of sugars or the ability to form them during the succeeding enzymatic hydrolysis, and avoid the formation of inhibitors for subsequent hydrolysis and fermentation processes. For the pretreatment of lignocellulosics, several physical, physical–chemical, chemical, and biological processes have been proposed and developed and every method has its own merits and demerits (Sun and Cheng 2002). During pretreatment and hydrolysis of lignocellulosic biomass, a great amount of compounds that can seriously inhibit the subsequent fermentation are formed in addition to fermentable sugars. Inhibitory substances are generated as a result of the hydrolysis of the extractive components, organic and sugar acids esterified to hemicellulose (acetic, formic, glucuronic, galacturonic), and solubilized phenolic derivatives. Hence, detoxification of the lignocelluloses hydrolysate is essential for better fermentation and product yields. Detoxification methods can be physical, chemical, or biological. As pointed out by Palmqvist and Hahn-Hägerdal (2000), these methods cannot be directly compared because they vary in the neutralization degree of the inhibitors.

9.3.1.5 Comparison of the Main Types of Feedstock

The selection of the suitable feedstock for ethanol production strongly depends on the local conditions. Evidently, North American and European countries have

based their ethanol industry on the starchy materials due to their agro-ecological conditions. These conditions are not appropriate for cultivation of sugarcane, the highest yielding feedstock. The use of starchy crops, specifically corn, for bioethanol production has provoked a hot debate on the suitability of these raw materials considering the energy input required for their production (Patzek et al. 2005; Pimentel 2003; Shapouri et al. 2003). For energy considerations crops yield should be analyzed. The calculated ethanol yield from corn is greater than that from sugarcane because of the higher amount of fermentable sugars (glucose) that may be released from the original starchy material (Table 9.2). However, the annual ethanol yield from each hectare of cultivated corn is lower than that for sugarcane. For the case of beet molasses, the yield per ton of feedstock is lower compared to corn, but as the beet productivity per cultivated hectare is considerably higher, the annual ethanol yield expressed in L/(Ha year) is higher related to starchy materials (beet: 6,600, dry-milled wheat: 3,214, wet milled wheat: 2,555) (Poitrat 1999). On the other hand, the high moisture content of cassava implies the use of a greater amount of feedstock to reach the same starch content related to corn. However, the crop yields of cassava are higher than that of corn. Moreover, the corn yield in some tropical countries is significantly lower than the corn yield in the USA favoring the use of cassava instead of corn in such countries. For instance, the yield of technified corn in Colombia reaches only 3.9 ton/Ha whereas the cassava yield can reach 30 ton/Ha. This yield leads to an ethanol yield of 5,400 L/(Ha year), greater than the expected yield from corn under Colombian conditions that reaches 4,329 L/(Ha year) (Agrocadenas 2006).

Lignocellulosic materials represent a promising option as a feedstock for ethanol production considering their output/input energy ratio, their great availability both in tropical and temperate countries, their low cost (primarily related to their transport), and their ethanol yields (Table 9.2). One of the advantages of the use of lignocellulosic biomass is that this feedstock is not directly related to food production. This implies the production of bioethanol without the need of employing vast extensions of fertile cultivable land for cropping cane or corn exclusively dedicated to the bioenergy production. In addition, lignocellulosics is a resource that can be processed in different ways for production of many other products like synthesis gas, methanol, hydrogen, and electricity (Chum and Overend 2001). The selection of the lignocellulosic feedstock is the interest of each country for transferring value to the produced wastes, especially for those wastes that do not have value as a food. For the case of the USA, corn stover is considered one of the most promising feedstock due to its wide availability. The total availability of this material in such a way that its recollection and use be environmentally sustainable, has been estimated in about 80–100 million dry tons per year becoming the most abundant agricultural residue in the world. Kadam and McMillan (2003), citing nonpublished data from the NREL, indicate that the theoretical yield of this material is 480 L EtOH/dry tons, assuming that both hexoses and pentoses can be fermented into ethanol. These authors point out that 33 million tons per year of corn stover would be necessary to ensure the total ethanol production of 11,000 million liters per year considering a more realistic yield of 330 L EtOH/dry

Table 9.2 Comparative indexes for the three main types of feedstock for fuel ethanol production

Item	Sucrose-containing materials	Starchy materials	Lignocellulosic biomass	Remarks	References
Feedstock yield, ton/Ha	70–122.9			Sugarcane; Brazil, Colombia	Agrocadenas (2006)
		20		Cassava	Agrocadenas (2006)
		35		Sweet sorghum	Agrocadenas (2006)
		1.5–3.0		Wheat	Agrocadenas (2006)
		6–10.07		Corn; USA	Agrocadenas (2006)
			19.6–34.40	Cane bagasse	Moreira (2000)
			6.59–11.06	Corn stover	Kim and Dale (2004)
			1.93–3.86	Wheat straw	Kim and Dale (2004)
Feedstock cost	US$/kg 0.0100			sugarcane; Brazil	Macedo and Nogueira (2005)
		0.0760 Corn;		USA	McAloon et al. (2000)
	0.0124	1,300		Cane/corn; Colombia	Quintero et al. (2007)
			0.0295	Dry corn stover, USA	Aden et al. (2002)
EtOH yield, l/ton	70			sugarcane juice	Moreira and Goldemberg (1999)
	100			Sugar beet	Berg (2001)
		180		Cassava	Agrocadenas (2006)
		86		Sweet sorghum	Agrocadenas (2006)
		340–350		Wheat	Agrocadenas (2006)
		370		Corn	Agrocadenas (2006)
		403.1		Corn, wet milling	Gulati et al. (1996)
		419.4–460.6		Corn, dry milling	Gulati et al. (1996)
			140	Cane bagasse	Moreira (2000)
			261.3	Wheat straw	Kim and Dale (2004)
			227.7	Corn stover	Kim and Dale (2004)
			330	Dry corn stover; USA	Kadam and McMillan (2003)

(continued)

Table 9.2 (continued)

Item	Sucrose-containing materials	Starchy materials	Lignocellulosic biomass	Remarks	References
Annual EtOH yield, L/(Ha year)	5,345–9,381			Sugarcane; Brazil, Colombia	Agrocadenas (2006)
	6,600			Sugar beet; France	Poitrat (1999)
			3,600	Cassava	Agrocadenas (2006)
			9,030	Sweet sorghum	Agrocadenas (2006)
			1,020–3,214	Wheat	Agrocadenas (2006), Poitrat (1999)
			6,600	Corn	Agrocadenas (2006)
Production costs, US$/L anhydrous EtOH	0.1980			Sugarcane; Brazil	Xavier (2007)
	0.2153	0.3381		Sugarcane/corn; Colombia	Quintero et al. (2007)
		0.2325	0.3963	Corn/corn stover; USA	McAloon et al. (2000)
Output/input energy ratio	8.0			Sugarcane	Berg (2001)
	1.9			Sugar beet	Berg (2001)
		1.34–1.53		Corn; USA	Berg (2001), Shapouri et al. (2003)
			6.0	Biomass	Berg (2001)
Possibility of co-generation	Yes	No	Yes		
Co-products	Concentrated stillage for fertilization	DDGS (dry milling)	Lignin as feedstock for chemicals		

Source Sanchez and Cardona (2008)

tons. The above-mentioned demonstrates the vast possibilities of biomass taking into account that, in this case, there will be no competition for cultivable land with crops dedicated to food production.

Other studies have been oriented to the use of rice straw for ethanol production (Kadam et al. 2000). Kim and Dale (2004) provide an interesting panorama on the size of the bioethanol feedstock resource at global and regional levels considering wasted crops (crops lost in distribution) and lignocellulosic biomass (crop residues and sugarcane bagasse). These authors estimate that the global potential ethanol production from these feedstock accounts 491 GL/year that is 16 times higher than current ethanol production. This amount of bioethanol could replace 32 % of global gasoline consumption. For them, rice straw is the feedstock that potentially could produce the largest amounts of ethanol, followed by wheat straw in countries like India and China. However, the great-scale ethanol production from lignocellulosic biomass could entail serious economic and environmental consequences (Berndes et al. 2001). These authors estimate that labor requirements for bioenergy production on a great scale in whatever country should not exceed 1 % of total manpower. Grassi (1999) points out that the development of bioenergy production technologies would represent the creation of 200,000 direct and indirect jobs and the reduction of 255 million tons per year of CO_2 in 2010. Once these technological limitations that are conversion processes of biomass into ethanol are overcome, lignocellulosic biomass will be the main feedstock for ethanol production.

A complete economic and environmental evaluation of the different feedstock is required in order to make decisions on the most appropriate raw materials for fuel ethanol production in each case. A useful approach for performing such evaluations is to employ simulation tools based on realistic data obtained from existing ethanol production facilities, pilot plants, or mathematical models. In addition, this approach allows the analysis of how different technological configurations (e.g., SHF or SSF) have influence on the indicators of the overall process. Examples of these comparative studies can be found in the works of McAloon et al. (2000) and Cardona et al. (2005) for corn and lignocellulosic ethanol, Quintero et al. (2007) for sugarcane and corn ethanol, and Aden et al. (2002) and Wooley et al. (1999) for lignocellulosic biomass. By comparing all three types of feedstock (sugar, starch, and lignocellulose), conversion of lignocellulosic biomass into ethanol is economical and sustainable technology through the usage of best hydrolysis methods (Table 9.3).

9.4 Biobutanol

Butanol is a four carbon alcohol ($C_4H_{10}O$). It contains more hydrogen and carbon compared to ethanol (Ramey 2004). Butanol fermentation was first discovered by Pasteur in 1861, and this work was followed by studies on the production of this chemical and feedstock by various investigators in the latter part of the nineteenth

Table 9.3 Research trends and priorities for improving fuel ethanol and butanol production from different feedstock

Issue	All feedstock	Sucrose-containing materials	Starchy materials	Lignocellulosic biomass
Feedstock	Reduction in costs of feedstock by improving crop yields, pest resistance, and cropping systems	Increase in crop productivity	Utilization of native starchy material other than cereal grains (cassava, indigenous roots, etc.)	Evaluation of the use of dedicated energy crops
				Genetic modification of herbaceous plants for changing their carbohydrate content
			Development of corn hybrids with higher extractable starch or with higher fermentable starch content	Economic utilization of different and alternative wastes like MSW
			Genetic improvement of corn (e.g. ''self-processing grains")	
Pretreatment		Removal of impurities and toxic substances from molasses	Reduction of energy costs of liquefaction	Reduction of milling power
				Optimization of steam explosion and dilute acid pretreatment
				Development of LHW, AFEX, and alkaline hydrolysis
				Reduced formation of inhibitors
				Recycling of concentrated acids
Hydrolysis			Low-temperature digestion of starch	Increase in specific activity, thermal stability, and cellulose-specific binding of cellulases (e.g. by protein engineering). Reduction of costs of cellulases production (10-fold reduction). Cellulases production by solid-state fermentation. Recycling of cellulases improvement of acid hydrolysis of MSW
Fermentation	Continuous fermentation with high cell density and increased yields and productivity	Reduction of inhibition by ethanol	Recombinant strains of yeasts with increased productivity and ethanol tolerance	Increase in conversion of glucose and pentoses into ethanol
		Microorganisms with increased osmotolerance or flocculating properties	High cell-density fermentation (e.g. immobilized cells, flocculating yeasts, membrane reactors, etc.)	Recombinant strains with increased stability and efficiency for assimilating hexoses and pentoses, and for working at higher temperatures
				Very high gravity fermentations
				Development of strains more tolerant to the inhibitors
				Increase of ethanol tolerance in pentose fermenting microorganism.

Source Sanchez and Cardona (2008)

century. In the early part of the twentieth century, Chaim Weizman (Manchester University 1912) isolated a bacterial strain capable of producing significant amounts of acetone and butanol that was named *Clostridium acetobutylicum*. Butanol is easier to blend with gasoline and other hydrocarbon products and also contains more heat energy than ethanol, which equates to a 25 % increase in harvestable energy (Btu's). It contains 110,000 BTUs per gallon, closer to gasoline's 115,000 BTUs, and is safer to handle with a Reid Value of 0.33 psi, which is a measure of a fluid's rate of evaporation when compared to gasoline at 4.5 and ethanol at 2.0 psi. Butanol is far less corrosive than ethanol and can be shipped and distributed through existing pipelines and filling stations. An 85 % butanol/gasoline blend can be used in unmodified petrol engines and butanol is much less evaporative than gasoline or ethanol, making it safer to use and generating fewer volatile organic compound (VOC) emissions (Qureshi et al. 2010a, b). Butanol contains 22 % oxygen making it a beneficiary fuel extender that is cleaner burning than ethanol (Ezeji et al. 2007). Ramey (2004) also reported that when consumed in an internal combustion engine it yields only carbon dioxide, making it more environmental friendly than biofuel.

9.4.1 Substrates for Butanol Production

Butanol producing cultures can utilize a wide variety of carbohydrates including lactose, sucrose (molasses), glucose, fructose, mannose, dextrin, starch, xylose, arabinose, and inulin derived from feed materials such as whey permeate, maize, millet, rye, sugar beet, wheat, oats, Jerusalem artichoke, and sulfite waste liquor (a by-product of paper industry that contains glucose, xylose, and arabinose). Xylose and arabinose are pentose sugars and are present in cellulosic substrates. The ability to use all these carbohydrates makes it possible to ferment nearly all the agricultural substrates such as woody biomass, agricultural residues, waste materials, and energy crops including switchgrass and miscanthus (Table 9.4).

9.4.1.1 Molasses and Whey Permeate

As a feedstock, cane molasses has numerous advantages compared to maize including easy handling and the fact that it contains sucrose that is easily hydrolyzed by solventogenic Acetone–Butanol–Ethanol (ABE) clostridia followed by the conversion of sugars into butanol. Additional advantages of molasses have been described elsewhere (Qureshi and Blaschek 2005). Whey permeate, a by-product of the dairy industry, is another valuable substrate that contains approximately 44–50 g/l lactose. Butanol-producing cultures can hydrolyze lactose to its component sugars followed by their use for producing butanol (Maddox et al. 1993). The culture does not require any additional exogenous enzyme for this hydrolysis. Whey permeates with this concentration of lactose is an ideal substrate

Table 9.4 Agricultural residues used for butanol production

Substrate	Culture	Max ABE concentration (g/l)	ABE yield (7)	ABE production (g/l.h)	Comments	References
Potato starch	*C. actobutylicum* DSM1731	12.1	0.241	0.24	Unhydrolyzed potato starch	Gutierrez et al. (1998)
Mixed Ag Res.	*C. beijerinckii* BA101	14.8	–	0.22	Actual agricultural waste	Jesse et al. (2002)
Corn fiber	*C. beijerinckii* BA101	9.3 + 0.5	0.39	0.10	Lime and XAD-4 treated	Qureshi et al. (2008)
DDGS	*C. saccharobutylicum* P262	12.1	0.31	–	Lime-treated DDGS	Ezeji and Blaschek (2008)
Wheat straw	*C. beijerinckii* P260	47.6	0.37	0.36	Simultaneous product removal	Qureshi et al. (2007)
Barley straw	*C. beijerinckii* P260	26.6	0.43	0.39	Lime-treated hydrolysate	Qureshi et al. (2010a, b)
Corn stover	*C. beijerinckii* P260	26.3	0.44	0.31	Lime-treated hydrolysate	Qureshi et al. (2010a, b)
Corn stover	*C. acetobutylicum* P262	25.7	0.34	–	Corn stover treated with SO2	Parekh et al. (1988)
Switchgrass	*C. beijerinckii* P260	14.6	0.39	0.17	Dilution with water	Qureshi et al. (2010a, b)
HW, CC, SS	*C. acetobutylicum2*	–	0.36	–	Large plant in Soviet Union	Zverlov et al. (2006)
Corncobs	*C. acetobutylicum* IFP913	20.5	0.31	0.96	48 m^3 size reactor	Marcha et al. (1992)
Inulin	*C. acetobutylicum* IFP9042	23–24	–	0.671	Inulinase was used	Marcha et al. (1985)

Source Qureshi (2010)

for butanol fermentation as the culture cannot use more than 50 g/l sugar for producing ABE in a batch process. This limitation is because of the butanol toxicity to the culture. Soya molasses is another substrate that can be used for butanol fermentation. Spray-dried soya molasses contain approximately 746 g carbohydrates per kg of which 434 g/kg (58 %) are fermentable sugars including glucose, sucrose, fructose, and galactose (Qureshi et al. 2001). The sugars, pinitol, raffinose, verbascose, melibiose, and stachyose cannot be fermented by *Clostridia*. However, their use should be possible after hydrolysis either using enzymes or dilute acid. Alternately, a solventogenic strain can be developed that can hydrolyze these sugars to monomeric carbohydrates and convert them into butanol. In addition to the above-mentioned substrates, substrates such as contaminated maize and fruit industry wastes have been demonstrated to be useful for this fermentation (Jesse 2002).

9.4.1.2 Starch

Since *Clostridia* are capable of hydrolyzing starch, potatoes and potato wastes have been evaluated as potential substrates for fermentation (Table 9.4). Starch concentration in the medium was limited to 45–48 g/l. To investigate if the hydrolytic step is required before fermentation, both hydrolyzed and unhydrolyzed potatoes were used for this fermentation. Unhydrolyzed potato starch resulted in the production of 12 g/l ABE, whereas after hydrolysis it resulted in the production of 10.4–11.4 g/l ABE suggesting that hydrolysis is not a requirement for using this substrate for ABE fermentation. Similarly, maize starch can be readily fermented to ABE.

9.4.1.3 Lignocellulose

Including the above traditional substrates, there are some novel and potential lignocellulosic substrates like maize fiber, maize stover, DDGE, wheat straw, rice straw, barley straw, switch grass, hemp waste, corn cobs, and sunflower shells available for ABE production (Table 9.4). Maize fiber is a by-product of the maize wet milling process and contains 60–70 % carbohydrates. One-fourth amount of butanol can be produced from available maize fiber. Maize fiber xylan is also evaluated for the production of ABE (Qureshi et al. 2006). Distillers dry grains and soluble (DDGS) is a by-product of the ethanol fermentation industry using dry milling process, where after fermentation, solids that contain maize fiber, cell mass, and other insoluble components are removed and dried. In a recent study, Ezeji and Blaschek (2008) used hydrolyzed DDGS for the production of butanol using *C. beijerinckii* BA101, *C. beijerinckii* P260, *C. acetobutylicum* 824, *C. sacchrobutylicum* P262, and *C. butylicum* 592.

Wheat straw is another potential substrate that can be used for the production of ABE. In a recent study, wheat straw was pretreated using dilute (1 %, v/v) sulfuric

acid followed by enzymatic hydrolysis (Qureshi et al. 2007). The hydrolysate obtained was subjected to butanol fermentation using *C. beijerinckii* P260. Fermentation was vigorous and up to 25.0 g/l ABE was produced with a productivity of 0.60 g/l.h. Switchgrass is an energy crop and can be used for producing fuels including ABE. For this study, switchgrass hydrolysates were prepared as described for wheat straw and barley straw hydrolysates. The resultant hydrolysate was subjected to fermentation without any additional treatment and it resulted in the production of 1.5 g/l ABE. As this ABE concentration is low, it was speculated that fermentation inhibitors were present in the hydrolysate. In order to produce ABE, the above three detoxification techniques were applied to the hydrolysate. Upon dilution with water, the culture was able to produce 14.6 g/l ABE (Qureshi et al. 2010a, b).

Researchers put their efforts to reduce the use of food or feed-grade substrates including molasses and rye flour; an attempt was made to utilize agricultural waste materials such as hemp waste, corn cobs, and sunflower shells (Zverlov 2006). These substrates are high in pentose sugars and low in hexoses such as glucose and galactose. These agricultural residues were hydrolyzed using dilute sulfuric acid treatment at temperatures ranging from 115 to 125 °C. The hydrolysates obtained were mixed with rye flour or molasses before fermentation, possibly because of an inability of the culture to grow in undiluted or untreated hydrolysates. In another extensive report on the conversion of corn cobs into ABE, Marchal et al. (1992) demonstrated successful corn cob hydrolysis and the production of butanol using *C. acetobutylicum*. Corn cobs were pretreated using steam explosion followed by hydrolysis using enzymes. Fermentation studies also performed in a 4 L bioreactor and scaled up to 50 m^3 system. In a 48 m^3 bioreactor, 20.5 g/l total ABE was produced with an ABE yield and productivity of 0.31 and 0.45 g/l.h, respectively. The Jerusalem artichoke is an agricultural crop with considerable potential as a carbohydrate substrate for butanol production by fermentation (Jones and Woods 1986). Marchal et al. (1985) investigated the use of Jerusalem artichoke juice for acetone butanol production and isolated a microbial strain that possessed inulinase activity. However, supplementation with additional inulinase enzyme for complete hydrolysis was necessary prior to butanol fermentation. With an optimized process, these investigators were able to produce 23–24 g/l acetone butanol. This process was tested at the pilot plant scale.

9.5 Biodiesel

Biodiesel is a widely used biofuel, defined as monoalkyl esters of long chain fatty acids derived from renewable feedstock, such as vegetable oil, animal fats, algae, etc. Biodiesel, considered as a possible substitute of conventional diesel fuel, usually consists of fatty acid methyl/ethyl esters, obtained from triglycerides by transesterification with methanol/ethanol respectively (Fig. 9.4). Biodiesel production capacity is growing rapidly, with an average annual growth rate from 2002

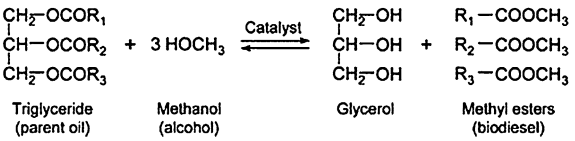

Fig. 9.4 Transesterification of oil to biodiesel (R_1–R_3 are hydrocarbon groups)

to 2006 of over 40 %. In the year 2006, the total world production was about 5–6 million tons, with 4.9 million tons processed in Europe (of which 2.7 million tons was produced from Germany), and most of the remainder from the United States. With the adoption of the Renewable Fuel Standards imposed in 2008, biofuels consumption is required to increase to 135 billion liters by 2022. The most common way of upgrading fats and oils to a transportation fuel is by transesterification. The first step in transesterification is production of diglycerides and alkyl esters, followed by monoglycerides, and finally alkyl esters and glycerol. All of these reactions are reversible, and excess alcohol (usually methanol) is used to drive the reaction to completion with yields of alkyl esters (Ma and Hanna 1999). Catalysts investigated for transesterification are either acids, bases, both liquid and heterogeneous, as well as free or immobilized enzymes (Haas et al. 2006; Kaieda et al. 1999; Komers et al. 2001; Ma and Hanna, 1999; Meher et al. 2006; Suppes et al. 2001, 2004). Enzymes are potentially useful compared to alkaline or acid catalyst, because they are:

1. More compatible with variations in the quality of the raw material and reusable.
2. Enzymes can produce biodiesel in fewer process steps using less energy and with drastically reduced amount of wastewater;
3. Are able to improve product separation and yield a higher quality of glycerol (Fukuda 2001; Kaieda et al. 1999; Kumari et al. 2007; Meher et al. 2006).

9.5.1 Potential Substrates Used for Biodiesel Production

9.5.1.1 Vegetable Oils

Some plants are efficient in the conversion of solar energy into reduced hydrocarbons or "oils". Soybean and palm trees as well as microalgae produce oils, which can be harvested and used in the production of a variety of biofuels. Vegetable oils have come to the fore for biodiesel production due to economy and feasibility. It is the similarities in the constitution of vegetable oils and petroleum-derived diesel that make vegetable oils suitable for conversion into biodiesel (Demirbas 2009; Bajpai and Tyagi 2006; Ma and Hanna 1999). Vegetable oils are naturally insoluble in water and are hydrophobic substances. Their general make-up consists of one glycerol to three fatty acids, thereby they are frequently referred to as triglycerides (Ma and Hanna 1999). The characteristics of the fat are influenced by the nature of the fatty acids attached to the glycerin; the nature of the fatty acids can have a knock-on effect on the characteristics of the biodiesel.

Vegetable oils include edible oils, nonedible oils, waste edible oils [sometimes called waste vegetable oil (WVO), and used vegetable oil (UVO)]. There are more than 50 papers cited in (Fukuda 2001) relating to biodiesel production from vegetable oils. A variety of vegetable oils such as sunflower oil, olive oil, and soybean oil have been used for production of biodiesel. The choice of vegetable oil feedstock depends on availability and is country specific. The technology for converting edible oil into biodiesel is well established (Table 9.5).

9.5.1.2 Tree Born Oils

The nonedible and tree born oils that were included are jatropha (*Jatropha curcas*), rubber seed (*Hevea brasiliensis*), castor (*Ricinus communis* L.), sea mango (*Cerbera odollam* or *Cerbera manghas*), Paradise Tree (*Simarouba glauca*), and Indian Beech Tree (*Pongamia pinnata*) (Table 9.5). There are potential problems with converting nonedible oil into biodiesel associated with high free fatty acid (FFA) content. *Biodiesel*. In excess of 350 oil bearing crops have been identified of which only a handful are considered viable for conversion into biodiesel; a table of these crops has been recently published by Demirbas (2009). The National Biodiesel Mission, formulated by the Planning Commission of the Government of India also emphasized the production of biodiesel from oils of nonedible crops

Table 9.5 Virgin oils used for biodiesel production

Oil name	References
Babassu	Merc on et al. (2000)
Borage seed	Stevenson et al. (1994)
Corn	Stevenson et al. (1994)
Cottonseed	Köse et al. (2002)
Jatropha curcas	Shah and Gupta (2007)
Karanj (*Pongamia pinnata*)	Modi et al. (2007)
Mahua (*Madhuca indica*)	Kumari et al. (2007)
Olive	Hoq et al. (1985)
Palm	Knezevic et al. (1998)
Palm kernel	Abigor et al. (2000)
Peanut	Stevenson et al. (1994)
Rapeseed	Linko et al. (1998)
Rice bran	Lai et al. (2005)
Safflower	Iso et al. (2001)
Soybean	Kaieda et al. (1999)
Sunflower	Mittelbach (1990)
Butterfat	Garcia et al. (1992)
Hoki liver oil	Stevenson et al. (1994)
Menhaden oil	Torres et al. (2003)
Tuna oil	Shimada et al. (2002)

Source Fjerbaek et al. (2008)

such as jatropha, jojoba, mahua, neem, karanja, wild apricot, cheura, kokum, *Simaroba*, and tung through a scheme called 'Integrated development of Tree Borne Oilseeds (TBO)'. Nearly half a dozen Indian states have set aside a total of 1.72 million hectares of land for jatropha cultivation and small quantities of jatropha biodiesel are already being sold to public sector oil companies (Tiwari et al. 2007).

9.5.1.3 Animal Fats

The most commonly considered animal fats are those derived from poultry, pork, and beef (Sharma et al. 2008). While one research group (Bajpai and Tyagi 2006) reported on the conversion of animal fats for biodiesel, other groups (Maa and Hanna 1999) have argued that although animal fats are mentioned regularly, their uses are limited as some of the methods for converting vegetable fats are not applicable to animal fats due to the natural differences between the two types of fats. Researchers have also produced biodiesel from salmon oil and waste animal fat. Although it would not be economical to raise fish or other animals simply for the fat, the use of fat by-products from hogs, cattle, and poultry adds value to the livestock industry (Reyes et al. 2006).

9.5.1.4 Microbial Oils

One concept currently under review is the use of algae as an oil producer for the manufacture of biodiesel. Oils of algae, fungi, and bacteria also have been investigated for biodiesel production (Schenk et al. 2008 and Strobel et al. 2008). Microalgae have a high potential as biodiesel precursors because many of them are very rich in oils, sometimes with oil contents over 80 % of their dry weight, although not all species are suitable as biodiesel production oils (Chisti 2008; Manzanera 2011). Besides, these microorganisms are able to double their biomass in less than 24 h, achieving a reduction between 49- and 132-fold in the medium culture time required by a rapeseed or soybean field. Furthermore, microalgae cultures require low maintenance and can grow in wastewaters, nonpotable water or water unsuitable for agriculture, as well as in seawater (Mata et al. 2010). The production of microalgae biodiesel could be combined with the CO_2 removal from power generation facilities (Benemann 1997) or the synthesis of several valuable products, from bioethanol or biohydrogen to organic chemicals and food supplements (Banerjee et al. 2002; Chisti 2007; Harun et al. 2010). Research has shown that the oil content of algae per hectare can be a staggering 200 times more than the most productive land-based crop (algae are the fastest growing photosynthetic organisms and have the potential to produce 46 tons of oil/hectare/year). This is a promising lead for new generation biofuels, without compromising with food supply as these can be cultivated on nonagricultural lands. However, microalgae biomass-based biofuels have several problems ranging from the optimization of

high density and large surface units of production to the location of the microalgae production unit. Anyway, the main decisions to take are the adoption of open or closed systems, and the election of batch or continuous operation mode. As will be discussed below, depending on the system and mode of operation choice, there will be different advantages and drawbacks.

Microalgae are not the only option to produce biofuels from oily biomass. Multiple prokaryotes and eukaryotes can accumulate high amounts of lipids. But, as occurred with microalgae, not all species are suitable for biodiesel production owing to differences in the kind of storage lipids. Thus, as stated by Waltermann and Steinbüchel (2010), many prokaryotes synthesize polymeric compounds such as poly(3-hydroxybutyrate) (PHB) or other polyhydroxyalkanoates (PHAs), whereas only a few genera show accumulation of triacylglycerols (TAGs) and wax esters (WEs) in the form of intracellular lipid bodies. On the other hand, storage TAGs are often found in eukaryotes, while PHAs are absent, and WE accumulation has only been reported in jojoba (*Simmondsia chinensis*). All these lipids are energy and carbon storage compounds that ensure the metabolism viability during starvation periods. Similar to the formation of PHAs, TAGs, and WE, synthesis is promoted by cellular stress and during imbalanced growth; for instance, by nitrogen scarcity alongside the abundance of a carbon source (Kalscheuer et al. 2004). The most interesting prokaryote genera in terms of accumulation of TAGs are nocardioforms such as *Mycobacterium sp*., *Nocardia sp*., *Rhodococcus sp*., *Micromonospora sp*., *Dietzia sp*., and *Gordonia sp*., alongside streptomycetes, which accumulate TAGs in the cells and the mycelia. TAGs storage is also frequently shown by members of the Gram-negative genus *Acinetobacter* (although, in this case, WE are the dominant inclusion bodies components) (Waltermann and Steinbüchel 2010). Within eukaryotes, with the exception of algae, yeasts of the genera *Candida* (non *albicans*) (Amaretti et al. 2010), *Saccharomyces* (Kalscheuer et al. 2004; Waltermann and Steinbüchel 2010), and *Rhodotorula* (Cheirsilp et al. 2011) are the most interesting ones to produce biodiesel feedstock. Steinbüchel and collaborators have worked on the heterologous expression of the nonspecific acyl transferase WS/DGAT from *Acinetobacter calcoaceticus* ADP1 in *S. cerevisiae* H1246 (a mutant strain incapable of accumulating TAGs) (Kalscheuer et al. 2004). These authors found that the yeast recovered the ability to accumulate TAGs, as well as fatty acid ethyl esters and fatty isoamyl esters. This finding showed that the *A. calcoaceticus* transferase had a high potential for biotechnological production of a large variety of lipids, either in prokaryotic or eukaryotic hosts. From this basis, as will be discussed in detail in Sect. 4.3, they worked on *Escherichia coli* TOP 10 (Invitrogen) and obtained an engineered strain able to produce fatty acid ethyl esters (biodiesel) directly from oleic acid and glucose (Kalscheuer et al. 2006).

Another possibility is combining the biomass obtained from microalgae and yeast, as recently proposed by Cheirsilp et al. (2011). These authors studied a mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* in industrial wastes. The used effluents, including both a seafood processing wastewater and molasses from a sugarcane plant. They found a synergistic

effect in the mixed culture. *R. glutinis* grew faster and accumulated more lipids in the presence of *C. vulgaris* that acted as an oxygen generator for yeast, while the microalgae obtained surplus CO_2 from yeast. The optimal conditions for lipid production were 1:1 microalga to yeast ratio initial pH of 5.0, molasses concentration at 1 %, 200 rpm shaking, and light intensity at 5.0 klux under 16:8 h light and dark cycles (Cheirsilp et al. 2011).

9.6 Latex Yielding Plants as a Source of Biofuels

Latex is usually an extractable complex mixture of triglycerides, waxes, terpenes, phytosterols, and other modified isoprenoid compounds from plants. When latex yielding plants are scraped or injured or a slit is made in the plant, high energy hydrocarbon fluids in the form of either latex or resin are exuded. Therefore, latex yielding plants are called 'hydrocarbon producing plants or petroleum plants or petroplants', and their crop 'petrocrop'. There has been much interest in cultivating plants rich in hydrocarbons or 'biocrude' as renewable sources of chemicals, for use as liquid fuel, and chemical feedstock (Buchanan et al. 1978 and Calvin 1979). For over three decades, many of the hydrocarbons yielding plants have been cultivated on an experimental basis in the United States, India, Japan, and a few other countries.

Bassham (1977) suggested that "energy farms of petroplants" be developed on arid and semiarid lands in the southwestern United States. These lands are potentially highly productive because of high solar radiation and a long growing season. Calvin (1977) reported that latex bearing plants are the most obvious alternative renewable source of fuel and chemical feedstock. Buchanan et al. (1978) evaluated 100 plant species found in the United States and suggested some as candidates for hydrocarbon production on energy farms. Johanson and Hinman (1980) stressed that developing biocrude farming and extracting facilities on marginal lands is desirable because they would not compete with food and fiber crops. Several species of plants were surveyed as potential feedstock for biocrude production in arid lands. Calvin (1982) considered *Euphorbia lathyris* (gopher plant) to be a kind of 'energy farm' capable of producing a mixture of reduced terpenoids which can be converted into a gasoline-like substance.

McLaughlin and Hoffmann (1982) surveyed about 400 accessions (195 species and varieties from 107 genera and 35 families) of either latex or resin producing plants from southwestern North America. Latex-bearing plants, particularly *Asclepias* spp. and *Euphorbia* spp., received the most attention. *Calotropis procera* (Ait.) R. Br. (Asclepiadaceae), commonly known as Aak, secretes latex containing high concentrations of extractable hydrocarbons which have been suggested as a substitute for conventional petroleum resources (Erdman and Erdman 1981). *C. procera* grows profusely with minimal care, a feature that reduces production costs. Furthermore, its ability to flourish on marginal arid and semiarid land is advantageous as its commercial development will not compete with other

conventional agricultural crops. Other members of Asclepiadaceae (milkweeds) have been proposed as renewable sources of fuel chemicals and chemical feedstock. The entire shoot of some species of *Asclepias* (*A. syriaca* L., *A. speciosa* Torr., *A. curassavica* L) have been examined for latex constituents and caloric value (Nemethy et al. 1979; Adams et al. 1983; Emon and Seiber 1985).

The Central Arid Zone Research Institute (Jodhpur. India) considered *C. procera* as a source material for biocrude. Several plant species yielding hydrocarbons were also tested as potential biocrude feedstock at the National Botanical Research Institute (Lucknow, India) in collaboration with the Indian Institute of Petroleum, Dehradun. Of the 400 species surveyed, 60 were identified as potentially useful based on availability, yield, and ease of extraction. This list was further reduced to 26 candidate species which are potentially exploitable commercially. The report was submitted as a basis for further research on petrocrops in India. In intensive studies of 10 latex euphorbs, isoprenes were quantified and compared with those of *Parthenium argentatum*, a known source of potentially exploitable hydrocarbons (Ratti et al. 1995). *Pedilanthus tithymaloides* Poit, which prefers marginal wasteland in northern and eastern India, was also evaluated as a renewable source of hydrocarbons. A white amorphous mixture of hydrocarbons comparable to gasoline was obtained by elution of the column by petroleum ether (De et al. 1997). Succulent latex-producing species like *E. tirucalli*, *E. antiquorum*, *E. nivula*, *E. milli*, and *P. tithymaloides* are grown in Karnataka and other parts of South India as hedge or ornamental plants. Nonsucculent latex producers such as *E. geniculata*, *E. corrigiodes*, *E. palcherrima*, *Synadenium grantii* are also grown in South India. In other parts of India, *E. antisyphilitica*, *C. procera*, and *Gyrostegia glandiflora* are grown, all of which are quite promising as sources of hydrocarbons. *Manihot esculenta* (tapioca) produces a latex yielding tuber which can be used for alcohol production, while other parts of the plant yield hydrocarbons (Nagendrappa 2000). The identification and characterization of latex yielding plants that may be potential sources of biocrude is one important contribution that plant biologists can make at present to the development of alternate biofuels. Research is also necessary to understand latex production at the whole plant level, to identify the main components of latex, and to elucidate the pathways of latex biosynthesis for the usage of these plants as potential renewable bioresources in the near future.

9.7 Conclusions and Future Perspectives

In conclusion, the above said liquid biofuels can be produced from various simple sugars, starch (starchy crops) lignocellulosic biomass, and oils. Bioethanol and Biobutanol are currently being produced from sugarcane and starch-containing substrates. It is definite that lingocellulosic biomass is the only option of substrate/feedstock for the production of both ethanol and butanol in the future. These plant-based substrates are abundant in nature, are outside the human food chain, and require low maintenance which makes them relatively inexpensive to grow. Many

interrelated aspects need investigation, but information about lignocellulosic biomass potential of a few species on a particular regional site opens the opportunity of selecting future biofuel candidates. However, the technical and economical challenges in bioconversion of lignocellulosic substrates are large. Although several options have been reported by different researchers for the lignocellulose-to-ethanol conversion process, the following factors are required to be carefully assessed in comparison with a well-established ethanol production using sugar or starch substrates. (1) Development of low-cost strategies of cellulose and hemicelluloses hydrolysis to soluble sugars. (2) A maximum fermentation-efficient process/fermenting organism converting a hydrolysate that contains both hexoses and pentose sugars including fermentation inhibitory compounds. (3) A consolidated bioprocess for the integration to minimize the demand of overall process energy. To overcome the above challenges and to produce sustainable biofuels the following five major research steps need to be taken:

(1) The process of enzymatic hydrolysis of agricultural substrates needs to be improved, which can be approached with the use of cheaper and of higher specific activity crude enzymes, by synthesis of enzyme in a process of reduced production cost, and by novel technology for the handling of large amounts of solids.
(2) The development of such microbial strains which are not only robust fermenting organisms, but also are at the same time more tolerant to inhibitors present in substrate-hydrolysates. These specially developed strains should be able to ferment all sugars available from the raw material in concentrated hydrolysates, giving high productivity of alcohols and withstanding high alcohol concentration in the medium.
(3) Investigate the possibility of breeding plants having desirable characters, especially low lignin contents to minimize recalcitrance to bioconversion along with increasing biomass yields. Improvements in genetics, agronomy, and the conversion process will undoubtedly help in the development of a feasible biofuel production system from biomass which can enhance and improve the feedstock availability and efficiency of biofuel production.
(4) A well-thought strategy for the process integration to reduce the number of steps involved in overall production process.
(5) Working on 3-R strategy: Recycling, Reduction, and Reuse of any by-products and wastes generated in the process to reduce the energy demand and protect the environment.

Currently, biodiesel is produced from vegetable oils, tree born oils, and animal fats. However, the economics of this process is still uncertain. The microbiological production process is extremely promising for the future usage. Algae grow as a thin surface layer in ponds, hence harvesting miles and miles of growth to get large amounts of biodiesel is needed. Huge ponds are required to grow microalgae in quantities that make the process commercially feasible. Growing of microalgae in natural lakes or ocean shores is suggestible. However, the invasiveness of algae could present an environmental hazard, since the grown algae will destroy and

overtake the ecosystem. I have pointed out the advantages that fatty acid-based biofuel production in cyanobacteria might offer and have explored the possible strategies that might be used in developing such systems. As biotechnology moves forward, genetic engineering to increase photosynthetic efficiency of the cyanobacteria and to adapt these organisms to the unnatural environment imposed by large-scale photo-bioreactors, will no doubt take central stage. Plenty of research funded by various national agencies, as well as multinational oil companies is very essential to start-up biotechnology companies to aim at making algal biodiesel a significant fraction of the diesel used in transportation in the next 20 years. Production of biofuels from hydrocarbon yielding plants is one of the novel and potential renewable bioresources for the future. The production of biofuels from second generation feedstock like latex avoids competition with agriculture, food processing technologies and ensures the global food safety.

It has been well accepted globally that there is much potential for the biofuel market and it is only matter of time before they are more available than petroleum-based fuels. The development and use of biofuels as an alternate to fossil fuels, still require a more advanced technological development, to increase their feasibility by enhancing the energy balance and reducing the emissions and production cost, are true alternatives that complete the biofuels' future scheme.

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Chapter 10
Second Generation Bio-Ethanol and Renewable Chemicals from Lignocellulosics

Sudip Kumar Rakshit

Abstract The emission of greenhouse gases (GHG) as result of consumption of fossil fuels and the finite nature of these resources provide the impetus for looking alternate sources of clean energy, which can be produced in a sustainable manner. As far as liquid fuels for transportation are concerned the production of bio-ethanol has been focus of considerable research. The use of starch-based (first generation) agricultural products as substrates for this conversion are possible but raises concerns about food security. The utilization of lignocellulosic residues for these purposes has been studied for a few decades. While it is possible to produce ethanol from such biomass, it is difficult to do so in an economically feasible way at the present cost of petroleum and ethanol. This chapter focuses on some of the innovative methods that are being attempted to overcome the bottlenecks to such processes. A broad overview of the potential use of lignocellulosics for the production of chemicals is also presented. The latter biorefining route will result in a materials with higher market value than ethanol, reduce our dependence on petroleum for a host of products used in day-to-day applications, and will also provide a sustainable alternative using the large amounts of lignocellulosic biomass found in many parts of the world.

10.1 Introduction

Biomass is produced as a part of the carbon cycle when carbon dioxide is fixed photosynthetically by plants. This can be used directly as fuels or after they are converted into fossil fuels after extended periods of time. Utilization of the

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biomass to produce energy (Saxena et al. 2009; Blottnitz et al. 2007; Huber et al. 2006) and value added products (Werpy and Petersen 2004a; Jong et al. 2012) is presently the focus of considerable research.

At the moment, there is nearly total dominance of fossil fuel-driven internal combustion engines for road transportation. This is because liquid fuels are easy to carry and convert into energy for the development of efficient engines and for the practicality of cars and vehicles that help us travel. However, as greenhouse gases (GHG) produce these fuels, the effect they have on climate and the finite nature of their availability indicate that development of alternate clean energy sources will be necessary in the near or middle term.

The alternatives that are being studied have to be renewable energy options which should have ease of application, meet the specifications of the engines that are being used, should be cost effective, and most importantly have a positive carbon balance. Electric motors will lead to the cleanest form of energy and bring the greatest benefit to the environment. However, their cost needs to be reduced further and their applicability for different classes of vehicles needs to be further developed. In all probability, transportation fuels will be a mix of a number of options (Pichon 2009).

Renewable liquid biofuels from biomass are not expected to require substantial modification of the existing motor engines and is thus considered to be an attractive proposition. Depending on the feedstock from which they are produced, these fuels have been designated to be first or second generation biofuels (Wikipedia Encyclopedia 2012). The starting materials for first generation biofuels are sugars and oil and include starch in corn, wheat, barley, and cassava, sucrose from cane sugar and vegetable oils from palm oil, jatropha, and so on. Second generation biofuels can be produced from the cellulose present in woody biomass, agricultural residues, and wastes. These materials are often associated with lignin and hence are called lignocellulosic biomass. Agricultural residues include the straw of wheat and rice, sugarcane bagasse, stem and roots from foodcrops, the top ends of trees like eucalyptus not used in paper manufacture, fast developing tall grass, and so on. The possibility of using wood from the huge forest industry, especially in North America and the Nordic countries, is also being explored. While considerable research had been carried out with these residues for more than two decades, the finite nature of fossil fuels and their effect on nature has led to considerable focus on second generation biofuels recently. This chapter will focus on the present situation, the bottlenecks to their development, and the future of these fuels which could potentially make a contribution to the development of a low carbon society. As the market price of ethanol is very low at present, the attempts being made to derive higher value renewable chemicals from the cellulose hydrolysis will also be presented.

10.2 First Generation Biofuels

Brazil, with rich sources of sugarcane, was the first country to industrialize the production of bioethanol from cane sugar. This was followed by the US, Germany, and Malaysia using sugarcane, corn, rapeseed oil, and palm oil, respectively. Countries like Thailand have now developed a road map for the increased production of first generation biofuels using locally produced cassava, sugarcane molasses, and so on. While these are either developed countries or rapidly developing economies, the lease of land to major multinational companies in less developed countries has opened considerable debate about long-term returns to the local communities. The availability of surplus agricultural produce, growing energy needs in the transport sector, and the possibility of reducing the oil import bill have been the driving forces for these developments. The environmental benefit of possible reduced GHG gas emission is more often an afterthought and not always substantiated. This is often evident from the fact that the producers of these fuels often do not take into account the effect of large water requirements for the production and the effect on the local communities. They are driven by the costs of the imported oil, the expected increase in their price, and the increasing demand for transportation fuels.

The major concern with first generation biofuel production is the effect on food security and the availability of the raw material in a sustained manner. Deforestation, change in land use patterns, and loss biodiversity are related concerns in this respect. Additionally, many governments are encouraging biofuel production by providing incentives in the form of subsidies. It is not clear whether this will benefit the economies in the long run. It will also require closer studies to determine the perceived GHG reduction benefits. For this, careful life cycle analysis (LCA) needs to be carried out, locally, taking into account the inputs like fertilizers required for their production and the transportation costs to the point of bioconversion among other parameters (BIO Intelligence Service 2010). The production of biodiesel from jatropha, which is not part of the food chain, is often encouraged. Biodiesel from jatropha is making considerable inroads in India (McGee 2006) and a number of developing countries as it has the potential of using arid and wastelands.

The effect of using food resources for fuel production in a local situation having a food surplus could lead to increased food pricing in the world market and increase the price bill for a food import in less developed countries of the world. Many of the sustainability issues raised for the production of first generation biofuels have indicated the potential benefits of alternative second generation biofuels. The latter, though technically a reality, have a number of barriers to overcome before they can be an economically feasible alternative.

10.3 Second Generation Biofuels

In this chapter, we refer to the bioconversion of lignocellulosic residues into ethanol as second generation biofuels. It must be emphasized here that a number of alternative second generation biofuels are in various stages of development. These include the Gas-to-liquid Fischer-Tropsh process which is also overall a biomass to liquid (BtL) process, biohydrogen involving gasification of the biomass and then reforming the methane produced, high temperature upgrading (HTU) of wet biomass, etc. As far as second generation biofuels are concerned most of the research investment is in the biochemical transformation of lignocellulosics into glucose and hence to bioethanol.

The major steps in the production of lignocellulosic bioethanol (Fig. 10.1) include first of all the separation of cellulose and (soluble) hemicelluloses from the woody or fibrous biomass. These sources of sugar are protected by lignin preventing its easy breakdown. A number of methods have been attempted over the years to pre-treat the lignocellulosic residues for this purpose (Kumar et al. 2009); Galbe and Zachi (2007);(Taherzadeh and Karimi 2008). These include treatment with acids, bases, solvents, steam explosion, ionic liquids, and so on. Depending on the substrate this has been efficient to different extents. However, nature has

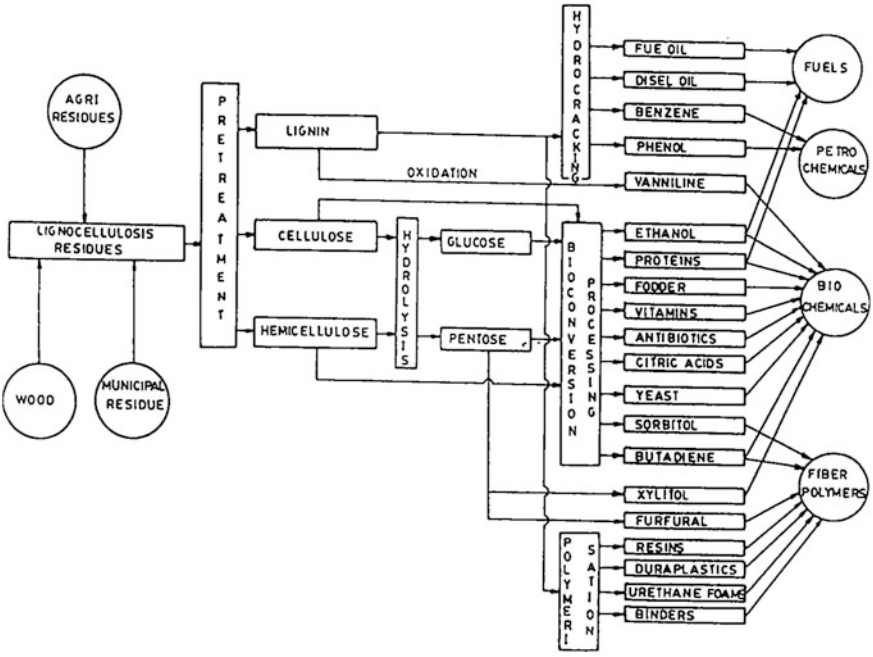

Fig. 10.1 The different steps in the bioconversion of lignocellulosic residues into ethanol. The possibility of using the different components obtained by pretreatment and hydrolysis to produce value added products by hydrocracking, bioconversion, and polymerization is also indicated

evolved this complex structure of lignin and cellulose to make the plants and trees sturdy. Hence some innovative or combination of methods, taking into account the complex structure of the natural biomass, will be required to make the cellulose present easily accessible to hydrolysis.

The cellulosic pulp extracted following pretreatment then has to be hydrolyzed to its component glucose monomer using different catalysts including acids and the cellulase enzyme complex. The severe conditions required for acid hydrolysis make construction of efficient reactors difficult. Besides, these conditions convert the sugars produced into inversion compounds which are difficult to ferment and convert into other products. The search for a source of cellulase enzyme which can break down cellulose easily and can itself be produced in a cost-effective manner has not been easy. The cost of the enzymes produced by fungal, yeast, and bacterial sources often contributes to nearly 40 % of the cost of producing ethanol by this route (Klein-Marcuschamer et al. 2012).

The sugar syrup obtained on hydrolysis has then to be fermented using yeast or bacterial strains to produce ethanol. Besides the glucose (C-6) content of cellulose from lignocellulosic substances, the possible co-fermentation of the components (C-5) of hemicellulosics to alcohol is also being explored. The slower fermentation of the C-5 components to alcohol by certain microorganisms and the inhibitory effects of the metabolites obtained by C-6 and C-5 fermentation further slow down co-fermentation (Yah et al. 2010).

Yeast fermentation of glucose to ethanol is relatively easy and ethanol concentrations of up to 12 % can be obtained. Like the first generation bioethanol, the fermented ethanol product has then to be distilled to 95 % concentration in order to make them suitable for blending with gasoline to produce gasohol. Azeotropic distillation required to get ethanol concentration to levels suitable for blending adds to the costs.

10.4 Advantages and Barriers to the Bioconversion of Lignocellulosics into Ethanol

10.4.1 Distribution of the Substrate

The major advantage of the use of lignocellulosic residues over the agricultural products used in first generation biofuels is the low cost and availability of the agricultural, forest, grass, and wastes. Their use has no impact on food security unless food crops are replaced by energy crops, especially in areas where they are not found in sufficient quantities. The fuel crops can also be grown in unutilized marginal and arid land without requiring substantial fertilizer inputs. They have higher yields in terms of energy per land area. With proper breeding methods and the use of biotechnological techniques this can be further improved in terms of yields and quality of the product.

The utilization of agricultural residues like wheat and rice straw and bagasse can be constrained by the need to put about 50 % of these residues back to the land for soil sustainability. The use of alternative feedstock like switchgrass, miscanthus, poplar, eucalyptus, and willow is presently under study. In our laboratory, we are working on the possibility of utilizing water hyacinth and typha grass which are clogs in many river streams especially in many tropical developing countries (Guragain et al. 2011). With its high water content and relatively simple composition water hyacinth can serve as a good substrate. Our own initial studies have shown that pretreatment of typha grass requires lower severity while using acid catalyzed organosolv methods. Genetic modification to produce plant biomass with reduced lignin protection of the cellulose and the cellulose itself being of a nature that it can be easily broken down to its monomer can be developed.

The bulk density of such lignocellulosic residues makes harvesting, treating, transporting, storing, and delivering large volumes of biomass feedstock require careful logistical analysis prior to plant investment and construction.

10.4.2 Pretreatment for Delignification

Different processes involved in the bioconversion of lignocellulosics, such as pretreatment and distillation after fermentation, are energy intensive. There is considerable research required to overcome the so-called biological barriers if cellulosic ethanol is to become a viable option. Overcoming the first major bottleneck to use of lignocellulosics is to find ways to barriers caused by lignin to the hydrolysis of the cellulose content. While the use of dilute acids, steam explosion, and organosolv methods are considered the most effective at the moment, a better understanding of the interactions between the different components of the different lignocellulosic resides will be required bring about delignification under less severe and cheaper ways. A number of forest biomass rich countries including Canada are focusing on the use of the lignin separated for a variety of applications and success in this could bring about a win–win situation as far as use of the cellulose for ethanol production is concerned.

10.4.3 Enzyme Hydrolysis

The second bottleneck is the need for a better and cheaper biocatalyst, an enzyme, which breaks down the cellulosics into glucose. While recycling and reuse of enzymes is considered important, the break of the solid substrate by a soluble has made this difficult. The use of traditional cellulose enzymes obtained from fungal and bacterial sources, even if they are produced in enhanced amounts by genetic engineering methods may not be enough. The effectiveness and cost of the enzymes still remain a problem. In our lab, we are discussed the possibility of

using the metagenomic approach to possibly screen for a new more potent cellulase enzyme which is used by bovine animals to break down cellulose (Nuguyen et al. 2012). This approach allows the separation of genomes from microorganisms which are unculturable in classical media and the expression of the cellullase gene in known hosts. Novel and innovative ideas like this need to be developed to produce better enzymes with increased conversion activity. Our own understanding of this is that in order to be as effective in breaking down these lignocellulosics, as the herbivorous, we will not just select one or a set of enzymes, but a whole combination of enzymes that are produced by a society of microorganisms in the rumen of these animals.

The simultaneous saccharification and fermentation (SSF) processes of the hydrolytic products is an area of considerable research. However, loss of enzymes increases costs in both the SSF and the two-stage process. In order to utilize both the pentose sugars in the hemicellulosic component and the glucose derived from cellulose, co-culture techniques are being developed. In order to overcome the problems of inhibition of one culture on another, the possibility of immobilizing one and using the other in free state is being explored. Genetic medication of yeast inherently capable of converting hexoses into ethanol to have the additional capability of converting pentoses will overcome many problems associated with co-cultures (Ho et al. 1998). The development of strains resistant to high levels of sugars and alcohols and inhibitors generated during the pretreatment process will also lead to more effective processes.

Research in this field has been considerable over the past few decades. Various types of biomass including forest products, agricultural residues, grass family lignocellulosics, and waste streams have been attempted as substrate. A number of pretreatment methods have been developed and compared. Enzymes from fungus (e.g. Trichoderma ressei), genetically modified microbes and mixture of enzymes from different sources have been tested. More than a thousand possible permutations and combinations have been estimated. While benefiting from the lessons learned from this, it is important to look for innovative new methods to overcome the major bottlenecks which make this process economically unacceptable at the moment.

The cost effectiveness of bioethanol from second generation is also dependent on the gasoline market. When the cost of crude oil reached USD 100–140 per barrel, second generation biofuels seemed suddenly to become competitive. But with the price falling back to 80–90 USD per barrel, the spurt of activity in the development of the industry has slowed down considerably. In Thailand, a pilot plant for the production of biofuel was set up rapidly when the price fluctuation of crude had reached its peak. While this might seem like an opportunity lost, in the long-term utilization and building of this facility may pay dividends. For example, the cost balance may be tilted more in favor of second generation biofuels if penalties or a carbon tax are imposed for higher CO_2 emissions.

The size and location of industrial level facilities will also require a detailed study of supply chains including availability and supply of raw materials and water, distances involved, etc. The optimal size of production facility will depend also on the ground reality in specific situations in developing countries.

Policies for the development of second generation biofuels may be as important as the technologies that need to be developed. While second generation biofuels can lead to mitigation and help reduce the effect of transportation on the environment, subsidy and support of first generation biofuels may push back its commercialization possibilities. A pro-second generation biofuel policy is realistically possible only if the strategy is to reduce carbon dioxide emissions over that of short-term benefits. As such a policy looks unrealistic at the moment, the option would be able to encourage both first and second generation biofuels with increased research and development incentives for the latter.

As in the case of first generation biofuels, adequate LCA needs to be done on a case-by-case basis. Present studies indicate that while second generation biofuels are better than first generation biofuels, the bioconversion of lignocellulosic residues can reduce GHG emissions by around 90 % when compared to fossil petroleum (EUCAR study 2007). A comparison of GHG impacts of transportation fuels against those for stationary applications indicated that under some conditions biofuels will be superior while in others biopower will be favored. Broad and unequivocal statements are difficult (Larson 2005).

It is clear that the industrialized world has considerable confidence that the existing hurdles for second generation biofuel technology can be overcome in a cost-effective way with greater research development and innovation. However, this will need greater collaboration and joint efforts. Evidence of this is the US Government's support to industry, aimed at making cellulosic ethanol cost-competitive with petrol. The activities under the EU's 7th Research Framework Programme which have an increased focus on second generation biofuels also indicate this prioritization.

10.4.4 Production of Renewable Chemicals

The number of chemicals and products that are dependent on the oil and natural gas industry drive the economics of the industry. While only 4 % of the crude oil inputs are used for the production of chemicals, it accounts for nearly 40 % of its profit margins (Werpy and Petersen 2004b). The finite nature of fossil fuels, the availability of large amounts of biomass, search for alternate used beyond pulp and paper, and the low cost of bioethanol, all suggest that the use of biomass for the production of chemicals holds a lot of potential. Some reports forecast that the world's renewable chemicals market will reach a level of US$76.8 billion by the year 2017 (Global Industry Analysts 2010).

The use of biological routes to produce renewable chemicals is attracting a lot of interest as they are carried out under much milder conditions and have a much lower impact on human health and environment. The growing opportunities in the renewable chemicals market will attract the interest of large conventional chemical

companies. This drive will be higher in the developed countries where environmental concerns are an overriding factor. This may not be the case in many developing and emerging economies where the improving living standards of the citizens is a greater priority and often environmental concerns are given lower importance.

Even today, only a small number of chemicals are produced from renewable resources by fermentation. The production of lactic acid, acetic acid, and ethanol are the only processes which can compete with petrochemical routes. Changing fermentation technologies together with genetic engineering can broaden the product spectrum of microorganisms. Recombinant microorganisms with altered sugar metabolism are able to ferment sugar to chemicals, which the corresponding wild-type strain does not produce (Danner and Braun 1999).

In the 1970s, ethanol was used to act as a basic building block in the organic chemical industry, when chemicals like ethylene and acetaldehyde were synthesized from fermented alcohol. In places like India where ethanol by fermentation was cheap, they were used for the production of chemicals like acetic acid, acetic anhydride, or ethyl acetate from fermentation ethanol (Danner and Braun 1999). This route remained unexplored with the fall in prices and availability of petroleum. However, the present impetus on climate change mitigation and the finite nature of fossil fuels have brought these developments into focus again.

The concept of a biorefinery to produce useful chemicals from biomass is thus an area of immense interest and investment. Similar to a petroleum refinery, a biorefinery would have to integrate conversion processes with the required equipments to biomass. The goal would be to produce high-value low-volume and low-value high-volume products. The high-value products need to enhance the profitability of the whole process (Fernando et al. 2006).

An NREL and PNNL study identified (Aden et al. 2004) 12 building blocks that have the most potential for success. The chemicals included 1,4-succinic, -fumaric, and -malic acids, 2,5-furan dicarboxylic acid, 3-hydroxy propionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, and xylitol/arabinitol. The study also found that biological transformations account for the majority of the routes from plant feedstocks to building blocks, but chemical transformations predominate in the conversion of building blocks into molecular derivatives and intermediates. They also highlight the R&D needs that could help improve the economics of producing these building blocks and derivatives.

As an example, succinic acid is of special interest, because it may serve as an intermediate in the production of chemicals like butane-1,4-diol, tetrahydrofuran, g-butyrolactone, or adipic acid (precursor to nylon). These in turn are used in the textile, plastics and resins, detergents, and the food industry. In the petrochemical route, it is produced by hydrogenation of maleic anhydride (Danner and Braun 1999). The biorefining route to such chemicals indicates that they may be economically feasible and are either commercialized or will be commercialized soon.

10.5 Conclusions and the Way Forward

It is clear that second generation bioethanol can have a more favorable GHG balance. They do not have any effect on food security as they use resources which do affect food supply. The use of grasses or other invasive plants or the use of genetically modified non-food and feed crops could make them more acceptable. However, the technological barriers have to be overcome to make the process cost-effective as compared to gasoline. A mix of different existing and innovative options and a rise in costs of fossil fuels may accelerate the drive toward second generation biofuels, especially bioethanol.

The biorefining concept will develop to the expected potential if there are no dramatic changes in the energy production sector and carbon sequestration remains a challenge. The variability of the products streams' from biochemical conversion, often intermediate products, has to be reduced. Integration into the existing chemical production plants or new chemical streams producing a whole new line of chemicals may be developed.

A combination of bioconversion and chemical processes can lead to a wide spectrum of products that can be used as solvents, fiber, and new polymers with different functional characteristics. With the continued expansion of this sector, it will be prudent for industry to develop a niche for themselves, while the market is still expanding.

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Part V
Biofuels, Co-products and Associated Technologies

Chapter 11
Fermentative Biohydrogen Production Using Microbial Consortia

Radhika Singh

Abstract Hydrogen offers the long-term potential for an energy system that produces near-zero emissions and is based on domestically available resources. Most of the hydrogen at the present technological development is generated from fossil fuels through thermochemical processes. Biohydrogen production is crucial to sustainable global clean energy supply and a promising alternative to fossil fuels. It has the potential to eliminate most of the problems the fossil fuels create. Biohydrogen production has become important because of its potential to become inexhaustible, low-cost and renewable source of clean energy. With the use of appropriate technologies, biohydrogen would be the desired clean product of the microbial process. Fermentative route of hydrogen production from carbohydrate-rich renewable sources such as biomass or waste materials is a feasible approach. Fermentative hydrogen can be produced either by dark fermentation or by photofermentation or by a combination of both (sequential and combined dark and photofermentation). Both sequential and combined dark and photofermentations have resulted in good hydrogen formation yields of 8 mol H_2 mol^{-1} glucose. Comparatively low hydrogen formation rates and yields are achieved in the combined fermentation because of adverse interactions and different nutritional needs of different bacteria.

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11.1 Introduction

Fossil fuels have been used as the main source of energy to fulfil the world wide demand. Coal, oil, and natural gas are the main reserves and their long-time use has resulted into resource depletion, environmental and public health problems. Global warming is the main evident as there has been an increase in the global average air and ocean temperature, melting of snow and ice and rising of the global average sea level (IPCC 2007). This has resulted in serious impacts on the ecosystems, food and water resources and hence on human health. To overcome this situation, there is a global need to introduce sustainable energy solutions to the society which are clean and renewable. According to the European Commission, renewable energy resources improve the energy security and there is decrease in the carbon dioxide emission (European Commission 2006). The renewable energy products mainly include biohydrogen, biogas or biomethane, bioethanol, biobutanol and biodiesel (European Commission 2003). These all are renewable and clean fuel source and do not add to global warming.

Biofuels are wide ranges of fuels which are derived from solid biomass, liquid fuels and various biogases. Biofuels are categorised into different generations according to the type of biomass used. Fermentation of sugars result in the first generation biofuels while the second generation biofuels are from non-food crops. Algae-based biofuels are in the third generation category and biofuels created by processes other than above will be in the fourth generation category. Consumption of biofuels produces no net carbon dioxide emissions and does not release sulphur. It has less toxic and particulate emissions than fossil fuels.

Hydrogen is the simplest element known to man. It is the most plentiful gas in the universe and also environment friendly and renewable. The atmosphere contains 0.07 % of hydrogen and the Earth's surface has 0.14 % of hydrogen. Hydrogen is lighter than air due to which it is mainly found as methane, water, etc. Hydrogen is a promising energy carrier of the future and can be derived from variety of energy sources. It is used in fuel cells with high efficiency (142.35 kJ/g). This means that on burning 1 gm of hydrogen, 142.35 kJ of energy is produced (Xianyan and Youcai 2009). It is categorised as a clean fuel—as its combustion produces only water—hence making it a non-polluting and carbon-free alternative. Its usage does not contribute to greenhouse gases. It has been reported that 50 million tonnes of hydrogen are traded globally per annum with a growth rate of almost 10 % per year (National Hydrogen Energy Roadmap 2002). Based on the National Hydrogen Program of the United States, the contribution of hydrogen to total energy market will be 8–10 % by 2025 (Kapdan and Kargi 2006). The US Department of Energy (2007) has declared that hydrogen power and transport systems will be available in all regions of the United States by the year 2040. With an increasing need for hydrogen energy, development of cost-effective and efficient hydrogen-production technologies has gained significant attention (Valdez et al. 2005a).

The only drawback of hydrogen as a fuel is that when it is burnt it releases such a large amount of energy that nitrogen and oxygen gases present in the atmosphere form different nitrogen oxides (NOXs) in traces. Combustion of hydrogen can also result in the formation of hydrogen peroxide as

$$H_2 + O_2 \rightarrow H_2O_2 \tag{11.1}$$

Hydrogen peroxide formed can discharge peroxide radicals to the atmosphere which can result in photochemical smog. Generally, the yield of hydrogen peroxide is extremely low. Catalytic converters, which can destroy hydrogen peroxide before it escapes into the atmosphere, can be installed wherever hydrogen is burnt as a fuel.

At industrial level, the most common methods for producing hydrogen include steam reformation of natural gas, coal gasification and splitting of water with electricity generated from fossil fuels. Carbon dioxide and greenhouse gases are released by these industrial methods of hydrogen production. Some microorganisms produce hydrogen naturally. Bio-hydrogen is production of hydrogen by the use of micro-organisms. Biohydrogen is one of the biofuels of the future, combining its ability to potentially reduce the dependence on foreign oil and contribute to lower the greenhouse gases (GHG) emission. Awareness regarding the future role of hydrogen as clean fuel for fuel cells producing near-zero emissions and as an intermediate energy carrier for storage and transport of renewable energy is increasing worldwide. Hydrogen is currently more expensive than other fuel options. It is likely to play a major role in the economy in the long run, if technology improvements succeed in bringing down costs. Biohydrogen production employing renewable biomass may be a potential answer to overcome some of the economic constraints to fulfil many of our energy needs. Sugarcane juice, molasses or distillery effluents can be used as substrates because they contain sugar in sufficient amount.

11.2 Biohydrogen Production Mechanisms

Hydrogen can be produced biologically by three mechanisms:

i. *Fermentative Hydrogen Production* Hydrogen can be produced by the fermentation of sugars by bacterial species such as *E.coli*, *Enterobacter aerogenes*, *Clostridium butyricum*, etc. This fermentation is classified as "dark fermentation" as it does not require light energy. Organic compounds can be fermented to produce hydrogen both in the presence and absence of light. Fermentative bacteria have high hydrogen evolution rates as compared to other biological hydrogen-production processes.

ii. *Hydrogen Production by Nitrogenase* Purple nonsulphur (PNS) photosynthetic bacteria such as *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides* can generate hydrogen in the presence of light under anoxic conditions. These bacteria contain nitrogenase enzyme and can generate hydrogen under nitrogen limited conditions. Few cyanobacteria species contain nitrogenase enzymes which can produce hydrogen as a byproduct of nitrogen fixation. They obtain electrons, required for reduction of protons to molecular hydrogen, from the oxidation of organic compounds. A simple reaction can be written as:

$$2H^+ + 2e^- \rightarrow H_2 \tag{11.2}$$

This reaction can be catalysed either by nitrogenase or hydrogenase enzymes (Falciatore and Bowler 2002).

iii. *Biophotolytic Hydrogen Production* Water can be microbially splitted into its elements hydrogen and oxygen in the presence of sunlight. Use of hydrogen in fuel cells can directly produce electricity with water as the byproduct. This carbon-free energy cycle can complement the electric grid for all energy usage: industrial, transportation and residential. Hydrogen production by biophotolysis involves water—which is a clean, renewable, carbon-free substrate available in inexhaustible amounts. Green algae and Cyanobacteria are commonly used for an efficient conversion of solar energy to hydrogen. Splitting of water during biophotolysis results in production of oxygen. Hydrogenase enzyme, responsible for formation of hydrogen, is sensitive to oxygen. Hydrogenase can be engineered and used in bioinspired nanostructures that maintain optimal conditions for hydrogen production.

Anaerobic fermentation is the degradation of organic materials by microorganisms in the absence of oxygen. It is a multistep biological process in which the biodegradable organics are finally converted to methane and carbon dioxide. Hydrogen and volatile fatty acids (VFAs) are formed in the intermediate step. Anaerobic digestion is widely used as a renewable energy source because the process produces biogas (a mixture of methane and carbon dioxide) suitable for energy production and this can substitute fossil fuels. The nutrient-rich digestate which is the leftover solid after digestion is a good organic fertilizer. As compared to methane, hydrogen is a more efficient fuel. Hydrogen can be produced via anaerobic fermentation by inhibiting methane formation or hydrogen consumption. VFAs formed as one of the intermediates can also be further degraded to form hydrogen and carbon dioxide. The carbon dioxide formed during the fermentation process is believed to enter the carbon cycle and is used during photosynthesis and hence does not contribute to global warming.

Fermentative route of hydrogen production from carbohydrate-rich renewable sources such as biomass or waste materials is a promising approach provided that the rates and yields of hydrogen formation are improved to economically feasible levels and large-scale operations are developed (Perera et al. 2010). Fermentative hydrogen can be produced either by dark fermentation or by photofermentation or by a combination of both.

11.3 Dark Fermentation

The oxidation of the substrate by bacteria generates electrons which need to be disposed off in order to maintain the electrical neutrality. Oxygen is the electron acceptor under the aerobic conditions while under the anaerobic or anoxic conditions other compounds, such as protons, act as the electron acceptor and are reduced to molecular hydrogen (Das and Veziroglu 2001; Levin et al. 2004). Carbohydrates, mainly glucose, are the preferred carbon sources for this process, which predominantly give rise to acetic and butyric acids together with hydrogen evolution (Eqs. 11.3 and 11.4; Nath et al. 2005).

$$C_6H_{12}O_6 + 2H_2O \rightarrow \underset{\text{Acetic acid}}{2CH_3COOH} + 2CO_2 + 4H_2 \tag{11.3}$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow \underset{\text{Butyric acid}}{2CH_3CH_2COOH} + 2CO_2 + 2H_2 \tag{11.4}$$

Here, glucose is initially converted to pyruvate (glycolysis), which is further oxidised to acetyl-CoA. Acetyl-CoA is converted to acetyl phosphate and results in the generation of ATP and formation of acetate. This oxidation to acetyl-CoA requires a ferredoxin (Fd) reduction. Reduced Fd is oxidised by hydrogenase which regenerates Fd(ox) and releases electrons as molecular hydrogen (Nath and Das 2004; Nath et al. 2005). The overall reaction of the processes can be described as follows:

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd(ox)} \rightarrow \text{Acetyl-CoA} + 2\text{Fd(red)} + CO_2 \tag{11.5}$$

and,

$$2H^+ + \text{Fd(red)} \rightarrow H_2 + \text{Fd(ox)} \tag{11.6}$$

The reactions given in Eqs. (11.3) and (11.4) are exothermic and do not require any external energy for its completion. Theoretically, 4 mol hydrogen can be produced per mole of glucose when acetic acid is the only VFA formed. Lower yields are practically obtained as some of the carbohydrate (glucose) is used up for growth and maintenance. Two moles of hydrogen are formed per mole of glucose if butyric acid is the only VFA formed and one mole hydrogen per mole of glucose is formed when propionic acid is the only VFA formed (Kim et al. 2006; Luo et al. 2010). Yields are low as the end products contain both acetate and butyrate (Nath et al. 2005). When both acetic acid and butyric acids are VFAs formed, one mole of glucose gives 2.5 mol of hydrogen (Krupp and Widmann 2008). Lower production yield are obtained in case of mixed nonsterile conditions (Arooj et al. 2008; Vazquez and Varaldo 2009; Argun and Kargi 2009). This is primarily due to the presence of hydrogen consumers such as methanogens (Vazquez and Varaldo 2009; Argun and Kargi 2009; Ray et al. 2010), homoacetogens, sulphate and nitrate reducing bacteria (Vazquez and Varaldo 2009; Guo et al. 2010). Although

hydrogen is produced at a higher rate, the yield of hydrogen from the fermentation process is lower than that of other chemical or electrochemical processes. Low conversion efficiencies of the substrate used is a constraint of the process.

Carbohydrates can be fermented under anaerobic conditions by a broad spectrum of heterotrophic bacteria to produce hydrogen, VFAs and carbon dioxide (Hawkes et al. 2007; Vazquez and Varaldo 2009; Dasgupta et al. 2010; Sagnak et al. 2010). Most common of these are spore forming *Clostridium* species, facultative *Enterobacter* species and *Bacillus* species (Kapdan and Kargı 2006; Bartels et al. 2010; Xu et al. 2010). Few thermophilic bacteria (Hawkes et al. 2007; Dasgupta et al. 2010; Hniman et al. 2011; Karadag 2011) and anaerobic acidogenic sludge (Kapdan and Kargı 2006; Hawkes et al. 2008; Oztekin et al. 2008; Vazquez and Varaldo 2009; Argun et al. 2009a) are also widely used. Formation of molecular hydrogen is catalysed by the enzyme hydrogenase (Nicolet et al. 2010; Trohalaki and Pachter 2010). Monosaccharides are normally the main carbon source (Argun et al. 2008a, 2009b; Vazquez and Varaldo 2009; Cai et al. 2010). Higher saccharides are hydrolysed by acid or enzymes to monosaccharides which are then fermented anaerobically (Sagnak et al. 2010).

Hydrogen gas formation rate and yield are the two most important criteria used in selecting the most suitable bacteria for fermentative hydrogen production. Hydrogen gas production rate (HPR) is defined as the amount of hydrogen (ml) produced per unit time and per unit reactor volume (volumetric rate) or per unit biomass (specific rate; SHPR) (Chen et al. 2008). The yield (HY) is defined as the amount of hydrogen produced per amount of substrate consumed (mol H_2/mol glucose) (Chen et al. 2008; Cakir et al. 2010). HPR, SHPR and HY can be expressed as (Eqs. 11.7–11.9):

$$\text{Volumetric hydrogen production rate (HPR)} = \frac{\text{Total amount of hydrogen produced (ml)}}{\text{Reactor volume (ml) x Time duration}} \tag{11.7}$$

$$\text{Specific hydrogen production rate (SHPR)} = \frac{\text{Total amount of hydrogen produced (ml)}}{\text{Mass of substrate used x Time duration}} \tag{11.8}$$

$$\text{Hydrogen production yield (HY)} = \frac{\text{Amount of hydrogen produced (mol)}}{\text{Amount of substrate consumed (mol)}} \tag{11.9}$$

The molar yield of hydrogen and the cost of the feedstock are the two main barriers for fermentation technology. The main challenge in fermentative production of hydrogen is that only less than 15 % of the energy from the organic source can typically be obtained in the form of hydrogen (Logan 2004). Major efforts are hence directed to increase the hydrogen yield. The US Department of Energy (2007) programme goal for fermentation technology is to realise yields of 4 and 6 mol H_2/mol of glucose by 2013 and 2018, respectively, as well as to

achieve 3 and 6 months of continuous operation for the same years. Additionally, some integrated strategies are now under development, such as the two-step fermentation process or the use of modified microbial fuel cells (Ueno et al. 2001; de Vrije and Claasen 2003; Logan and Regan 2006). Through these coupled processes, more hydrogen or energy per mol of substrate can be achieved in the second stage.

Different types of wastes like organic fraction of municipal solid wastes (OFMSW) are important feedstocks for hydrogen production. On an average, almost 50 % of the municipal solid wastes of under developed countries consist of fermentable and biodegradable fraction (Valdez et al. 2005b). Food wastes constitute a major OFMSW. The OFMSW has a significant potential of biological hydrogen production, which also depends on its composition (Okamoto et al. 2000). Lay et al. (2003) have reported that the hydrogen-production potential of carbohydrate-rich high solid organic wastes (HSOW; rice and potato) is about 20-fold higher than of fat-rich HSOW (fat meat and chicken skin) and protein-rich HSOW (egg and lean meat). An increase in alkalinity has been observed on addition of sewage sludge to the food waste. This has enhanced overall hydrogen-production potential most probably due to high protein content in sewage sludge. Ammonia is produced from proteinaceous substances such as peptone, which neutralises volatile fatty acids which would otherwise reduce the pH (Cheng et al. 2002; Mohanakrishna et al. 2010). Hence, a good buffering microenvironment is probably maintained, which supports fermentation and also provides micronutrients, organic matter and microbial biomass.

Crucial roles of nutrients like Nitrogen (N), phosphorous (P), Iron (Fe) and sulphur (S) have been reported for hydrogen production by dark fermentation (Hawkes et al. 2007). Nutritional requirements of acidogenic bacteria depend on the type of bacteria and the experimental conditions. Optimal C/N and C/P ratios in dark fermentation ranged between 11.4/1 and 200/1 for COD/N and between 73/1 and 970/1 for COD/P ratios, respectively (Hawkes et al. 2007; Argun et al. 2008a; Sreethawong et al. 2010).

A huge spectrum of substrates can be used for biohydrogen production ranging from simple monosaccharides like glucose (Li et al. 2008), sucrose (Antonopoulou et al. 2007), organic wastewater (Show et al. 2011), starch containing wastewater e.g. cassava wastewater (Yokoi et al. 2001, 2002; Sangyoka et al. 2007), dairy wastewater (Venkata Mohan et al. 2007), sweet potato starch residue (Lay et al. 2012), cheese whey (Kargi et al. 2012a, b), food waste (Ruknongsaeng et al. 2005; Bansal et al. 2011, 2012). It takes longer fermentation time to degrade starch and cellulose as they have to be first hydrolysed into monosaccharide before being used for hydrogen production. Biohydrogen production by fermentation of carbohydrate-rich renewable materials reduces the release of carbon dioxide (Van Ginkel et al. 2005; Refaat and Sheltawy 2008).

Biomass is a stored source of solar energy initially collected by plants during photosynthesis. Carbon dioxide is captured in the process, which is then converted to complex molecules like cellulose, hemicelluloses and lignin. Biomass term includes a wide range of organic materials produced from plants and animals that

feed on plants. Crop residues (primary residues), forest and wood process residues (secondary residues), animal and human wastes, organic municipal solid wastes, food processing wastes (secondary and tertiary residues), purpose grown energy crops and short rotation forests are examples of biomass (IEA 2008). Wastes and biomass rich in sugars and complex carbohydrates can be used as efficient substrates for the generation of biohydrogen (Ntaikou et al. 2010). This process converts waste to energy which also helps in the stabilization of the waste.

Primary residues and energy crops constitute "green wastes" which have large amount of microbial convertible carbohydrates. Woody wastes contain about 70 % cellulose and hemicelluloses (dried weight), and 25–35 % lignin which covers the polysaccharides (Take et al. 2006). Direct conversion of green wastes to biohydrogen by anaerobic fermentation is difficult because of their complex composition and polymeric structure (Ren et al. 2009a; Guo et al. 2010; Ntaikou et al. 2010). Cellulose and hemicelluloses can be anaerobically fermented to biohydrogen, but lignin is not degraded under anaerobic conditions. Lignin restricts the degradation of cellulose and hemicelluloses as the bonding in lignocelluloses resist mobilisation. Also, lignin is often inhibitory to microbial growth (de Vrije and Claasen 2003). Hence for effective biohydrogen production, it is necessary to pretreat green wastes to destroy the polymeric bonding structure of lignin. Delignification of green wastes can be done by physicochemical treatment and enzymatic treatment. Hydrogen can be produced economically from wastes and wastewater as a useful product and this would effectively reduce waste treatment and disposal costs (Van Ginkel et al. 2005). Dark fermentation of the organic fraction of solid wastes (Bansal et al. 2011, 2012) also produces hydrogen. Cellulose is an important constituent of agricultural waste and waste generated by the pulp and paper industry (Cheng et al. 2011).

Biohydrogen production by dark fermentation has been carried out using both pure and mixed cultures. Pure cultures of *Clostridium* species, such as *Clostridium butyricum*, *Clostridium acetobutyricum*, *Clostridium beijerinckii*, *Clostridium thermolacticum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium pasteurianum*, etc. efficiently convert carbohydrates to acetate, butyrate, hydrogen, carbon dioxide and organic solvents (Chong et al. 2009). Few thermophilic bacteria like *Thermotoga neapolitana*, *Thermotoga elfii* and *Caldicellulosiruptor saccharolyticus* can produce biohydrogen by dark fermentation (de Vrije et al. 2002; Nguyen et al. 2008; Ivanova et al. 2009).

Several unidentified mixed anaerobic bacteria have been used to produce biohydrogen from wastewaters and renewable raw materials (Wu et al. 2009; Bansal et al. 2012; Mohanakrishna et al. 2010). Production of biohydrogen by anaerobic fermentation using mixed cultures is easy to operate and control (Wang and Wan 2009). Hence, mixed culture serves as an ideal system for biohydrogen production from wastes and wastewaters. They can be used under anaerobic or microaerobic conditions and are more robust and not easily contaminated. Biohydrogen can be produced from complex substrates using mixed cultures as they have the capability to adapt to a variety of carbon sources. Utilising mixed culture is extremely important and suitable for nonsterile, ever-changing, complex environment of the

wastewater. Few anaerobic mixed cultures produce hydrogen which is simultaneously consumed by hydrogen consuming microorganisms e.g. methane producing bacteria. Hence, hydrogen consumers should be inhibited for an efficient production of biohydrogen. The physiological differences between hydrogen producers and hydrogen consumers govern different methods used for the enrichment of hydrogen producing inoculum. Mixed cultures are pretreated by exposing to extreme environments such as high temperature, extreme acidity and alkalinity, etc., which inhibits or inactivates hydrogen consumers and only robust spore forming hydrogen producing bacteria survive. Mixed culture can be taken from different natural sources like cow dung (Bansal et al. 2012) or soil (Logan et al. 2002).

Pretreatment of the inoculum partially or totally eliminates or inhibits hydrogen consumers. Pretreatment of the parent inocula is said to accelerate the hydrolysis step which reduces the impact of the rate-limiting step and increase hydrogen production (Zhu and Bèland 2006). Hydrogen gas is produced in the acidogenic stage of anaerobic metabolism (Kapdan and Kargı 2006; Vazquez and Varaldo 2009). Optimal hydrogen production has been observed between pH 5.5 and 6.5 (Kapdan and Kargi 2006; Mu et al. 2006; Wei et al. 2010). Solvent formation has been observed at low pH of 4.5. It has been observed that *Clostridium acetobutylicum*, *Clostridium butylicum* and *Clostridium beijerinkii* can produce ethanol, butanol and acetone at low pH ranges hence reducing hydrogen formation (Datar et al. 2007; Ezeji et al. 2007). Dark fermentation has also been carried out at alkaline pH (Zhao et al. 2010). High pH of 10 was taken to avoid formation of propionic acid and also to inhibit methanogens or hydrogen consumers. Hydrogen production by dark fermentation can be under mesophilic (25–40 °C), thermophilic (40–65 °C) or hyperthermophilic (greater than 80 °C) conditions (Levin and Chahine 2010). Fermentation in the thermophilic range gives higher yield of hydrogen most probably because of complete or partial elimination of hydrogen consumers and increase in metabolic activity of bacteria (Karadag 2011). Some of other pretreatment methods studied are sterilization (Kotay and Das 2009), microwave-assisted radiations (Guo et al. 2010) and ultrasonication (Venkata Mohan et al. 2008). Oxidation–reduction potential (ORP) is also an important parameter affecting hydrogen production by dark fermentation. For example, if ORP values are out of the range of −200 and −250 mV, which is optimal value for *Clostridium* sp., a decrease in hydrogen production has been observed.

11.3.1 Batch Reactor Studies

Majority of the dark fermentation studies for hydrogen gas production have been carried out in batch reactors with different types of bacteria and substrates. Batch fermentations are usually subject to substrate and product inhibitions yielding low hydrogen gas productivities. The reported batch studies were mainly aimed to identify conditions enhancing hydrogen production by substrate and culture selections and adjustment of operation parameters.

Ntaikou et al. (2008) have used *Ruminococcus albus* as inoculum culture for hydrogen production from sweet sorghum biomass. Vegetable waste supplemented with sewage has been used for hydrogen production by dark fermentation (Mohanakrishna et al. 2010; Bansal et al. 2011, 2012). High total volatile fatty acid (TVFA) concentrations (of which 65 % was acetic acid) resulted in reduction in pH and hence adversely effected hydrogen gas formation. Hydrogen can also be produced by batch dark fermentation using hydrolyzates produced from steam explosion of corn stover (Datar et al. 2007).

Heat pretreated anaerobic sludge was used as inoculum culture and hydrogen, carbon dioxide, acetic acid and butyric acids were the major products formed. Increase in the substrate concentration beyond 25 g L^{-1} resulted in decrease in hydrogen formation rate, because of the inhibitory effect of VFAs in the medium (Datar et al. 2007; Lee et al. 2008). Hydrogen gas production by thermophilic–anaerobic fermentation was found to be more effective than mesophilic fermentation and high hydrogen yields close to the theoretical yield of 4 mol H_2 per mol glucose have been obtained (Commission of European Communities 2009; Zeidan and van Neil 2010). Product inhibition on hydrogen gas formation in dark fermentation was investigated by increasing acetate concentration in the fermentation medium (Wang et al. 2008). Almost 50 % of the substrate was converted to VFAs consisting of 60 % butyric acid. Increase in acetate concentrations gradually decreased the rate and yield of hydrogen formation. A noncompetitive product inhibition model was used to describe the product inhibition.

Hydrogen was produced from carbohydrate-rich organic wastewater in a sequencing batch reactor (Chen et al. 2009). Effects of pH (4.9–6.7), and cyclic duration (4, 6, and 8 h) were investigated by using pretreated anaerobic sludge. pH of the fermentation medium decreased because of accumulations of VFAs. pH and durations of the cycles affect hydrogen gas production by sequencing batch operation.

Effects of C/N and C/P ratios on hydrogen gas formation rate and yield by dark fermentation of wheat powder solution has been studied by Argun et al. (2008a). Nitrogen and phosphorous were externally added to the fermentation media in the desired concentrations. Optimal yield was obtained at C/N/P ratio of 100/0.5/0.1 (w w^{-1} w^{-1}).

Both sterile and nonsterile inoculum cultures have been used for hydrogen production from various sources through dark fermentation. Different yields were reported when complex and pure carbon sources were used. Yields around 3 mol H_2 mol^{-1} glucose were reported at low glucose concentrations (less than 5 g L^{-1}) resulting in low volatile fatty acids formation with no considerable product inhibition (Datar et al. 2007; Lin et al. 2007; Ntaikou et al. 2008). Hydrogen formation yields at higher initial carbohydrate concentrations, above 10 g L^{-1}, were around 1.0–2.0 mol H_2 mol^{-1} glucose; which is probably due to the inhibition caused by VFAs. On the other hand, hydrogen formation rate had an increasing trend with an increasing initial substrate concentration up to 20 g glucose L^{-1} followed with a decrease at higher substrate concentrations (Datar et al. 2007; Lee et al. 2008; Argun et al. 2008b).

11.3.2 Continuous Reactor Studies

Dark fermentation studies carried out in a continuous reactor resulted in a constant product quality, production rate and the yield with high productivities at the steady state as compared to that in a batch reactor. Suspended and immobilized cultures were used in a continuous stirred tank reactors (CSTRs) and upflow anaerobic sludge blanket (UASB) reactors for biohydrogen production (Van Ginkel and Logan 2003; Van Ginkel et al. 2005; Ren et al. 2006; Krupp and Widmann 2008; Azbar et al. 2009). Effects of HRT (1, 2.5, 5 and 10 h) and glucose loading rate (0.5–18.9 g COD h^{-1}) on the rate and yield of hydrogen gas formation were studied by Van Ginkel and Logan (2003) using heat shocked agricultural soil as inoculum in dark continuous fermentation. High feed glucose concentrations at low HRT levels resulted in flocculation which adversely affected hydrogen formation.

Continuous operation was found to be more advantageous for immobilized-cell systems (Zhang et al. 2008). Lee et al. (2007) have used a membrane cell recycle reactor (MCR) along with a CSTR. MCR provided high cell concentrations in the fermenter allowing operation at high dilution rates. 20 g COD L^{-1} of fructose was taken as the carbon source. Agitated granular sludge bed reactor (AGSB) fed with starch has been operated in a continuous mode (Cheng et al. 2008). Anaerobic sludge immobilized on powdered activated carbon was used as inoculum and effects of pH (5.5 and 6) and HRT (0.5, 1, 2, 12 h) on hydrogen gas formation was investigated. Presence of *Clostridium* species and *Bifidobacterium* species in the microbial consortia was reported to play crucial role in hydrogen gas formation.

Higher hydrogen formation rates (up to 7600 ml H_2 L^{-1} h^{-1}) as compared to batch systems (max. 1119 ml H_2 L^{-1} h^{-1}) with relatively lower yields (less than 3 mol H_2 mol^{-1} glucose) have been obtained in continuous reactors as compared to batch systems (Lee et al. 2008; Zhang et al. 2008). High biomass concentrations in immobilized-cell reactors provided considerable advantages over CSTR such as high loading rates, low HRTs with extremely high hydrogen formation rates (Lee et al. 2007). Suspended cell CSTRs provided higher yields when compared to immobilized systems. CSTRs could not tolerate high substrate loadings at low hydraulic retention times (HRTs); less than 10 h, due to rapid biomass flocculation adversely affecting hydrogen-production performance (Van Ginkel and Logan 2003). Immobilized-cell reactors are more advantageous than suspended cell CSTR with respect to high hydrogen productivities. However, lower hydrogen yield obtained in immobilized-cell reactors is an important disadvantage (Cheng et al. 2008). Neither continuous suspended cell nor immobilized systems were found to be capable of providing both high hydrogen yields and high rates under high substrate loading rates and low HRT levels.

11.3.3 Fed-Batch Reactor Studies

High cell density fed-batch operation has considerable advantage as compared to batch and continuous operations and is usually used to overcome substrate/product and toxic compound inhibitions encountered at high substrate concentrations. In a fed-batch reactor, metabolic rates can be adjusted by adjusting the feed flow rate and composition of the substrate. The substrate solution is added with a rate sufficient to support the bacterial community and to eliminate the substrate and product inhibitions with no effluent removal. The reactor substrate concentration reaches a low quasi steady-state level when the substrate consumption rate is equal to the feeding rate (Argun and Kargi 2011). When the reactor is full, the contents are allowed to settle. The supernatant containing the products are removed and then another cycle of fed-batch operation is started. Cell-retention time can be adjusted by removing a fraction of settled bacteria. Biohydrogen production in a fed-batch reactor has not been much explored. Chin et al. (2003) reported hydrogen production by fed-batch operation with an extremely high feed glucose concentration (500 g L^{-1}). A long-term fed-batch operation resulted in a constant hydrogen formation yield of 2 mol H_2 mol^{-1} glucose and a rate of 930 ml H_2 h^{-1}. Kargi and Parmukoglu (2008) investigated hydrogen production from boiled waste wheat powder (WP) by fed-batch operation and studied effects of substrate loading rate on rate and yield of hydrogen gas. Maximum hydrogen produced was 3.1 mol per mole of glucose at a rate of 36 ml H_2 h^{-1}. Feed contained 20 g L^{-1} WP with a loading rate of 4 g WP d^{-1}.

Hydrogen production from sweet potato starch in repeated fed-batch reactors using pure culture of *Clostridium butyricum* and co-culture of *Clostridium butyricum* and *Enterobacter aerogenes* was studied by Yokoi et al. (2001). They compared hydrogen-production capabilities in the presence of 0.1 % polypeptone as nitrogen source. Co-culture of *C. butyricum* and *E. Aerogenes* was found to be more effective than pure culture of *C. Butyricum*. Yield of hydrogen obtained was 2.3–2.4 mol per mole of glucose up to 2 % feed starch concentration. A similar co-culture approach through repeated fed-batch operation in the presence of corn steep liquor as nitrogen source has been reported to give a yield of 2.1 mol H_2 mol^{-1} glucose (Yokoi et al. 2002).

11.4 Photo-Fermentation

Photosynthetic nonsulphur (PNS) bacteria can convert VFAs to H_2 and CO_2 under anaerobic conditions (Das and Veziroğlu 2001; Levin et al. 2004; Kapdan and Kargi 2006; Westermann et al. 2008). PNS bacteria also have the ability to use carbon sources like glucose, sucrose and succinate rather than volatile fatty acids for hydrogen production (Fang et al. 2006; Jeong et al. 2008; Li et al. 2009). Commonly used PNS bacteria for photofermentative hydrogen production are

Rhodobacter sphaeroides 0.U001, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*-RV, *Rhodobacter sulfidophilus*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* (Basak and Das 2007). Both nitrogenase and hydrogenase enzymes are present in PNS bacteria (Das and Veziroğlu 2001; Dasgupta et al. 2010). Nitrogenase is the main enzyme responsible in molecular hydrogen production under anaerobic conditions (Koku et al. 2002; Dasgupta et al. 2010). Iron (Fe) and molybdenum (Mo) are known to be the most important cofactors required by the nitrogenase enzyme in hydrogen production (Fascetti et al. 1998). The limited use of nitrogen source is of special importance since every 20 μM of ammonia was reported to cause inhibition on the nitrogenase enzyme responsible for hydrogen formation (Koku et al. 2002). The malate/glutamate ratio greater than 1 for effective hydrogen formation by photofermentation has been recommended (Koku et al. 2002).

Hydrogen gas formation from acetic acid by photofermentation can be represented by the following Eq. (11.10) (Manish and Banerjee 2008; Uyar et al. 2009). Due to positive free energy change, the reaction is not spontaneous requiring external energy input in the form of light which could be provided as artificial or solar light source (Rocha et al. 2001; Chen et al. 2010; Argun and Kargi 2010b). As presented in the equation, theoretically 4 mol H_2 can be produced from 1 mol acetic acid when acetic acid is the only VFA formed.

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad \Delta G^0 = +104\,kJ \tag{11.10}$$

Efficient hydrogen production by photofermentation is sensitive to environmental conditions (Argun et al. 2008c; Tuna et al. 2009; Ozmihci and Kargi 2010a). Optimal pH and temperature ranges were reported to be 6.8–7.5 and 31–36 °C respectively (Koku et al. 2002). Suitable wave length and light intensities for photofermentation were reported to be between 400 and 1000 nm (Koku et al. 2002; Akkerman et al. 2002), 6 and 10 klux, respectively (Basak and Das 2007). Major hurdles in photofermentation were reported to be lack of preferred carbon sources such as malate and lactate, nonuniform light distribution through fermentation broth and metabolic shift from hydrogen production to polyhydroxy butyrate synthesis (PHB) (Koku et al. 2002; Basak and Das 2007; Das and Veziroğlu 2008).

Hydrogen-production performance of PNS bacteria has been evaluated on the basis of the hydrogen yield and the light efficiency (Akkerman et al. 2002). The yield coefficient is the ratio of the amount of produced hydrogen to the consumed carbon source. The light efficiency denotes the ratio of generated hydrogen energy to the supplied light energy. Depending on the carbon source, hydrogen formation yields up to 80 % of the theoretical yield were reported in the literature (Fascetti and Todini 1995; Akkerman et al. 2002; Basak and Das 2009; Bretner et al. 2010). Light conversion efficiencies varied between 0.2 and 9.3 % (Akkerman et al. 2002; Koku et al. 2002). A light efficiency of 10 % for PNS bacteria corresponds to the theoretical maximum of photochemical efficiency (Akkerman et al. 2002; Bianchi et al. 2010).

According to the following relationship (Akkerman et al. 2002), the light efficiency can be increased by reducing the supplied light energy to the reactor (Eq. 11.11):

$$\text{Light efficiency } (\%) = \frac{\text{Hydrogen production rate } \times \text{ Hydrogen energy content}}{\text{Absorbed light energy}} \tag{11.11}$$

Light efficiencies around 10 % level were obtained only at low light intensities and low hydrogen rates which is dependent on the fraction of energy absorbed by the bacteria and the energy loss during the several steps of excitation and electron transfers (Akkerman et al. 2002). Dasgupta et al. (2010) proposed genetic strain development by modification of light-harvesting antenna complexes responsible for capturing solar energy, reduction in pigment content of bacteria, improvement in nitrogenase enzyme efficiency and reduction in uptake of hydrogenase enzymes. Reactor geometry and light distribution are the key factors for efficient conversion of solar energy to hydrogen (Koku et al. 2002; Basak and Das 2007; Berberoglu and Pilon 2010). Light distribution inside photobioreactors constitutes the most important parameter effecting hydrogen-production rate (Akkerman et al. 2002). Optimisation of light distribution with high reactor surface area is an essential factor to enhance the light efficiency in photofermentation (Akkerman et al. 2002; Dasgupta et al. 2010). Operating parameters also affect the photofermentation process efficiency.

Net energy ratio (NER) is used to determine the process efficiency (Eq. 11.12), which is the ratio of total energy produced to energy required for plant operations like mixing, pumping, aeration and cooling (Burgess and Velasco 2007). NER greater than one can be obtained by improving the light conversion efficiency to hydrogen (Dasgupta et al. 2010).

$$\text{NER} = \frac{\sum \text{ Energy produced (biomass/hydrogen)}}{\sum \text{ Energy input (mixing, aeration, pumping, cooling etc.)}} \tag{11.12}$$

Immobilisation of PNS bacteria on solid matrix yields higher hydrogen formation rates than the suspended culture (Levin et al. 2004).

11.4.1 Batch Reactor Studies

Majority batch studies were carried out using pure carbon sources in sterile and nutrient-rich fermentation media investigating optimum operating conditions, culture selections, nutrient concentrations and inhibitory conditions. Most of photofermentation experiments utilising solar irradiation as light source were studied indoor.

Fang et al. (2006) used *R. sphaeroides* as biocatalyst to study photofermentative hydrogen production from glucose. Nitrogen and light sources were sodium

glutamate and tungsten lamp (light intensity of 135 Wm^{-2}), respectively. Hydrogen gas was produced from starch by a new strain called *Rubrivivax gelatinosus* which was reported to be capable of utilising a wide range of carbon sources (Li and Fang 2008). Tao et al. (2008) have used a PNS strain ZX-5 which produces hydrogen most efficiently from butyrate as compared to succinate, lactate, malate, acetate, pyruvate, valerate, isobutyrate, xylose, fructose, maltose, sucrose, propionate, D-mannitol and glucose. The ZX-5 strain is also a potential hydrogen producer from wastewaters utilising wide range of carbon sources.

Hydrogen production was enhanced through more efficient light penetration using an annular photobioreactor (Basak and Das 2009). The reported hydrogen yield corresponds to 75 % of the theoretical yield which is 6 mol H_2/mol malate (Uyar et al. 2009). Eroğlu et al. (2008) have used a temperature controlled flat-plate solar bioreactor (8 L) for batch hydrogen production from malate, lactate, acetate and olive mill wastewater (OMW). *R. sphaeroides* OU 001 was used as the inoculum culture and outdoor experiments were carried out. Acid-hydrolysed wheat starch was subjected to photofermentation using three different *Rhodobacter* species. *R. sphaeroides*-RV yielded the highest hydrogen-production yield and rate among the other strains tested. Hydrogen gas formation increased with total sugar concentration up to 8.5 g L^{-1} and the optimum was found to be 5 g L^{-1} resulting in the highest rate and yield (Kapdan et al. 2009).

11.4.2 Continuous Reactor Studies

Hydrogen yields and rates varied in continuous photofermentation depending on the substrate, inoculum culture and experimental conditions. Yields up to 80 % of the theoretical yield have been reported depending on the substrate used (Fascetti and Todini 1995). Long hydraulic retention times HRTs of 25 h (Fascetti et al. 1998) to 120 h (Jeong et al. 2007) are required indicating slow conversion of VFAs to H_2 and CO_2 by PNS bacteria.

Continuous hydrogen gas production experiments from carbon monoxide (CO) using *R. rubrum* have been carried out (Najafpour et al. 2003). CO was oxidised to CO_2 while water was reduced to H_2. Hydrogen gas was produced at the rate of 397.5 ml H_2 g^{-1} h^{-1} and was 80 % of the theoretical yield. Fermentation of lactic acid by *R. sphaeroides*-RV was carried out in two-stage chemostat for hydrogen production (Fascetti and Todini 1995). The first reactor was used for nitrogen removal and for bacterial growth while the second reactor was for hydrogen production. Increasing nitrogen concentration adversely affected hydrogen formation due to inhibition of the nitrogenase enzyme. Fascetti et al. (1998) investigated hydrogen production from dark fermentation effluent (DFE) of source selected municipal solid wastes with *R. sphaeroides*-RV by continuous photofermentation. 1 L photobioreactor was operated at HRT = 25 h and was illuminated with 100 klux tungsten lamp to yield the highest SHPR of 100 ml H_2 g^{-1} h^{-1}. Continuous hydrogen production in a pneumatic flat panel photobioreactor was

operated by using *Rhodobacter pseudomonas* as an inoculum culture (Hoekema et al. 2002). The reactor was first run in batch mode followed by continuous operation at HRT = 28.5 h, 175 Wm^{-2} illumination, 30 °C and pH = 6.8–7.0. Mixing was provided by recirculating argon gas through the reactor. Hydrogen production was not observed from batch operation due to high residual ammonium levels and high acetate concentrations. CO_2 was identified as an essential nutrient for growth of PNS bacteria and should not be removed by continuous gas sparging (Hoekema et al. 2002).

Studies for continuous photofermentation have been carried out mostly by suspended cultures rather than using immobilized cells. High hydrogen gas formation rate of 1300 ml H_2 L^{-1} h^{-1} with 75 % substrate conversion efficiency was reported in a study where *R. sphaeroides*-RV was immobilized on porous glass indicating an advantage over suspended culture (Tsygankov et al. 1994).

11.4.3 Fed-Batch Reactor Studies

Almost 80 % of the theoretical yield of biohydrogen was produced from acetate in a fed-batch mode using *Rhodopseudomonas faecalis* strain RLD-53 (Ren et al. 2009b). The reactor was illuminated by 4 klux incandescent lamp source. pH and temperature were controlled at 7.0 and 35 °C, respectively. An 80 L pilot scale photobioreactor was operated in the fed-batch mode in outdoor conditions in a green house. The highest conversion efficiency was 1 % (Boran et al. 2010).

11.5 Sequential Dark and Photofermentations

Dark fermentation results in low hydrogen yields due to accumulation of VFAs in the medium (Brentner et al. 2010). The VFA fermentation capability of PNS bacteria provides a unique opportunity for valorization of DFE as a substrate for photofermentation (Ozmihci and Kargi 2010a; Perera et al. 2010; Chen et al. 2010; Su et al. 2010; Laurinavichene et al. 2010; Afsar et al. 2011). When dark and photofermentations are operated simultaneously, the maximum theoretical hydrogen yield increases to 12 mol H_2 mol^{-1} glucose when acetic acid is the sole product in the dark fermentation (Chen et al. 2010; Su et al. 2010; Ozmihci and Kargi 2010b). For effective photofermentation TVFA and NH_4^+ concentrations in the DFE must be below 2500 and 40 mg L^{-1}, respectively (Argun et al. 2008c; Ozmihci and Kargi 2010a, b; Su et al. 2009a, b; Ozgur et al. 2010; Cheng et al. 2011). Dilution, ammonium stripping, centrifugation and sterilization of DFE have been used as pretreatment steps to reduce TVFA and NH_4^+ below certain limits (Argun et al. 2008c; Argun and Kargi 2010a). Residual glucose in DFE results in a shift of glucose to VFA fermentation by PNS bacteria which takes a long time resulting in low hydrogen gas productivities (Argun and Kargi 2010b). Hence,

DFE should to be favourably ammonia and glucose deficient with desirable VFA concentration (less than 2500 mg L^{-1}) for effective hydrogen gas production by photofermentation (Argun et al. 2008c). Biomass can be acid hydrolysed before fermentation or biohydrolysis step can be incorporated into dark fermentation. Direct photofermentation of carbohydrates derived from acid hydrolysis of biomass can also be carried out. Pretreatment of DFE prior photofermentation and neutralisation after acid hydrolysis are important (Argun and Kargi 2011).

Sequential dark and photofermentation of glucose can be represented by following reactions (Manish and Banerjee 2008) when acetic acid is the only VFA produced.

Dark fermentation:

$$C_6H_{12}O_6 + 2H_2O \underset{\Delta G^\circ = -206\,kJ}{\longrightarrow} 2CH_3COOH + 4H_2 + 2CO_2 \quad (11.13)$$

Photofermentation:

$$2CH_3COOH + 4H_2O \underset{\Delta G^\circ = 104.6 \times 2 = 209.2\,kJ}{\longrightarrow} 8H_2 + 4CO_2 \quad (11.14)$$

Sequential or combined dark and photofermentation (overall reaction):

$$C_6H_{12}O_6 + 6H_2O \underset{\Delta G^\circ = 3.2\,kJ}{\longrightarrow} 12H_2 + 6CO_2 \quad (11.15)$$

The overall maximum theoretical yield in sequential fermentation is 12 mol H_2 mol^{-1} glucose when acetic acid is the only VFA produced (Eqs. 11.13 and 11.14). Actual yields are much lower than that due to formation of a mixture of VFAs and utilisation of part of the substrate for growth, maintenance and PHB formation (Argun et al. 2008c; Argun and Kargi 2010a). An overall yield of minimum 8 mol H_2 mol^{-1} glucose is aimed for an economically viable process (Chen et al. 2010).

Hydrogen gas production studies from waste and pure carbon sources by sequential dark and photofermentation using different operational modes have been reported to have considerably higher yields than single-stage dark or photofermentation. However, hydrogen formation rates in sequential fermentation are lower than those of dark fermentations alone. PNS bacteria require long HRTs (up to 5 days) for efficient conversion of VFAs to hydrogen in a continuous reactor. The highest hydrogen productions are obtained using repeated-batch operation mode where the substrate concentrations varied between 5 and 25 g glucose L^{-1}. Highest hydrogen formation yield of 7.2 mol H_2 mol^{-1} glucose is reported by Yokoi et al. (2002) where medium containing 10 g L^{-1} sweet potato starch was fermented by sequential dark and photofermentation operated in a fed-batch mode. Co-culture of *E. aerogenes* HO-39 and *Clostridium butyricum* has been used in dark fermentation producing hydrogen and VFAs. *R. sphaeroides* efficiently converts VFAs present in DFE by photofermentation and addition of sodium molybdate and EDTA was stated to be crucial in enhancing photofermentation yield. Yokoi et al. (2001) earlier used this approach and obtained a total yield of 7.0 mol H_2 mol^{-1} glucose from sweet potato starch in sequential dark and photofermentation.

Sequential dark and photofermentations increased hydrogen yield by minimum 5 mol H_2 mol^{-1} glucose. Reduced hydrogen productivity was obtained due to slow hydrogen gas production by photofermentation. DFE should be pretreated prior to photofermentation. Increase in cell density and high substrate loading, for example by using immobilized-cell reactors and fed-batch operations, need to be extensively explored.

11.6 Combined Dark and Photofermentation

Dark and photofermentation can be carried out in a single reactor in which VFAs produced by dark fermentation can be converted to hydrogen and carbon dioxide by photofermentation. Theoretically, 12 mol hydrogen per mol of glucose can be obtained by combined dark and photofermentations (Eq. 11.15). Thermodynamically, 600 °C and 34.5 MPa are required for the above conversion (Ni et al. 2006). This conversion can be carried out at room temperature and atmospheric pressure (Kapdan and Kargi 2006) by combined fermentation at a slower rate (Levin et al. 2004).

It has been reported than an ideal combined fermentation proceeds with less than 5 g L^{-1} glucose, at 30 °C and pH 7.00. Using a proper biomass ratio of PNS/dark fermentation bacteria, suitable light/dark illumination cycle and supplementation of Fe(II) and Mo aid in the combined fermentation (Argun and Kargi 2010b; Kargi and Ozmihci 2010). PNS bacteria are capable of fermenting carbohydrates along with dark fermentation bacteria to produce VFA. A long lag time is taken by PNS bacteria to shift from carbohydrate fermentation to VFA fermentation. This creates a hindrance in the combined dark and photofermentations (Argun and Kargi 2010c). Accumulation of VFAs takes place hence causing inhibition of dark fermentation and PNS bacteria (Ozmihci and Kargi 2010b). This problem can be partially solved by utilisation of low carbohydrate concentration and simultaneous removal of VFAs from the medium. A pH between 7 and 7.5, ORP of −150 Mv, temperature of 30 °C and HRT more than 6 days are optimal conditions for combined fermentation (Argun and Kargi 2010c). PNS bacteria are more sensitive to changes in environmental conditions; hence, the operating parameters assigned are more towards to that of photofermentation than dark fermentation. Higher hydrogen yields are obtained in combined fermentation as compared to single-stage dark or photofermentation. However, lower hydrogen formation rates are obtained as compared to single-stage dark fermentation (less than 35 ml H_2 L^{-1} h^{-1}). Majority of the combined fermentation studies have been carried out by suspended and immobilized cultures in batch reactors. Conventional batch reactors can normally operate with 5 g substrate/L, but this concentration can be increased by 10 folds if the reactor is operated in repeated fed-batch mode (Yokoi et al. 1998).

Asada et al. (2006) have reported the highest yield of 7.1 mol H_2 mol^{-1} glucose in combined fermentation. A co-culture of *Lactobacillus delbrueckii* NBRC 13953 and *R. sphaeroides*-RV were used. Hydrogen production in combined fermentation from sucrose under 4 klux light illumination was enhanced using statistical

experiment design methods (Sun et al. 2010). Highest hydrogen-production yield was reported to be 10.16 mol H_2 mol^{-1} sucrose equivalent to 5.08 mol H_2 mol^{-1} hexose. Yokoi et al. (1998) have produced hydrogen in fed-batch reactor by combined fermentation. *Rhodobacter* sp. M-19 and *C. Butyricum* were used as inoculum cultures in an initial ratio of 10:1. A high yield of 6.6 mol H_2 mol^{-1} glucose was obtained.

A hybrid annular bioreactor was operated in a continuous mode for combined dark and light fermentation. *Clostridium beijerinkii* DSM 791 and *R. sphaeroides*-RV were used as microbial strains with a biomass ratio 1:3.9. Low hydrogen formation rates and yields were reported because of accumulation of VFA and also the suboptimal conditions for dark and light fermentations (Argun and Kargi 2010c).

11.7 Conclusions

An ideal process scheme for fermentative hydrogen production should give a cheap, simple and robust operation with high hydrogen formation rate and yield. Commonly used batch reactors are seldom inhibited because of high initial substrate and final product concentrations. This can be avoided by slow feeding of substrate and continuous removal of products like VFAs, solvents and hydrogen. Continuous operation results in constant quality product at steady state.

Highest hydrogen formation rate (7.5 L H_2 L^{-1} h^{-1}) has been reported in a single-stage continuous dark fermentation immobilized reactor operated at low HRT of 15 min. Low hydrogen yields (maximum 3 mol H_2 mol^{-1} glucose) were obtained in the continuous mode. Higher yields can be achieved in a batch reactor at low initial substrate concentrations (less than 5 g glucose L^{-1}). Higher yields (about 3 mol H_2 mol^{-1} glucose) and rates can be achieved at high speed substrate concentration in fed-batch operation mode.

DFE can undergo photofermentation to produce hydrogen gas from VFAs by using PNS bacteria (mainly *Rhodobacter* sp.). Photofermentation requires strict control of environmental conditions, unusual nutrient requirements (Fe, Mo, V, glutamate, vitamins etc.) and uniform light intensities. PNS bacteria are more sensitive to changes in ambient conditions. Lower hydrogen-production rates are achieved by *Rhodobacter* sp. (0.17 L H_2 L^{-1} h^{-1}) as compared to that of dark fermentation. Almost 80 % of the theoretical yields have been achieved under low light intensities and low hydrogen formation rates. PNS bacteria cannot tolerate NH_4^+ and VFAs above 40 and 2500 mg L^{-1} respectively due to substrate inhibition. These limitations reduce hydrogen gas productivity in batch fermentation. Although continuous suspended cultures have slightly better hydrogen yields, immobilisation of PNS bacteria on solid matrices (e.g. porous glass) increases the rates of hydrogen formation appreciably (3600–3800 ml H_2 L^{-1} h^{-1}). High cell density fed-batch culture with controlled feeding and environmental conditions has been the most suitable operation mode resulting in both high hydrogen formation rates and yields.

Both sequential and combined dark and photofermentations have been experimented to improve hydrogen formation yields to the economical level of 8 mol H_2 mol^{-1}glucose. Sequential fermentations require larger fermenter volumes and separation/pretreatment units in between the two stages. In combined fermentation, both dark and photofermentation take place in the same reactor which makes it easier to operate. However, higher hydrogen gas yields and productivities are obtained in sequential mode than the combined one due to the longer lag times between the dark and light fermentation in the latter case. Also, adverse interactions and different nutritional needs of different bacteria result in low hydrogen formation rates and yields in the combined fermentation and hence making the sequential fermentation a preferable one.

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Chapter 12
Biohydrogen as Biofuel: Future Prospects and Avenues for Improvements

Jahangir Imam, Puneet Kumar Singh and Pratyoosh Shukla

Abstract Biological hydrogen production is one of the most imperative and demanding areas of research and technology development as a clean, efficient, and sustainable energy option to be considered as imminent fuel. The successful biohydrogen production needs technology improvement, use of updated microbial technologies to generate, and developing innovative proficient methods of bio-hydrogen production. This review explains the various possibilities toward the advancement of biohydrogen production methods, microbial technology involved in different methods with their benefits and shortcomings. It also spotlights on the avenues for enhancement in biohydrogen production and the future prospects of exploiting biohydrogen as prominent biofuel.

Keywords Bio-hydrogen · Hydrogenase · Biophotolysis · Photofermentation · Dark fermentation · Genetically modified microorganisms · Bioreactors

12.1 Introduction

Today in this twenty-first century there is large demand of chemical hydrogen as energy fuel. But the world is also having challenges to meet the demand. Fossil fuels serve as the primary source of energy to fulfill world's energy requirement

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and are the major energy provider (80 %). These fossil fuels are exhaustible and also releases greenhouse gases (CO_2, CH_4 and CO) in the environment resulting in global warming and pollution. Therefore, the concept of sustainable energy development was evolved for a livable future where human needs are met while keeping the balance with nature (Vijayaraghavan and Soom 2006). There are lots of interests among technologists to generate clean and sustainable energy from renewable carbon source (Bhat 2000).

Presently, 96 % of all hydrogen are derived from fossil fuels, with 48 % from natural gas, 30 % from hydrocarbons, 18 % from coal, 4 % from electrolysis, and about 1 % is produced by biomass (Fig. 12.1). Hydrogen is considered as the most important fuel as it has low emission, it is environment friendly, renewable, does not evolve any greenhouse gases, and also contain high energy content per unit mass of any known fuel (143 GJL^{-1}). It can also easily converted into electricity by fuel cell and on combustion gives water as the only by product (Das et al. 2008).

Hydrogen produced from renewable sources such as water, organic waste or biomass, either biologically or photobiologically is termed as biohydrogen (Vijayraghavan and Ahmed 2006). Microbial production of hydrogen by anaerobic fermentation or by photosynthesis has been reported (Fig. 12.2). The anaerobic fermentation process is a choice for biohydrogen production over photosynthesis as it does not require sunlight. But now microorganisms like algae, cyanobacteria are also used for the production of biohydrogen.

Therefore, this review will focus on the different methods available for the biohydrogen production, its application and future aspects. We will also discuss the important improvement which can be exploited for the biohydrogen production.

12.2 Different Methods of Biohydrogen Production

Many microorganisms produce hydrogen as their byproduct during anaerobic fermentation processes. Some microorganisms like green algae, cyanobecteria produce enzymes to convert water into hydrogen in the presence of thermal energy source such as sunlight. This process is known as photobiological process. In biological hydrogen production process, hydrogen formation and consumption are

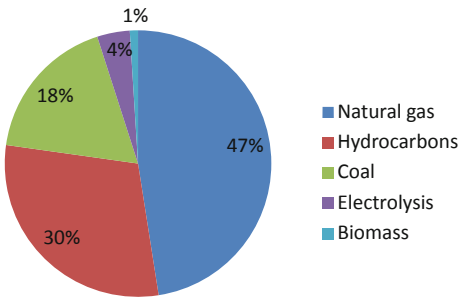

Fig. 12.1 Different sources of hydrogen production

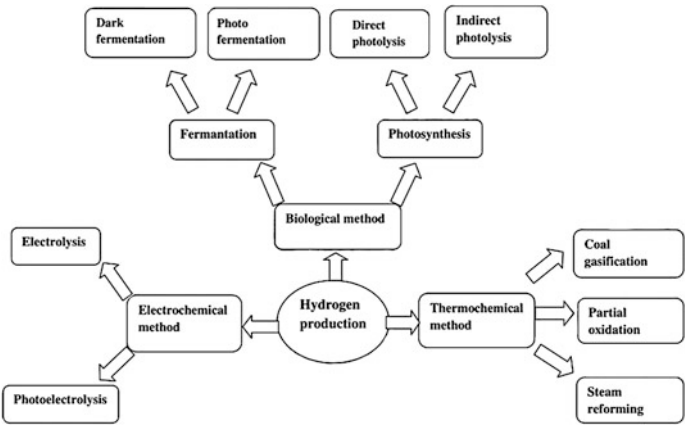

Fig. 12.2 Different methods of hydrogen production

uncoupled, so that hydrogen is available as the final product (Reith et al. 2003). There are various biological methods available for hydrogen production. Table 12.1 gives an overview of biological hydrogen production process, which is being explored in fundamental and applied research.

12.2.1 Direct Biophotolysis

This biological process utilizes solar energy and photosynthesis system of microalgae to convert water into hydrogen. It uses the same photosynthesis system as present in plants and algae but instead of generating carbon containing products, it produces hydrogen. Here, both Photosystem I (PS I) and Photosystem II (PS II) photosynthetic systems are involved and with the help of sunlight, it converts the most easily available substrate, water into oxygen and hydrogen (Fig. 12.2). This can help in unlimited production of hydrogen in this methods becomes successful in future (Fig. 12.3).

In direct biophotolysis, the hydrogen production time is very less since the hydrogenase enzyme activity is sensitive to simultaneously oxygen production at high level. This is a major setback of using this method for biohydrogen production. This problem has been overcome by the use of green algae, *Chlamydomonas reinhardtti* which maintain low O_2 level (Melis et al. 2000) but the H_2 produced is very low (Benemann 2000).

12.2.2 Indirect Biophotolysis

The sensitivity of H_2 evolution to O_2 is been minimized in indirect photolysis by splitting O_2 and H_2 production. In this, CO_2 is first fixed as storage carbohydrate

Table 12.1 Overview of different biological hydrogen production methods with their advantages (Reith et al. 2003; Kaushik Nath and Debabrata Das 2004)

S.No.	Methods	General reaction	Microorganisms used	Advantages
1	Direct biophotolysis	$2\ H_2O + \text{light} \rightarrow 2H_2 + O_2$	Microalgae	Can produce H_2 from water and sunlight Solar conversion energy increased by 10 folds as compared to trees and crops
2	Indirect biophotolysis	Overall reaction: $12H_2O + \text{light} \rightarrow 12H_2 + 6O_2$	Microalgae, Cyanobacteria	Can produce H_2 from water Has the ability to fix N_2 from atmosphere
3	Photofermentations	$CH_3COOH + 2\ H_2O + \text{light} \rightarrow 4\ H_2 + 2\ CO_2$	Purple bacteria, Microalgae	A wide spectral light energy can be used by these bacteria Can use different waste material such as distillery effluent, waste etc.
4	Dark fermentation	$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2$	Fermentative bacteria	It can produce H_2 all day long without light A verity of carbon source can be used as substrate It produces valuable metabolites such as butyric, lactic and acetic acids as by products It is anaerobic process so there is no problem of O_2 limitation
5	Hybrid fermentation technology (Two-Phase $H_2 + CH_4$ Fermentation)	(a) $C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$ (Stage I) (b) $2CH_3COOH \rightarrow 2CH_4 + 2CO_2$ (Stage II)	Fermentative bacteria + Methanogenic bacteria	Two-stage fermentation can improve the overall yield of hydrogen

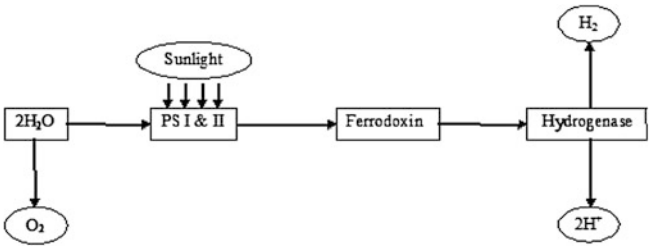

Fig. 12.3 Direct biophotolysis

with the evolution of O_2 which is followed by its conversion to H_2 by dark anaerobic fermentation processes (Fig. 12.4).

$$6H_2O + 6CO_2 + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2$$

$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2$$

Over all

$$12H_2O + \text{Light} \rightarrow 12H_2 + 6O_2$$

Cyanobacteria and various types of green algae fixes both, the CO_2 and the nitrogen from the atmosphere with the help of PS II and nitrogenase enzyme. Since the nitrogenase enzyme is localized in heterocyst, it provides an oxygen free environment for hydrogen production.

12.2.3 Photofermentation

In photofermentation, H_2 production occurs under oxygen deficient condition using high energy and organic acids. These reactions are performed by purple nonsulfur bacteria because of the presence of nitrogenase enzyme. The overall pathways for the photofermentation process can be expressed as (Fig. 12.5):

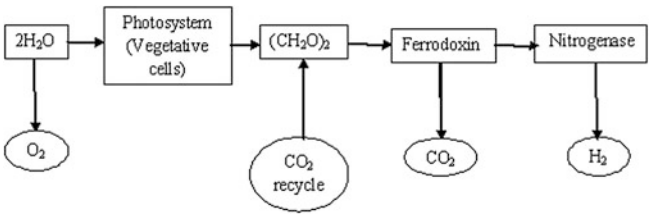

Fig. 12.4 Indirect biophotolysis

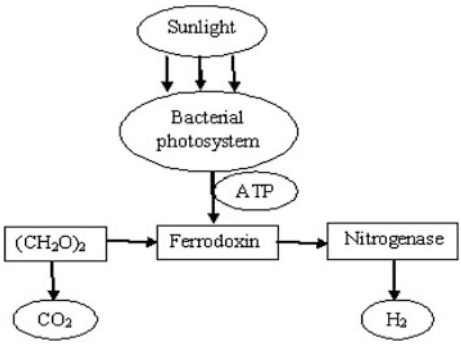

Fig. 12.5 Photofermentation process by photosynthetic bacteria

These photosynthetic bacteria lack PSI which is an advantage as it eliminates the sensitivity of H_2 production to high O_2 concentration. These phototropic bacteria can easily convert the light energy into H_2 using cheap organic substrate (Boltan 1996; Fedorov et al. 1998; Tsygankov et al. 1994). Both the batch (Zurrer and Bachofan 1979) as well as continuous culture (Fascetti and Todini 1995) processes can be employed for H_2 production. In spite of having these advantages, the photochemical efficiency is low (3–10 %) (Das et al. 2008). This may be due to two reasons:

1. Inhomogeneous light distribution in the bioreactor.
2. Low nitrogenase activity.

This can be improved by improving the design of bioreactor, so that homogeneous light distribution occurs in bioreactor and may improve photoconversion efficiency microorganisms as well as maintaining the maximum nitrogenase activity. The major bottleneck of this method of H_2 production in practical application is that the process requires a large surface area to collect light energy, and the construction of such large bioreactor is usually cost expensive.

12.2.4 Dark Fermentation

These phenomena occur under anaerobic condition. In this, the bacterial oxidation of the substrate generates electron which is then accepted by proton, an electron acceptor and get reduced to molecular H_2 (Das and Veziroglu 2001; Levin et al. 2004). Dark fermentation has high H_2 evolution rate but the yield is low in comparison to other chemical and electrochemical process. The reason for this low H_2 yield is that the end product contains both acetate and butyrate. Moreover, if H_2 yield is increased the reaction becomes thermodynamically unstable (Das et al. 2008, Fig. 12.6).

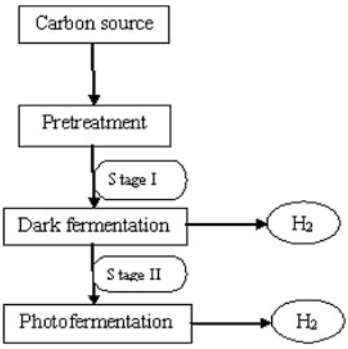

Fig. 12.6 Dark fermentation

12.2.5 Hybrid Fermentation Technology

This technology is based on combining the two fermentation process to enhance H_2 production yield (Das et al. 2008). The synergy of the process lies in the maximum conversion of the substrate which otherwise fails to achieve a complete conversion due to thermodynamic limitation (Liu et al. 2005). There were two combined systems developed on the basis of hybrid fermentation technology:

1. Dark and photofermentative bioreactors.
2. Bioelectrochemically assisted microbial bioreactors.

12.2.5.1 Dark and Photofermentative Bioreactors

In this system, the light independent bacteria and photosynthetic bacteria provide an integrated system for maintaining the H_2 yield (Yokoi et al. 1998). In this two stage fermentative system, limit dark fermentation occurs in which carbohydrate is converted into H_2 by photosynthetic bacteria in photobioreactor. This combines fermentation process results in the production of 12 ml of H_2 from 1 ml of glucose. This hybrid fermentation process is highly efficient in a sense that it requires only one substrate and it releases H_2 in both the stages.

12.2.5.2 Bioelectrochemically Assisted Microbial Bioreactors

This bioreactor technology is based on electrochemical cell, with the involvement of microbial fuel cells (MFC). In this, MFC produce photons and electrons due to the oxidation of organic matter by the bacteria (Ishikawa et al. 2006; Schotz and Schroder 2003) and the photon (H_2) get evolved at cathode. In Bioelectrochemically assisted microbial reactor (BEAMR), H_2 is evolved at the cathode (Liu 2005) using biodegradable material. Both the MFC and BEAMR systems are efficient

and have similarities; therefore scientists are trying to develop system where both the systems could be forced or exploited for each other.

The different method described above for biohydrogen production has its own advantage and disadvantage. No single method can produce H_2 in large amount to fulfill the requirement of world's H_2 demand. Even though these technologies have greatly advanced during the last two decades, the general commercialization is still lagging. We need to improve our R&D and industrial sector and also manage their research to improve biohydrogen production at commercial scale.

12.3 Biohydrogen Producing Microorganisms

There are several groups of microorganisms that can produce hydrogen. These are basically divided into two broad groups, one is heterotrophic bacteria which require supply of chemically bound energy in the form of household waste, industrial waste, effluents etc., and they are independent of sunlight. The other group is mainly the photosynthetic bacteria which utilize sunlight for hydrogen production. Another group of photosynthetic bacteria is cyanobacteria, which has nitrogen fixation properties and produces hydrogen as byproduct. The common class of hydrogen producing microorganisms is strictly anaerobic. Some facultative bacteria have been identified for hydrogen production if the enzyme hydrogenase is present in the bacteria. Recently, aerobic bacteria have also been reported to produce hydrogen. Eukaryotic green algae also possess hydrogenase enzyme and can be used to produce biohydrogen (Table 12.2).

12.4 Bottleneck in Biohydrogen Production

In the coming time, the world will face the energy crisis as well as the degradation of the environment. The fossil fuel based energy will deplete and the human kind will be in great danger. This can be only stopped by increasing our dependence to H_2 energy-based economy instead of fossil fuel based economy. But the transition from fossil fuel to H_2 energy is not easy because of the following bottlenecks which are listed below:

1. Low H_2 yield from any available method.
2. The H_2 production pathways in microorganisms are still not completely elucidated.
3. Low availability of cheap substrate.
4. Cheap methods should be developed to process the raw material industrially.
5. Presently, there is not a single microorganism available which can produce more than 4 mol H_2/mol of glucose.
6. Highly efficient bioreactor is still not developed.

Table 12.2 List of various groups of microorganisms with their advantages and disadvantages

Group	Microorganisms	Advantages	Disadvantages
I	Green algae	Can produce H_2 from water and sunlight	Sunlight dependent
			Sensitivity of hydrogenase to high oxygen concentration
		CO_2 is the sole carbon source	Require H_2O as an additional substrate
		Presence of hydrogenase enzyme	
II	Cyanobacteria	Can produce H_2 from water and sunlight	Sunlight dependent
			Inhibitory effect of oxygen on nitrogenase
		CO_2 is the sole carbon source	Requirement of CO_2 gas
		Nitrogenase enzyme is involved in H_2 production	
III	Photosynthetic bacteria	Utilizes sunlight as energy source	Sunlight dependent
			Low photochemical efficiencies (3–10 %)
		Can utilize cheap organic or inorganic wastes	Inhomogeneity of light distribution in bioreactor
		Versatile metabolic capabilities	
		Lack of PS II	
		No O_2 inhibition of hydrogen production	
IV	Fermentative bacteria	Light independent	Require treatment of fermented broth
		Different and cheap raw materials can be used	CO_2 is required
		Produces valuable by-products	
		No O_2 inhibition problem	

7. The H_2 production reaction is thermodynamically unstable. Hence, the system must be developed to keep the partial H_2 pressure low (Thauer et al. 1977, Vijayaraghavan and Soom 2006).
8. Sensitivity of hydrogenase enzyme to O_2 and H_2 partial pressure effect in H_2 production yield.
9. Little knowledge about the H_2 producing microorganisms.
10. Lack of sensibility, poor economy, and much dependence to fossil fuel energy source.
11. H_2 purification and storage.

12.5 Avenues for Improvement in Biohydrogen Production

Many methods and processes must be improved and advanced in near future to overcome the bottleneck in biohydrogen products and to increase the H_2 yield to fulfill the world's demand.

12.5.1 Genetically Modified Microorganisms

Today, genetic engineering is the key area of focus for the improvement of strains for H_2 production (Das et al. 2008). In photofermentation, the presence of pigment in photosynthetic bacteria hinders the high distribution in photobioreactor. By genetically modifying the potential pigment protein expression, we can allow the efficient light absorption (Miyake et al. 1998). Miyake et al. (1998) found a mutant strain of *Rhozobacter sphaeroids* by or irradiation mutation in which LH1/LH2 (Light Harvesting 1/Light Harvesting 2) ratio was altered and he found that the H_2 production is increased to 1.4 times. Akhtar and Jones (2008) have made a synthetic operon of hydrogenase in a single plasmid to overcome the problem of heterogeneous gene expression. One another approach is to change the *E.coli* host system which has faster growth ratio and can easily commercialized.

Currently, genetically modified microorganisms use is a challenge because of the possibility of horizontal gene transfer and plasmid instability.

12.5.2 Metabolic Engineering of Microorganisms

The genetic manipulation of some eukaryotes, regulatory or transport pathways can be done to increase the H_2 production yield. Some examples of metabolic engineering for H_2 production are:

1. Blocking the formation of alcohol and some acids by redirecting their pathways.
2. H_2 production can be increased by directing the carbon flow into synthesis of formate (Yoshida et al. 2006).
3. By increasing the substrate utilization efficiency of microorganisms.

So by understanding the metabolic pathways and function it can be targeted and manipulated easily to regulate the H_2 production.

12.5.3 Modification of Hydrogenase Enzyme

Hydrogenase enzyme is one of the important aspects of research, since it is the key enzyme of the biohydrogen production. it provides the overexpression of this enzymes may lead to reduced yield of H_2 (Morimoto et al. 2005).

12.5.4 Improvement of Bioreactor Design

To fulfill the need of making biohydrogen as a fuel in near future, it chiefly depends on success of converting the process from lab scale to industrial scale. In

Table 12.3 List of modified bioreactors for hydrogen production

Microorganisms	Raw materials	Type of modification	Reference
Rhodobacter sphaeroides RV	Basal medium with lactate and glutamate	Multi-layered photobioreactor (MLPR)	Kondo et al. (2006)
Rhodopseudomonas palustris WP3-5	Acetate	internal optical fiber illumination	Chen and Chang (2006)
Sewage sludge	Sucrose	Fixed bed bioreactor with activated carbon	Chang et al. (2002)
Activated sludge and digested sludge	Glucose	Anaerobic fluidized bed reactor	Zhang et al. (2003)
Anaerobic sludge	Sucrose	Polymethy methacrylate(PMMA) immobilized cells	Wu and Chang (2007)
Sludge from wastewater treatment plant	Sucrose	Carrier-induced granular sludge bed (CIGSB)	Lee et al. (2006)
Sludge from wastewater treatment plant	Sucrose	Fluisized bed reactor (FBR)	Wu et al. (2007)
Enterobacter cloacae IIT-BT 08	Sucrose	Rhomboidal bioreactor	Kumar and Das (2001)

this, bioreactor plays a very important part; in bioreactor design its efficiency, robustness, and reliability are important parameters which need to be addressed. Different types of bioreactors have been modified to design a suitable bioreactor which can be used for H_2 production (Table 12.3).

12.5.5 Utilization of Inexpensive Raw Material

For large-scale industrial production of biohydrogen , use of inexpensive raw material is desirable. The main criteria for the selection of a substrate for H_2 production are its availability, cost, carbohydrate content, and biodegradability (Kapdan and Kargi 2006). Therefore, research should also be focused on cheap, easily available, and low-cost raw material which can be used in industrial scale without compromising in the production yield of H_2.

12.6 Future Prospects

For our energy requirements, we are almost totally dependent on carbon-containing fossil fuel sources like oil, coal, and natural gas. These fossil fuels have been formed over millions of years from plant biomass. With the rapid use of these fossil fuel resources, it will be exhausted in the near future. The other major

concern of continuous use of fossil fuels is, on combustion they releases greenhouse gases like CO_2 and other which is the main reason of global warming and climate change. Therefore, many research groups all over the world have focused their research on biological hydrogen production from cheap raw material with the help of microorganisms.

The biohydrogen is considered as a future dream fuel because it has many advantages over the existing energy sources. It is renewable, does not release any greenhouse gases, has high energy content, can be converted into electricity and most importantly on combustion it releases water as the byproduct (Das et al. 2008).

Today, there is lot of focus on biological hydrogen production at small-scale mainly because of two reasons (1) can utilize renewable energy resources, and (2) usually operated at ambient temperature and atmospheric pressure. The small-scale biohydrogen production at location where the raw materials are easily available is the ideal choice for biohydrogen production. In this, the biohydrogen gas produced can be either used directly at the point of production ("Stand-alone system") or supplied at another location ("grid-connected production system"). The potential of biohydrogen production is not only decided by the characteristics and cost of the production process, but also by its integration into the overall energy infrastructure (Reith et al. 2003).

The transition from fossil fuel based energy sources to biohydrogen-based energy sources is not easy and also not feasible in the short term because of the above mentioned problems. This work has to be addressed at different levels from lab scale to industrial scale to its commercialization to make biohydrogen the 'future dream fuel'. To make this possible, we also have to focus our research in the following areas:

1. Improvement of H_2 production processes using cheap raw material.
2. Improvement of microbial strain for the utilization of various carbon substrates.
3. Use of mixed culture consortium for better uptake of substrate and more H_2 production.
4. Improvement of various bioreactor designs.
5. Standardization of scaling up of H_2 production.

12.7 Conclusions

Biohydrogen is most commonly produced by algae and bacteria and is regarded as prospective biofuel. It is also considered as the 'energy carrier of the future' being a clean energy source with high-energy content as compared to hydrocarbon fuels. Various improvement areas of research are presently envisaged toward increasing the efficiency by developing oxygen-tolerant FeFe-hydrogenases and boosted H_2 production rates by advanced electron transfer. Further, few aspects viz., augmentation of chlorophyll (Chl) antenna size in green algae to maximize

photobiological solar conversion efficiency resulting in better light exploitation and superior photosynthetic productivity by the green alga mass culture are on the spotlight these days. Nevertheless, the majorities of issues are improving bioreactor design through following approaches:

- Restricting photosynthetic H_2 production by buildup of a proton gradient.
- Inhibition of photosynthetic H_2 production by CO_2.
- Bicarbonate binding at PS II for proficient photosynthetic activity.
- Efficient conversion of solar energy into chemical energy stored in molecular H_2.

However, unlike fossil fuels, petroleum, natural gas, and biomass, hydrogen is not readily available in nature and diverse methods can be applied for biohydrogen production. This appraisal describes the latest idea of this challenging field of research which can provide suitable options to researchers to develop their learning toward experimenting with biohydrogen production utilizing modern state-of-the-art technologies. Despite the fact that each method has its own merits and demerits but in entirety if biohydrogen has to be used as marketable fuel and it has to be cost-effective and effortlessly available.

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Chapter 13
Biohydrogen Production from Microalgae

Dheeraj Rathore and Anoop Singh

Abstract Algal biohydrogen is thought to be the ideal energy source being free from air pollution and global warming as energy consumption is arrested with this technology and the environment has been protected in an ecofriendly and sustainable manner. For sustainability assessment, a life cycle assessment approach is most relevant to avoid issues in problem shifting and also assess the environmental impacts and resources used throughout a product's life cycle and consider all attributes or aspects of natural environment, human health, and resources. The LCA of algal biohydrogen can play a major role in technological improvement and decision making for development of policies.

13.1 Introduction

Finding sufficient supplies of clean energy for the future is one of the most daunting challenges for humanity and is intimately linked to global stability, economic prosperity, and quality of life (Singh et al. 2010a, b, 2011a). Presently, most of the energy demand is fullfiled by fossil fuels. The global petroleum demand has increased steadily from 57 million barrels a day^{-1} in 1973 to 1985 million barrels a day^{-1} in 2009 and will continue to increase in line with the

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world's economy (Saraf and Hastings 2011). The increasing energy demands will speed up the exhaustion of finite fossil fuels. United Arab Emirates, one of the major oil export countries, wil fail to meet the share in the oil and natural gas demands by 2015 and 2042, respectively (Kazim and Veziroglu 2001). The fossil fuel resources in Egypt will be exhausted within one to two decades (Abdallah et al. 1999). Using petroleum-based fuels creates atmospheric pollution during combustion. Apart from emission of greenhouse gas (GHG) CO_2, air contaminants like NO_X, SO_X, CO, particulate matter, and volatile organic compounds are produced (Klass 1998). The commitment made by signatories of the Kyoto Protocol (1998) and their future expansion in the up-coming Copenhagen meeting to reduce greenhouse gas emissions to mitigate global warming has specifically targeted combustion CO_2 from fossil fuels released into the atmosphere.

The continued use of fossil fuel is now widely recognized as unsustainable because of depleting supplies and the contribution of these fuels to accumulation of carbon dioxide in the environment. Renewable, carbon neutral, energy resources are necessary for environmental and economic sustainability (Prasad et al. 2007a, b; Singh and Olsen 2011). In the past few years energy researches have come up with several alternate options for a renewable and sustanable source of energy (Nigam and Singh 2011; Singh et al. 2011b, 2012). As described by Hoffert et al. (2002) future reductions in the ecological footprint of energy generation will reside in a multifaceted approach that includes the use of hydrogen, bio-energy, wind, nuclear, solar power, and fossil fuels from which carbon is sequestered.

Hydrogen gas is seen as a strong candidate for future energy carrier by virtue of the fact that it is renewable, does not evolve the greenhouse gas CO_2 in combustion, liberates large amounts of energy per unit weight in combustion, and is easily converted into electricity by fuel cell (www.oilgee.com 2012). Hydrogen is a clean and renewable energy source that does not produce carbon dioxide as a byproduct when used in fuel cells for electricity generation. Progress in the late 1990s contributed to a breakthrough in terms of sustainable hydrogen production. However, the problem is with the way it is produced. Although hydrogen biogas can be efficiently produced at the laboratory level, there is no known commercially operating hydrogen from biomass production facility in the world today (Zhu and Beland 2006).

H_2 production is primarily the domain of algae and bacteria. Nature has created biological reactions that use sunlight for the oxidation of water (oxygenic photosynthesis), and enzymes that use electrons for the generation of H_2 (hydrogenases). Oxygen adversely affects the function of the hydrogenase enzyme (Erbes et al. 1979; Ghirardi et al. 2000) and acts as a positive suppressor of hydrogenase gene expression (Florin et al. 2001; Happe and Kaminski 2002). Hans Gaffron discovered the hydrogen metabolism in unicellular green algae, which are eukaryotic organisms of oxygenic photosynthesis (Gaffron 1939, 1940). Gaffron observed that, under anaerobic conditions, the green alga *Scenedesmus obliquus* can either use H_2 as electron donor in the CO_2 fixation process in the dark (Gaffron 1944), or evolve H_2 in the light (Gaffron and Rubin 1942).

This chapter is an insight into the investigation so far conducted in the field of suitability of H_2 as replacement of conventional fossil fuel, ways of its efficient bioproduction from micro algae, and the barriers in the field.

13.2 Suitability of H_2 Production from Algae

Algae are one of the oldest life-forms. They are primitive plants, with thalloid body structure, naked reproductive cells, and have chlorophyll '*a*' as their primary photosynthetic pigment (Lee 1980). Algae structures are primarily for energy conversion without any development beyond cells, and their simple development allows them to adapt to prevailing environmental conditions and prosper in the long term.

Interest in green algae emanates from the fact that, in principle, they can employ the highly efficient process of photosynthesis to produce hydrogen (H_2), a valuable fuel, from the most abundant of the natural resources, sunlight and water (H_2O) (Melis and Happe 2004). Under optimal growth conditions, green algae grow with remarkable rates, reaching biomass duplication times of 6 h in the laboratory (Smith et al. 1990) and 24 h under mass culture ambient conditions (Ben-Amotz and Avron 1990). The first scientific investigation of H_2 evolution by microalgae was reported by Gaffron and Rubin (1942), who demonstrated that after a period of dark anaerobic 'adaptation', the green alga *S. obliquus* produces H_2 in the dark at low rates, with H_2 production greatly stimulated in the light, though only for relatively brief periods. Other noteworthy observations were that uncouplers and low CO_2 concentrations stimulated light-driven H_2 production in green microalgae.

Stuart and Gaffron (1972) wrote 'We conclude that all of the algae tested are able to photo produce H_2 via noncyclic electron flow through photosystem I to hydrogenase.' Other investigators, many of them associates of Gaffron, contributed with observations on photosynthetic hydrogen production in other unicellular green algae, including *Chlorella* (Spruit 1958; Kessler 1973), *Chlamydomonas* (Frenkel 1952; Frenkel and Lewin 1954; McBride et al. 1977; Greenbaum 1982, 1988; Maione and Gibbs 1986a, b) and other related organisms (Miura et al. 1986, 1992). So far, cyanobacteria and green algae are the only known organisms with both an oxygenic photosynthesis and a hydrogen production (Schutz et al. 2004). While H_2 production in cyanobacteria is mostly coupled to nitrogen fixation, unicellular green algae utilize photosynthetically generated electrons for H^+ reduction. Kessler (1974) summarized the relevant information in a review article, showing that many but not all green algal species are equipped for molecular H_2 metabolism. Nevertheless, the ability of green algae to photosynthetically generate molecular H_2 has captivated the fascination and interest of the scientific community because of the many *fundamental* aspects and the *practical* implications of the process (Melis and Happe 2001).

13.3 Production Pathways

Hydrogen has the largest energy content per weight of any known fuel, and can be produced by various means (Hallenbeck and Benemann 2002; Levin et al. 2004). Unfortunately, both the main methods for hydrogen production, electrolysis of water and thermocatalytic reformation of hydrogen-rich compounds, usually require high-energy inputs obtained from non-renewable resources (Levin et al. 2004). Biological production of hydrogen solves this problem by using microorganisms to convert biomass or solar energy into hydrogen gas (Hallenbeck and Benemann 2002; Das and Veziroglu 2001). Although Gaffron and Rubin (1942) considered both water and carbohydrates as sources of electrons for H_2 evolution, they favored the latter as the main source. Spruit (1958) found evidence supporting water as the main electron donor in such a reaction.

A schematic representation of the photosynthetic reactions in the chloroplast thylakoid membrane of green algae is demonstrated in Fig. 13.1. In photosynthesis, excitation energy from sun is transferred from antenna pigments of light-harvesting complexes (LHC) of photosystem II to photoactive chlorophyll molecule (P680) of the reaction center. As a result, strongly oxidizing cation radical $P680^+$ is formed which catalyzes oxidation of water in the oxygen evolving complex (OEC) of PS II through a series of redox active components including the tyrozine Z residue (YZ) and Mn_4O_4Ca cluster located at the luminal side of the thylakoid membrane (Goussias et al. 2002; Kern and Renger 2007; Barber 2008). Electron released from $P680^+$ accepted by a negatively charged radical of the Mg-free chlorophyll pigment pheophytin (Pheo). The reduced Pheo quickly passes an extra electron to the PS II primary quinone molecule (QA). The QA is tightly bound to PS II and acts as a single-electron carrier. From QA, an electron is transferred to the secondary quinone molecule (QB), a two-electron and two-proton acceptor. In double reduced and protonated form, QBH2 is loosely bound to PS II and thus it can be exchanged with the oxidized QB from the plastoquinone (PQ) pool. After that, an electron is transported further to PS I via the PQ pool, cytochrome b6f complex (cyt b6f), and plastocyanin (PC). Subsequent to light excitation PS I passes an electron to the soluble protein ferredoxin (Fd) through a series of electron carriers including chlorophylls P700 and A0, quinone A1, and iron-sulfur clusters FX, FA, and FB. In the stroma, an electron is transferred from the reduced Fd to the $NADP^+$ with the formation of NADPH by a process catalyzed by the ferredoxin-NADP-reductase (FNR). In chloroplast NADPH is used as reducing power for fixation of carbon dioxide in the dark reaction (Bassham et al. 1950). This electron pathway corresponds to the linear electron transport (LET) (Antal et al. 2011). However, electrons can be alternatively redirected from the reduced Fd backward to the PQ pool through the putative ferredoxin-quinone-reductase (FQR) in the so-called cyclic electron transport around PS I (CET; Moss and Bendall 1984). Such CET can be induced when FNR becomes inactive or carbon fixation proceeds at a slow rate which takes place in dark adapted plants or under different environmental stresses (Golding and Johnson 2003; Breyton et al. 2006).

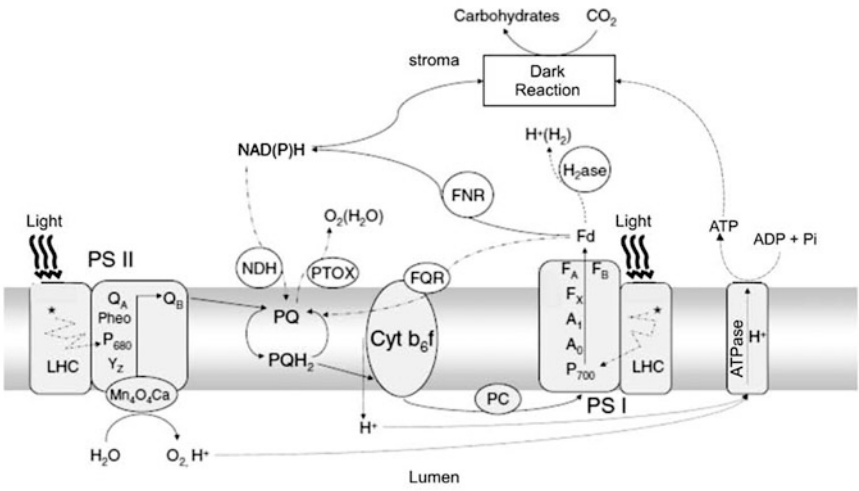

Fig. 13.1 A scheme of the photosynthetic reactions in the chloroplast thylakoid membrane of green algae. Dash–dot line shows excite ion migration from antenna to the reaction center. The major electron transfer pathways are indicated by solid lines. The pathways involved in chlororespiration, cyclic electron flow around PS I, and the Mehler reaction are shown by dash–dot–dot lines. The dashed line designates the oxygen-sensitive electron transport rout induced under anaerobic conditions. Reactions coupled to the generation of proton gradient and to the ATP synthesis are depicted by dotted lines (*Source*: Antal et al. 2011)

Production of H_2 from algae is mainly based on the photolysis of water molecule in the process light reaction of photosynthesis. Hydrogen production is a property of many phototrophic organisms (Weaver et al. 1980; Appel and Schulz 1998; Asada and Miyake 1999; Boichenko et al. 2001), and the list of H_2 producers includes several hundred species from different genera of both prokaryotes and eukaryotes (Boichenko and Hoffmann 1994). Oxygenic photosynthetic organisms, such as plants, green, red, brown, and yellow algae, and cyanobacteria, use water as a source of electrons and protons in photosynthesis. Among these organisms only green microalgae and cyanobacteria have been shown to sustain hydrogen production (Hall et al. 1995; Melis et al. 2000; Sakurai et al. 2004). The enzyme mediating H_2 production in green algae is the reversible (or bidirectional) hydrogenase that catalyzes the following ferredoxin (Fd)-linked reaction in the absence of ATP input (Boichenko and Hoffman 1994):

Hydrogenase

$$2H^+ + 2e^- \leftrightarrow H_2$$

The available H_2 energy production processes from algal biomass can be divided into two general categories: thermochemical and biological processes. Combustion, pyrolysis, liquefaction, and gasification are the four thermochemical

processes. Direct biophotolysis, indirect biophotolysis, biological water–gas shift reaction, photo-fermentation, and dark-fermentation are the five biological processes (Ni et al. 2006). This chapter is limited to bioproduction of H_2 from the algae; therefore only direct photolysis and indirect photolysis are covered in detail.

13.3.1 Direct Photolysis

The German plant physiologist Hans Gaffron (1939) discovered hydrogen metabolism in green algae. Direct photolysis based on the efficacy of algae to splitting of water molecule directly into hydrogen and oxygen. Protons and electrons extracted via the water-splitting process are recombined by a chloroplast hydrogenase to form molecular hydrogen gas with a purity of up to 98 % (Hankamer et al. 2007). Both green algae and cyanobacteria depend on photosynthesis as the starting point for all subsequent hydrogen production. The conversion of solar energy into hydrogen starts with antenna pigments such as chlorophylls, carotenoids, and phycobilisomes (Prince and Kheshgi 2005). In most oxygenic photosynthetic organisms including green micro-algae light-harvesting pigments are associated with two photosystems, PS I and PS II (Fig. 13.1; Nelson and Yocum 2006). The process of light harvesting, electron transfer, and proton production in micro green algae was reviewed by Antal et al. (2011). In green algae, hydrogen evolution is mediated by (FeFe)-H_2ase (reviewed by Ghirardi et al. 2007) which crystal structure has been recently reported (Stripp et al. 2009). The enzyme catalyzes a simple reversible reaction:

$$2H^+ + 2Fd_{reduced} \leftrightarrow H_2 + 2Fd_{oxidized}$$

where equilibrium is strongly shifted to the right side. (FeFe)-H_2ase is highly efficient in hydrogen generation, each molecule of the enzyme being able to produce up to 10^4 gas molecules per second at room temperature (Hatchikian et al. 1992).

Unfortunately, the reversible hydrogenase in green algae is highly sensitive to O_2, which irreversibly inactivates the enzyme's activity within minutes (Ghirardi et al. 1997). As a consequence, the direct photoproduction of H_2 from water in algal cultures is difficult to sustain. The sensitivity of hydrogenase to O_2 generated by normal photosynthesis has until now precluded consideration of green algae for possible use in applied H_2-producing systems. Both chemical and mechanical methods have been developed to remove O_2 produced by the photosynthetic activity of the algal cells. These have included the addition of O_2 scavengers (Healey 1970; Randt and Senger 1985), the use of added reductants (Randt and Senger 1985), and the purging the cultures with inert gases (Greenbaum 1982; Gfeller and Gibbs 1984). However, all these methods are expensive upon scale-up and realistically may not be applicable to applied systems.

13.3.2 Indirect Photolysis

The problem of H_2ase sensitivity to oxygen is solved by separating in space or time reactions of water splitting and hydrogen evolution. There are many different ways that indirect biophotolysis is done, but in most strategies, the first step involves growing the photosynthetic organism in large quantities to obtain biomass rich in carbohydrates. The second step varies, but usually involves using biomass in a hydrogen-producing fermentation (Hallenbeck and Benemann 2002; Levin et al. 2004; Zaborsky 1998). The first step fixes CO_2, providing biomass and carbohydrate stores, and the second step produces hydrogen from those stores (Juanita and Wang 2009). During the second stage, the algae are deprived of sulfur thereby inducing anaerobic conditions and stimulating consistent hydrogen production (Melis and Happe 2001). However, this process is not as energetically efficient as direct biophotolysis (Prince and Kheshgi 2005). Gaudernack (1998) explained indirect biophotolysis in four steps process which include: (1) biomass production by photosynthesis, (2) biomass concentration, (3) aerobic dark fermentation yielding 4 mol hydrogen/mol glucose in the algae cell, along with 2 mol of acetates, and (4) conversion of 2 mol of acetates into hydrogen, respectively.

Melis et al. (2000) reported a H_2 production process uses the green alga *Chlamydomonas reinhardtii* in which separation of the photosynthetic O_2 evolution from H_2 production in time is achieved via sulfur deprivation of the cells. Under these conditions, the biosynthesis of the photosynthetic proteins is obstructed and the alga starts to respire the earlier photosynthetically evolved O_2. As O_2 is consumed a whole array of various fermentative pathways may also turn on, including the degradation of the damaged proteins and stored carbohydrates to yield H_2.

13.4 Algae Engineering

Understanding the molecular fundamentals of hydrogen production and utilization in biological systems is a goal of supreme importance for present energy research. Hydrogen was a vital energy source for organisms during the early stages of our planet but under reducing atmospheric conditions (i.e., in the absence of a substantial amount of oxygen), this process gradually lost its importance with the development of a photosynthetic machinery that was able to exploit light energy more efficiently, particularly when Photosystem I (PS I) and Photosystem II (PS II) were combined into one light-triggered photosynthetic electron transport (ET) chain. This photosystem was even capable of oxidizing water in PS II using light, providing plenty of energy. Hydrogen-dependent processes therefore lost their central role as a necessity for the survival of most cells. In consequence, there was no strong evolutionary pressure for the design of oxygen-resistant hydrogenases and the increasing oxygen content of the atmosphere produced by the water-splitting process led to these enzymes being switched off (Horner et al. 2002).

Today, hydrogenases still exist in bacteria and microalgae (i.e., cyanobacteria and unicellular green algae) but their genes are mostly activated under anaerobic conditions and their main function is twofold: either to provide an alternative electron source to aid survival under suboptimal conditions or to capture electrons as a kind of security valve to prevent dangerous over-reduction of the electron transport chain (Appel and Schulz 1998). Among the three principally different types of hydrogenases in nature—[Fe-only]-type, [NiFe]-type and [Fe-S-cluster-free]-type (Lyon et al. 2004) hydrogenase belongs to the [Fe-only]-type are usually the simplest hydrogenases (only one subunit in the case of *Chlamydomonas*) and the most active hydrogenases known (up to 2000 H_2S_1) present in green alga (Happe et al. 2002; Girbal et al. 2005).

Hydrogenase, the unique class of enzymes, catalyzes the formation and decomposition of the simplest molecule occurring in biology: H_2. The seemingly uncomplicated task is solved by sophisticated macromolecular machinery. Hydrogenases are metalloenzymes harboring Ni and Fe, or only Fe atoms, arranged in unique structure in their active center. Redox metalloenzymes in general are extremely sensitive to inactivation by oxygen, high temperature, CO, CN, and various additional environmental factors. Oxygen inactivation is thought to occur by the direct interaction of O_2 with the [2Fe-2S] center on the catalytic H-cluster (Adams 1990; Chen et al. 2002). The optimization of H_2 photoproduction requires identification of an O_2-tolerant H_2ase as the active site metal cluster of HydA is O_2 labile (Beer et al. 2009).

Recent advances with respect to the identification of genes involved in [FeFe] H_2ase (Posewitz et al. 2004; Girbal et al. 2005; McGlynn et al. 2008) and [NiFe] H_2ase (Schubert et al. 2007; Ludwig et al. 2009) maturation and regulation, and the development of heterologous expression systems for both classes of enzyme (Posewitz et al. 2004; Girbal et al. 2005; Lenz et al. 2005; King et al. 2006; Sybirna et al. 2008), make it feasible to biochemically characterize H_2ases by heterologous expression in organisms that do not possess endogenous H_2ase machinery. Target genes identified through bioprospecting efforts can therefore be characterized genetically and biochemically using heterologous expression studies. Moreover, the natural diversity of genes encoding other key enzymes with relevance to bioenergy can be examined, and exploited using similar approaches (Beer et al. 2009).

[Fe]-hydrogenase genes (termed HydA) were identified in different green algae (Florin et al. 2001; Wunschiers et al. 2001; Winkler et al. 2002, 2004; Forestier et al. 2003). The HydA coding sequence showed a typical mosaic structure of a nuclear-encoded gene with seven introns and eight exons. Hydrogenase genes have also been cloned and reported in the literature from the unicellular green algae *S. obliquus* (Florin et al. 2001; Wunschiers et al. 2001), *Chlorella fusca* (Winkler et al. 2002) and *Chlamydomonas moewusii* (Winkler et al. 2004). In *S. obliquus*, the genomic DNA of the HydA gene contains five introns and six exons, encoding a protein with a molecular mass of 44.5 kDa (Florin et al. 2001).

All [Fe]-hydrogenases incorporate a [2Fe-2S] cluster bridged by a cysteine residue to a [4Fe-4S] cluster at the catalytic site (the H-cluster), and have unusual ligands such as CO, CN, and di(thiol)methylamine (Fan and Hall 2001; Nicolet et al. 2001). Three other conserved cysteine residues bind the H-cluster to the protein matrix. Most of these enzymes also contain additional iron–sulfur centers that act as electron relays to and from donor and acceptor carriers (Peters 1999; Nicolet et al. 2000). However, these cofactors are absent from the green algal enzymes (Florin et al. 2001; Forestier et al. 2001; Wünschiers et al. 2001; Happe and Kaminski 2002; Winkler et al. 2002).

Recently, there has been considerable progress in identifying relevant bioenergy genes and pathways in microalgae, and powerful genetic techniques have been developed to engineer some strains via the targeted disruption of endogenous genes and/or transgene expression. Collectively, the progress that has been realized in these areas is rapidly advancing our ability to genetically optimize the production of targeted biofuels (reviewed in Beer et al. 2009). Some studies indicated that mutagenesis can be used to decrease the O_2 sensitivity of the hydrogenase and thus eventually lead to a system that produces H_2 under aerobic conditions (McTavish et al. 1995; Ghirardi et al. 1997; Seibert et al. 2001; Flynn et al. 1999, 2002). One of the most significant advances in algal genetics is the development of improved gene silencing strategies in *C. reinhardtii*. High-throughput artificial miRNA (amiRNA) techniques for gene knockdown, which are highly specific and stable, were recently reported (Molnar et al. 2009; Zhao et al. 2009).

13.5 Sustainability

Sustainable development, although a widely used phrase and idea, has many different meanings and therefore provokes many different responses. In broad terms, the concept of sustainable development is an attempt to combine growing concerns about a range of environmental issues with socio-economic issues. The sustainable development implies smooth transition to more effective technologies from a point view of an environmental impact and energy efficiency (Dincer 2008). New hydrogen powered fuel cell technologies in both its high and low-temperature derivatives are more effective and cleaner than conventional energy technologies, and can be considered one of the pillars of a future sustainable energy system (Kwak et al. 2004). Biological hydrogen production presents a possible avenue for the large-scale sustainable generation of hydrogen needed to fuel a future hydrogen economy.

Hydrogen is an ideal, clean, and potentially sustainable energy carrier for the future due to its abundance and non-polluting nature. Numerous bacteria, cyanobacteria, and algae are capable of producing hydrogen from water, solar energy, and a variety of organic substrates. H_2 has been identified as one of the most promising fuels for the future (Abraham 2002; EU Commission 2002; Koizumi 2002). The US, European Union, and Japan have already embarked on establishing

H_2 fuel stations, and in parallel, car manufacturers have invested extensively in the development of H_2 fuel cell-powered cars (Hankamer et al. 2007).

The energy content of hydrogen has the highest for per weight of any known fuel, and can be produced by various means (Hallenbeck and Benemann 2002; Levin et al. 2004). A by-product of hydrogen, when used either in combustion or as fuel cell, is only pure water, making it an attractive non-polluting energy carrier (Oh et al. 2003; Lin and Lay 2004).

13.6 Life Cycle Assessment

LCA is a tool to assess the environmental impacts and resources used throughout a product's life cycle and consider all attributes or aspects of the natural environment, human health, and resources (Singh et al. 2010c; Korres et al. 2010). Rodríguez et al. (2011) have concluded in an LCA study that an LCA-based indicator might be an effective tool to compare alternative energy routes in terms of environmental impact and indirect natural resource costs toward different services and commodities. The overall energy balance of using hydrogen as vehicle fuel does indeed seem to be less beneficial than gasoline. But being the only non-carbon fuel it may still make sense to produce hydrogen with algae if some of the obstacles are solved. Investigations of the environmental benefits and impacts in a life cycle perspective are scarce. Only a few LCA-studies has been performed specifically on biohydrogen production.

LCA allows the possibility to compare different biohydrogen production approaches and identify the environmental "hot spots" of the whole process. Inventory analysis and the results of different researchers in this field permit to find values of selected ecoindicators in order to evaluate the biohydrogen production efficiency with the selection of the best initial data for life cycle analysis. These ecoindicators weigh the resources needed for biohydrogen production system (Romagnoli et al. 2011).

Romagnoli et al. (2011) conducted an LCA study to identify and evaluate the life cycle environmental and human health impacts of the entire supply chain associated with the production of biohydrogen from photosynthetic process technology. In this study, six biohydrogen production methods were analyzed, i.e.,

1. Cycling photobiological hydrogen production from *C. reinhardtii* with forced sulfur deprivation.
2. Oxygenic photosynthesis coupled to H_2 production by hydrogenase enzyme in green algae.
3. Oxygenic photosynthesis coupled to H_2 production by nitrogenase enzyme in cyanobacteria.
4. Nonoxygenic photosynthesis coupled to H_2 production by nitrogenase-catalyzed enzyme in cyanobacteria.
5. Indirect microalgal biophotolysis (*C. Reinhardtii*).
6. Fermentation (2 stage bioreactor).

The results of this study indicated the need for further improvements in order to reach reasonable industrial biohydrogen production. Romagnoli and co-workers concluded that using biohydrogen to produce electricity offers more environmental benefits than using a fossil fuel-based source and they claimed positive results in terms of climate change and human health categories. The LCA studies can further be used as a tool for identification of the main environmental improvements in technology development, policy decisions, and as an additional strength in the growing hydrogen market. The LCA of algal biohydrogen is very important before taking them into consideration for commercial scale production and making a policy for that purpose.

13.7 Technical Barriers

According to Edwards et al. (2006) the following are the possible scientific and technical challenges associated with the hydrogen economy:

- Lowering the cost of hydrogen production to a level comparable to the energy cost of fossil fuel.
- Development of a CO_2-free route for the mass production of sustainable hydrogen at a competitive cost.
- Development of a safe and efficient national infrastructure for hydrogen delivery and distribution.
- Development of viable hydrogen storage systems for both vehicular and stationary applications.
- Dramatic reduction in costs and significant improvement in the durability of fuel cell systems.

13.8 Future Perspective

Vincent Rosner highlighted the following points for future development in algal biohydrogen production to make it a sustainable energy source during the presentation of the 19th World Hydrogen Energy Conference (Rosner 2012).

- Development of design cells with increased production rate (>100x);
- Design of a commercial production plant;
- Implementation of biomass utilization;
- Energetic improvement of process components;
- Involvement of illumination over sunlight;
- Assessment and benchmark.

The process of hydrogen production by green algae has practical implications because it pertains to the question of energy supply and demand for the entire

world. The algal hydrogen production promises to positively alter the equation on energy supply and demand, alleviate global warming, and mitigate environmental pollution, as hydrogen is generated from water since water is the only by-product of hydrogen combustion. The manifestation of photosynthetic hydrogen production from algae will bring about technological developments in many directions and will find many applications (Melis and Happe 2004). Algal biohydrogen is currently more expensive in comparison to fossil fuel, so it is likely to play a major role in the economy in the long run, if technological development succeeds in bringing down costs. The algal biohydrogen production may be a potential answer to overcome some of the economic constraints to fulfill many of the energy needs.

13.9 Conclusion

Bioproduction of H_2 is considered as the ultimate cleanest energy carrier to be generated from renewable sources. Most of the algae and cynobacteria are able to photoproduce H_2 with the help of water and CO_2 as raw materials using a unique class of enzyme hydrogenase. Although a number of advances have been made recently, the practical development of biohydrogen production is a long-term prospect, commensurate with the time frame required to adopt hydrogen as a major fuel source. There are a number of technical challenges in each area that must be overcome before these technologies can be adopted on a practical large scale. Extensive R&D in this area is underway worldwide. An LCA will help in accessing the sustainability of algal biohydrogen production and adopting the appropriate policies for that.

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Chapter 14
Microbial Fuel Cells for Sustainable Bioenergy Generation: Principles and Perspective Applications

S. Venkata Mohan, S. Srikanth, G. Velvizhi and M. Lenin Babu

Abstract The energy gain in microbes is driven by oxidizing an electron donor and reducing an electron acceptor. Variation in the electron acceptor conditions creates a feasibility to harness energy. In order to support the microbial respiration, electrons will transfer to the exocellular medium toward the available electron acceptor, especially in the absence of oxygen. The microbes can use a wide range of electron acceptors such as metals, nutrients, minerals, etc., including solid electrodes. When the microbes use the solid electrode as electron acceptors, the setup is called microbial fuel cell (MFC) and the electrons can be harvested and used for different applications. MFC can be defined as a microbially catalyzed electrochemical system which can facilitate the direct conversion of substrate to electricity through a cascade of redox reactions, especially in the absence of oxygen. Linking the microbial metabolism to anode and then transmitting the electrons to cathode generates a net electrical power from the degradation of available electron donor. This concept of MFC operation has expanded considerable interest in the recent research due to its application in the energy recovery from wastewater. Microbes in MFC can also use variety of organic or inorganic electron donors as well as acceptors to produce a surfeit of desirable biofuels or biochemicals which is termed as microbial electrosynthesis. Apart from the electrogensis, the applications of MFC are widespread in different fields including waste/wastewater remediation, toxic pollutants/xenobiotics removal, recovery of commercially viable products, sequestration of CO_2, harvesting the energy stored in marine sediments, desalination, etc. In this chapter, an attempt was made to bring out all the existing applications of MFC into one platform to make a comprehensive understanding on the inherent potential of microbial metabolism, when the designated electron acceptor is present.

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14.1 Introduction

Increasing global energy demand and depleting fossil fuels has put forward the necessity for searching alternative fuels. Microbial fuel cell (MFC) is one such alternative route where the microbial membrane potentials will be utilized for harnessing power (bioelectricity) by introducing the artificial electron accepting conditions. MFC can be defined as a biologically catalyzed electrochemical (bioelectrochemical) system which can facilitate direct conversion of chemical energy (from substrate/fuel) to electrical energy through a cascade of redox reactions in the absence of oxygen (Kjeldsen et al. 2002; Logan 2008, 2010; Franks and Nevin 2010; Venkata Mohan and Srikanth 2011; Venkata Mohan 2012). The microbial respiration (reduction) separated from fermentation (oxidation) by ion selective membrane [mostly proton exchange membrane (PEM)] in a defined fuel cell introduced with the artificial electron acceptor/carriers (electrodes) can harness bioenergy in the form of bioelectricity. MFC application for bioelectricity generation from various substrates including waste/wastewater has gained prominence in recent bioenergy scenario due to its sustainable nature. Apart from power generation, application of MFC can also be extended to the production of other commercially viable reduced end products such as organic acids, aldehydes, alcohols, etc. (Logan 2010; Rabaey and Rozendal 2010). On the other hand, removal of various toxic pollutants and remediation of waste is one of the areas, where much of the MFC application is being observed more recently (Venkata Mohan et al. 2009a; Mohanakrishna et al. 2010a). Considering these toxic pollutants and waste/wastewater as electron donor or acceptor, in the MFC will pave a path to overwhelm the existing difficulties in treating them along with the generation of sustainable energy. A paradigm shift from the waste remediation to the energy generation is gaining importance in the recent scenario of bioenergy research. MFC also plays a major role in carbon cycle by reducing carbon dioxide (CO_2) to various viable fuel alternatives viz., ethanol, butanol, etc., in its terminal reduction reactions along with the power generation, contributing its part in reducing the carbon footprints on the Earth (Rabaey and Rozendal 2010). This chapter cohesively outlines and describes the basic principles associated with MFC operation with particular emphasis to sustainable power generation along with its other potential applications.

14.2 Principles of MFC

14.2.1 Mechanism of Power Generation

Microbes in nature carry their metabolic activities (both anabolism and catabolism) either in presence of oxygen (aerobic) or in its absence (anaerobic). Irrespective of the nature of metabolism, all the microbes will involve in the

utilization of available substrate (fermentation) generating the reducing equivalents [protons (H^+) and electrons (e^-)] in the form of redox carriers (NAD^+, FAD^+, FMN^+, etc.) which will help in generating the energy [adenosine triphosphate (ATP)] during respiration (Kim and Gadd 2008; Nelson and Cox 2008). Glycolysis is O_2 sensitive and hence it is common in both the aerobic and anaerobic metabolisms which convert the six-carbon glucose into two molecules of three-carbon pyruvate. Aerobic metabolism further proceeds with the tricarboxylic acid (TCA) cycle and oxidative phosphorylation generating CO_2 and H_2O, whereas anaerobic metabolism continues with the interconversion reactions generating CH_4, CO_2, and H_2O. Both the processes generate the reducing equivalents attached with the redox carriers which are high-energy molecules. These reducing equivalents generated during fermentation will always move through a series of redox components toward an available terminal electron acceptor (TEA) generating a proton motive force that helps in generating energy-rich phosphate bonds ATP which is useful for the microbial growth and subsequent metabolic activities. However, the function of TEA is based on the thermodynamic hierarchy of the electron acceptors available in the system (Kim and Gadd 2008). In the presence of O_2, due to maximum reduction potential in the biological system and strong electronegative nature, the reducing equivalents will pass through a redox cascade of respiratory/electron-transport chain (ETC) toward O_2 where ATP is generated through oxidative phosphorylation (Kim and Gadd 2008; Nelson and Cox 2008). In the absence of O_2, other electron accepting molecules available in the system will drive the electron flow through the redox cascade. However, the electron flow slows down due to the thermodynamic feasibility of the reaction and the reducing equivalents enter a series of interconversion reactions resulting in the formation of energy rich reduced end products. Due to the less positive redox potentials of oxidants, the energy gain from the anaerobic metabolism is considerably low compared to the aerobic metabolism for the microbe (Schroder 2007; Nelson and Cox 2008). However, during anaerobic metabolism, there is a feasibility of harnessing the electrons available in the system in various forms of bioenergy or valuable bioproducts. MFC function mainly based on harnessing these available electrons by artificially introduced electrodes as intermediary electron acceptors (Fig. 14.1).

14.2.2 Electron Discharge from the Biocatalyst

Microbes with high electron discharge capability are considered to be electrochemically active and are crucial in the MFC operation. Electron transfer from the biocatalyst metabolic activities to the anode (intermediary electron acceptor) is catalyzed by two mechanisms, viz., direct electron transfer (DET) and mediated electron transfer (MET), based on the electron carrier involved (Fig. 14.2). DET is possible by means of physical contact of the bacterial cell with the anode and without the involvement of any redox species or mediator. Some of the membrane

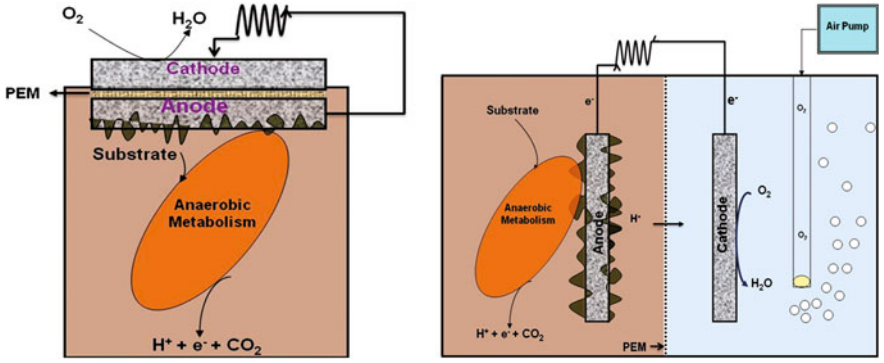

Fig. 14.1 Schematic representation of single (*air cathode*) and dual (*aerated cathode*) chambered MFC depicting the anodic oxidation and cathodic reduction reactions

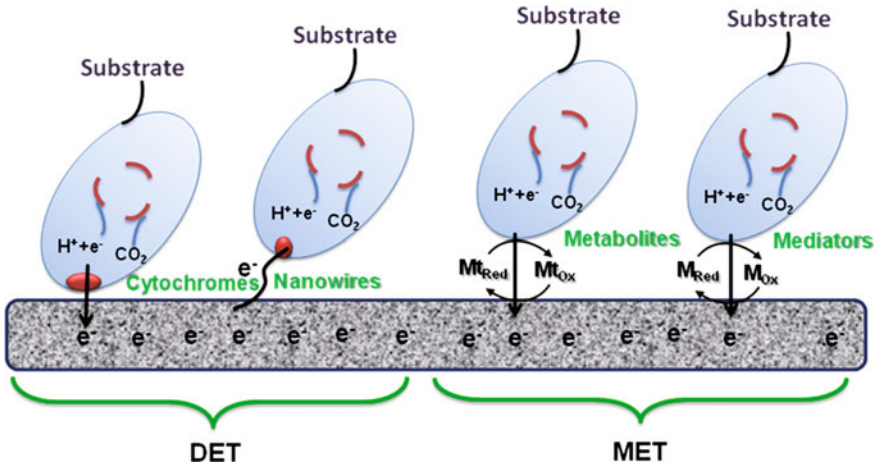

Fig. 14.2 Schematic representation of electron transfer mechanism from microbial metabolism to the anode

bound cell organelles viz., cytochromes, nanowires, etc., will help in the electron transfer from outer membrane of the bacterial cell to the external electron acceptor (anode). *Geobacter*, *Rhodoferax*, and *Shewanella* are the most-studied bacterial species for their exocellular electron transfer through membrane bound organelles (Kim et al. 1999; Chaudhuri and Lovley 2003; Holmes et al. 2004; Lovley 2006; Chang et al. 2006; Schroder 2007).

The name MET itself indicates the involvement of mediators for the exocellular electron transfer from the biocatalyst to the anode. The mediators may be artificially added or naturally excreted soluble shuttlers or primary and secondary metabolites from bacterial metabolism (Schroder 2007). Both the natural and

artificial mediators are available for the electron transfer in a wide range, viz., inorganic compounds such as iron or magnesium containing metal complexes, organic compounds such as quinine and phenazine derivatives.

Microbes grown under electron acceptor depleted conditions and distant from the anode will also tend to release low molecular weight electron shuttling compounds through secondary metabolic pathways, e.g., pyocyanine and 2-amino-3-carboxy-1, 4-naphthoquinone (ACNQ), phenazine-1-carboxamide, etc., (Newman and Kolter 2000; Hernandez and Newman 2001; Newman 2001). *Pseudomonas* group of microbes secretes pyocyanin and pyovirdin like colored compounds for the electron shuttling. Phenazines, phenoxazines, quinines, etc., are the natural mediators investigated in the literature for their suitability as mediators (Bennetto et al. 1983; Roller et al. 1984; Bennetto 1990; Park and Zeikus 1999; Newman and Kolter 2000; Park and Zeikus 2000; Rabaey et al. 2004; Schroder 2007).

14.2.3 Fuel Cell Behavior and Half Cell Potentials

The change in fuel cell behavior under varying external resistances (polarization) helps to understand the performance of fuel cell in terms of maximum power density, cell design point, half cell potentials, etc. (Logan et al. 2006; Venkata Mohan et al. 2008a). Polarization is a powerful tool for the analysis and characterization of fuel cells. Polarization curve is plotted by considering the change in current density over cell voltage and power density with the function of external resistance. Decrease in resistance will increase the electron flow through the circuit generating higher currents which lowers the potential difference between anode and cathode. However for higher power output, both the current and voltage should be high because power is the product of both. The resistance at which both the current and voltage are optimum resulting in higher power is called cell design point beyond which the fuel cell can be operated effectively. Operation of MFC below the cell design point will cause instability because of higher currents and lower voltages (Venkata Mohan et al. 2008b). Change in half cell potentials for anode and cathode over varying external load is also crucial to assess the start-up of electron discharge from the biocatalyst to the anode, reaction rates of cathodic reduction, interaction between cathode and TEA, etc. (Srikanth et al. 2010a).

Voltage curve of polarization helps in depicting the internal losses of the system that hamper the electron flow from biocatalyst to the anode. An S-shaped voltage curve will be obtained for an ideal MFC where the initial part depicts the activation overpotentials, middle part depicts ohmic losses, and the terminal part depicts concentration losses (Fig. 14.3). Certain amount of energy is required to carry out any biological reaction which is considered as activation energy. All the reactants must cross an activation energy barrier to form as products. Similarly, oxidation at the anode or reduction at the bacterial surface or interior requires certain activation energy which incurs potential loss accounting for the activation overpotential (Larminie and Dicks 2000; Velvizhi and Venkata Mohan 2012).

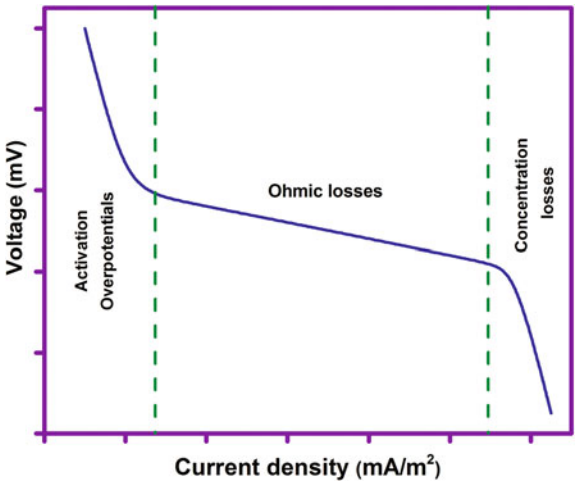

Fig. 14.3 Voltage curve of polarization against current density with the function of varying external resistance depicting the possible internal losses during the electron transfer from microbe to the anode

Activation overpotentials are important in the lower current density zones (below 1 mA/cm^2) and hence they are considered as the most important parameter/factor during MFC operation (Rabeay et al. 2005; Velvizhi et al. 2012). These overpotentials can be overcome by increasing the operation temperature, anode surface area, concentration of redox shuttlers, etc.

Ohmic losses are caused by the electrical resistances of the electrodes, solution–electrode interface, and the electrolyte–membrane interface. Controlling the ohmic losses is important to harness higher power densities because these losses occur at the middle zone where the optimum voltage and current are generated. These losses can be overwhelmed by increasing the electrical conductivity of the electrolyte or by using highly conductive electrode materials. Usage of noble metal electrodes (such as platinum, titanium, etc.) increases the economics of MFC operation. However, considering waste as anodic fuel increases the electrolyte conductivity and manages the economics (Rabeay et al. 2005; Velvizhi et al. 2012). Concentration losses occur due to the large oxidative force of the anode, where the electron donor is being oxidized at faster rate releasing more number of reducing equivalents than they can be transported to the anode surface and then to the cathode. However, this is important at higher current densities where the MFC becomes unstable and hence, it is not necessary to consider these losses in MFC operation. Only in the case of thick nonconductive biofilm formation on anode, hampering of electron transfer occurs to the anode surface, where the concentration polarization will be an obstruction (Rabeay et al. 2005; Velvizhi et al. 2012). However, electrochemically active MFC with higher current outputs will prefer the formation of thin and open biofilm, and hence it is not a significant problem. Apart from the above-mentioned losses, the electrons will also be lost during the competitive metabolic activities which require similar precursor compound, termed as electron quenching (Sect. 14.3.2.1).

14.3 Applications of MFC

The reducing equivalents generated during MFC operation have multiple applications in the energy generation as well as waste remediation areas. Broadly MFC application can be classified as a power generator, wastewater treatment unit, and system for the recovery of value-added products. Reducing equivalents generated from substrate metabolism gets reduced in presence of an electron acceptor at physically distinct component of MFC (cathode) and results in power generation. Alternatively, when the waste/wastewater functions as an electron donor or acceptor, its remediation gets manifested either through anodic oxidation or cathodic reduction under defined conditions. Similarly, when some of the oxidized metabolites act as electron acceptors during MFC operation and forms as reduced end products having commercial importance. Apart from these three, several other diverse applications are reported for MFC based on their configuration and mode of operation which also will fall in either or all of these three categories.

14.3.1 Power Generator

Protons and electrons are generated during microbially catalyzed oxidation at anode and the protons will migrate to the cathode through PEM creating a potential difference between the anode and cathode against which the electrons will flow through the circuit where they get reduced in presence of TEA generating power. Microbially catalyzed oxidation of substrate takes place at anode Eq. (14.1) generating the reducing equivalents, while their reduction takes place at cathode Eq. (14.2). Overall reaction occurring with MFC is represented by Eq. (14.3).

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \text{ (Anode)} \tag{14.1}$$

$$4e^- + 4H^+ + O_2 \rightarrow 2H_2O \text{(Cathode)} \tag{14.2}$$

$$C_6H_{12}O_6 + 6H_2O + 6O_2 \rightarrow 6CO_2 + 12H_2O \text{(Overall)} \tag{14.3}$$

The PEM introduced between the fermentation (anode) and respiration (cathode) will mimic the function of external membrane generating a potential gradient, while the electrodes will act as redox components of the cell assisting in the electron flow toward TEA. Electron transfer from its source (metabolism) to the sink TEA will be driven by the potential difference between the redox components of the microbe and fuel cell (Fig. 14.4). The membrane potential across the cascade of membrane components is called proton motive force due to which the reducing equivalents will reach the intermembrane space. The electrons will reach anode creating a negative anodic potential, while the protons will go to the cathode creating a positive potential. The difference between positive cathodic and negative anodic potentials is considered as cell voltage which drives the electron flow

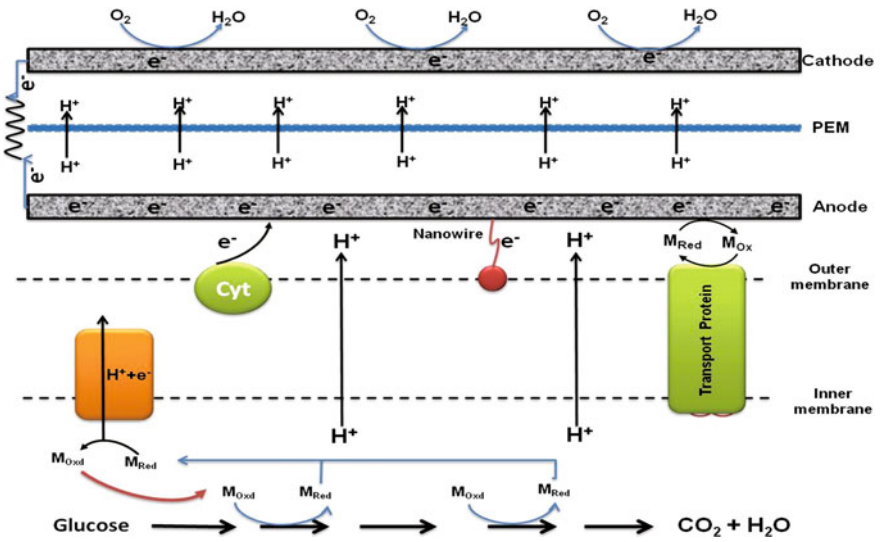

Fig. 14.4 Schematic representation of reducing equivalents generation and their transfer to TEA including the principle and mechanism of electron mobility from its source to sink [M_{Ox} oxidized mediator; M_{Red} reduced mediator]

from anode to cathode [electron motive force (*emf*)]. Overall, the electron transfer from its source to sink is purely based on the differences in the redox potentials of the components of the fuel cell irrespective of their nature (biological or chemical or physical). MFC function as power generator was well established by using a wide range of substrates as electron donors and acceptors in the anode and cathode chambers, respectively. However, electrogenic activity of MFC is governed by several physical, biological, and operational factors.

14.3.1.1 Physical Factors

MFC depends on the physical components including fuel cell configuration, anolyte volume, electrode materials, membrane, etc., which are to be considered crucial in regulating the power generation. Basically MFC configurations can be classified into two types based on the physical separation of fermentation (anode) and respiration (cathode) viz., dual and single chambers (Fig. 14.1). In the dual chamber, both the anode and cathode are immersed in liquid medium supporting the electron donor and acceptor conditions, respectively. While in the single chamber configuration, only anode is immersed in the liquid medium (anolyte) supporting the electron donor conditions and the cathode was exposed to ambient air. Initially, MFC operation was mostly with double chamber configuration (Venkata Mohan et al. 2007a, 2008a, b, c; Zhang et al. 2009a) and later shifted to single chamber operation (Venkata Mohan et al. 2008d, 2009b) to reduce the

construction costs and increase the ease of operation. The electrogenic efficiency of MFC will vary based on the electron acceptor conditions. Oxygen (O_2) is considered to be the best-known electron acceptor in the biological redox system. Apart from O_2, Fe^{3+} (potassium ferricyanide), and Mn^{2+} (potassium permanganate) are most-studied electron acceptors in dual chamber MFC as catholytes (You et al. 2006; Venkata Mohan et al. 2008b, c, e). Fe^{3+} depicted higher power generation over other electron acceptors which might be due to its strong oxidizing nature and greater mass transfer rate along with lower activation energy required for the cathodic reaction. The usage of metals as electron acceptors has a drawback of replenishment metal ions after exhaustion and their discharge in the environment also toxic. Hence, it is suggested to use O_2 (aerated/open air cathode) as electron acceptor in MFC. The performance of single chamber is relatively low compared to double chamber in terms of electrogenic activity due to the inherent limitations in the cathodic reduction reactions. However, single chamber configuration is similar to the existing conventional wastewater treatment units, and hence can be easily implemented for the full-scale integrated wastewater treatment (Venkata Mohan et al. 2008a). Apart from these basic configurations, U-tube MFC, serpentine MFC, and few other configurations were also studied. Irrespective of the configuration, MFC performance was independent of reactor volume because the possible theoretical potential in MFC is around 1.2 V [NAD^+ (-0.32 V) and (O_2 +0.816 V) are available higher electron donor and acceptors in the biological system]. Henceforth, stacks of smaller MFCs connected in series will result in a cumulated voltage that can be used for real-time applications (Venkata Mohan et al. 2011b).

The nature of solid electron acceptors (electrodes) used in MFC can also influence the power generation efficiency due to its role as intermediary electron transferring agent from the source to sink. The growth of electrochemically active microbes also depends on the anode properties which can effectively accept the electrons from the microbial metabolism (Cheng et al. 2006). The materials used in MFC as electrode should have characteristics of being electrically conductive, biocompatible, and chemically stable in the reactor solution, efficient electron discharge and should sustain its properties with time. The other characteristics viz., high porosity, nonfouling nature, geometrical appearance, easily accessible, and should fit for large scale (Chaudhuri and Lovley 2003; Srikanth et al. 2011a), which help in the increased performance. Carbon based materials viz., graphite, graphene, and carbon were most widely used materials as anode in various forms such as cloth, brush, plate, etc. Graphite in its native form and modified with nanomaterials and other catalysts has functioned as good anode material in many of MFC studies but it has its limitations for large-scale applications (Srikanth et al. 2011a; Mohanakrishna et al. 2012). The synergistic interaction of the microbes with different anode materials was also evaluated to understand the change in electron discharge and microbial growth patterns of MFC (Srikanth et al. 2011a). Apart from carbon-based materials, application of platinum, titanium, vanadium, nickel, stainless steel, aluminum, brass, and copper were also studied among which nickel and stainless steel can be effectively used in the economic point of view.

Ion exchange membrane used to separate both the chambers is also an important physical factor effecting MFC performance. Initially MFC studies were carried out with salt bridge between anode and cathode for the exchange of ions. In the later stages, PEM was used and extensively studied for its function in exchanging the protons specifically to the cathode chamber (Venkata Mohan et al. 2008b, c, d, e, f). PEM is a sulfonated tetrafluorethylene copolymer, consisting of a hydrophobic fluorocarbon backbone (–CF2–CF2–) to which hydrophilic sulfonate groups (SO^{3-}) are attached. The presence of negatively charged sulfonate groups in the membrane explains the high level of proton conductivity of PEM (Rozendal et al. 2006). Apart from PEM, different other ion exchange membranes viz., glass wool, cation and anion exchange membranes, were also studied in MFC (Venkata Mohan et al. 2008a; Pandit et al. 2012). PEM depicted higher potential development over other membranes due to the selective proton exchange which avoids the interference of other reactions in MFC causing losses. However, the usage of PEM increases the cost of MFC construction and decreases its application feasibility in the wastewater treatment.

14.3.1.2 Biological Factors

Apart from physical factors, nature of microbes used along with their growth and synergistic interaction with the electrode, mechanism of electron transfer from the microbes and the role of electron shuttlers, etc., will also govern the power generation in MFC. Microbes used in the anodic oxidation play a major role in the MFC performance. The extracellular electron transfer rate is influenced by the potential difference between the final electron carrier and the anode, irrespective of the mechanism (Newman and Kolter 2000; Marsili et al. 2008; Lee et al. 2009). The cell compartmentalization and the more complicated architecture of cell respiratory chains give an advantage of harnessing the energy from biocatalyst. Initial studies of MFC were carried out with pure cultures and later the research was shifted toward the application of mixed consortia as biocatalyst in MFC where the synergistic association among the individual strains will result in higher power output. Most of the reported electrochemically active microbes are facultative and metal reducing in nature. Metal reducing species have the special ability to act as self-mediators, transferring electrons to the anode through physical contact. These organisms have an outer membrane cytochrome oxidase type *c* protein that allows transfer of electrons from the interior of the bacteria cell membrane to the exterior of the cell membrane. Some species will be able to discharge electrons using electron shuttlers (either naturally secreted or artificially added) to the anode (Sect. 14.2.2). Most widely studied strains include *Geobacter sulfurreducens* (Dumas et al. 2007), *Shewanella haliotis* ATCC 49138 (Raghavulu et al. 2012), *Pseudomonas aeruginosa* (Raghavulu et al. 2011a), *Rhodoferax ferrireducens* (Chaudhuri and Lovley 2003), *Desulfuromonas acetoxidans* (Bond et al. 2002), *Klebsiella pneumonia* (Zhang et al. 2008), etc. *Shewanella, Geobacter* and *Pseudomonas* are the most widely studied strains in MFC for their electrochemical activity and

Table 14.1 Table representing MFC operation with mixed culture as biocatalyst

Source of the inoculums	Electron donor	MFC configuration	Power (mW)	Reference
Anaerobic mixed consortia	Designed synthetic wastewater with glucose	Dual chamber	0.086	Venkata Mohan et al. (2008c)
Anaerobic mixed consortia	Composite chemical wastewater	Dual chamber	0.421	Venkata Mohan et al. (2009a)
Mixture of aerobic and anaerobic sludge	Azo dye with glucose	Single chamber	0.09	Sun et al. (2009)
Anaerobic sludge from wastewater plan	Ethanol	Dual chamber	0.025	Kim et al. (2007)
Mixed bacterial culture	Glucuronic acid	Single chamber	1.18	Catal et al. (2008)
Leachate and sludge	Landfill leachate	Dual chamber	0.0004	Greenman et al. (2009)
Rumen microorganism from rumen of a cow	Cellulose	Dual chamber	0.02	Yazdi et al. (2007)
Mixed aerobic activated sludge and anaerobic sludge	Phenol	Dual chamber	0.1	Luo et al. (2010)
Mixed bacterial culture	Xylose	Single chamber	0.74	Catal et al. (2008)
Anaerobic sludge	Designed synthetic wastewater with glucose and glutamate	U-shape	0.02	Zuo et al. (2008)

electron transfer mechanisms. *Saccharomyces cerevisiae* (Yeast), a lower eukaryote can also be considered as a potential biocatalyst in MFC (Raghavulu et al. 2011b). However, the sort of flexibility with mixed culture seems beneficial for MFC application and provides a definite advantage toward power generation, especially when waste is considered as electron donor. The syntrophic association of microbes present in mixed culture can be considered as mixotrophy as the mutual interactions among their metabolic activities will have an added advantage of harnessing the electrons from the multiple components of waste. The electron flux through a series of metabolic reactions will result in a chance of harnessing them as bioelectricity. Several studies have been reported on the application of mixed culture in MFC with diverse wastewater (Table 14.1). Biofilm formation on the surface of the anode will significantly influence the current generation in MFC irrespective of microbe nature (Bond et al. 2002; Logan and Regan 2006). Understanding the formation of biofilm and its function in MFC is also important as it correlates with the amount of active biomass that can potentially use the electrode as the electron acceptor. Thick biofilm coverage on anode will have negative impact on power generation because the external layers of biofilm experience difficulty in discharging the electrons onto the anode. Hence, the anode of MFC always prefers thin and open biofilms unlike conventional biological systems to avoid the obstruction of electron flow (Wang et al. 2009). However, the increasing biofilm growth on anode showed increased MFC performance (Venkata Mohan et al. 2008d). Selective enrichment of electrochemically active biofilm on

the anode surface might be the reason behind this which may be thin but enriched with the electroactive strains.

14.3.1.3 Operational Factors

MFC performance is also regulated by the operational factors viz., nature of electron donor, organic load, retention time, redox condition (pH), microenvironment, etc. MFC can use a diverse range of substrates (anolyte fuel) as electron donors in the anodic oxidation to generate the reducing equivalents. Among the simple substrates, glucose and acetate are the most widely used electron donors (Table 14.2). Diverse range of waste/wastewater can also be used as anodic fuel which is an asset to the existing waste remediation approaches. Simple domestic sewage to the complex industrial wastewater has been studied as anodic fuel in MFC (Venkata Mohan et al. 2011a; Srikanth and Venkata Mohan 2012a, b; Behera et al. 2010). However, the power generation of MFC depends on the characteristics and biodegradability of the waste used (Sect. 14.3.2.1). Further, the power generation in MFC depends on the organic load of wastewater. Highly biodegradable waste can be loaded at higher loading rates, while low biodegradable waste will interfere with the metabolism of microbes at higher loading rates. The retention time of the MFC also varies based on the loading of anolyte. Higher loadings can continue the generation of reducing equivalents for longer periods, while lower loading rates can retain the electrogenesis for less time (Reddy et al. 2010; Velvizhi and Venkata Mohan 2011; Jadhav and Ghangrekar 2009).

Neutral pH is the optimum condition for many bacterial growth and metabolic activities. Most of the enzymes function near neutral pH and the biomolecules of the cell become unstable at extreme pH. Hence, the internal pH of microbe is maintained at neutral redox microenvironment irrespective of the external pH (Kim and Gadd 2008). However, the external pH can bring alterations in the microbial activities including synthesis of biomolecules and ion transport across membrane. MFC performance also depends on the redox conditions of the fermentation (anolyte). External acidophilic pH is defended by the excessive production of acid shock proteins that consume the H^+ entered the cell and bring the

Table 14.2 Simple substrates used as electron donors in single chambered MFC

Simple substrate	Power density (mW/m^2)	Conversion efficiency (%)	Reference
Glucose	401	10	Sharma and Baikun (2010)
Acetate	368	16	Sharma and Baikun (2010)
Starch	242	21	Min and Logan (2004)
Butyrate	305	7.8	Liu et al. (2005a)
Dextran	150	17	Min and Logan (2004)
Peptone	269	6	Heilmann and Logan (2006)
Ethanol	302	–	Kim et al. (2007)

internal pH to near neutral. On the contrary, the extreme alkalophilic pH can be defended by the function of Na^+/H^+ antiport pump which converts the proton motive force to the sodium motive force (Kim and Gadd 2008). The exocellular electron transfer to the anode is dependent on the operating pH which creates a proton gradient between cell interior and surrounding environment. Higher performance was reported at acidophilic pH over neutral and basic pH in diverse configurations of MFC (Raghavulu et al. 2009a, b; Jadhav and Ghangrekar 2009). The electron transfer is independent of external pH when the circuit is connected against resistance. The closed circuit creates a strong proton motive force on anode due to the continuous reduction of protons with the electron acceptor in the terminal reaction. Moreover, the metabolic activities of biocatalyst will be higher and waste remediation also favors neutral pH.

Most of the MFC research was confined to the operation of anodic chamber under anaerobic microenvironment but few reports are also available on the application of aerobic metabolic function at anode (Ringeisen et al. 2007; Rodrigo et al. 2007; Venkata Mohan et al. 2008f). If a low level of oxygen is allowed in the anode chamber which cannot neutralize all the electrons generated in the system, remaining electrons can be harnessed. This has an added advantage of higher treatment efficiency including some toxic compounds (dyes and colored compounds) which needs sequential alternative microenvironments. Overall, high carbon concentration, low oxygen levels, and least possible distance between anode and cathode are the prerequisites for the power generation from aerobic MFC. However, detailed studies pertaining to the process understanding are required to establish the advantages of oxygen presence at anode.

14.3.2 Bioelectrochemical Treatment

More recently MFC documented good efficiency of waste remediation compared to conventional anaerobic treatment process (Kim et al. 2000; Bond et al. 2002; Rabaey et al. 2003; Liu et al. 2005a; Hu et al. 2008; Lee et al. 2008; Biffinger et al. 2008; Sun et al. 2009; Aelterman 2009; Chae et al. 2009; Luo et al. 2010; Oh et al. 2010; Velvizhi and Venkata Mohan 2011; Venkata Mohan and Chandrasekhar 2011a). Due to its function as wastewater treatment unit, MFC can be also called as bioelectrochemical treatment system (BET) when wastewater treatment was prime motto. The principle of BET relies on the fact that electrochemically active microorganisms can transfer electrons from a reduced electron donor to an electrode and finally to an oxidized electron acceptor generating power. Therefore, it can be presumed that coupling of bioanode to a counter electrode (cathode) will have definite positive influence on the overall wastewater treatment efficiency, which has to be tapped (Venkata Mohan and Chandrasekhar 2011a). During BET operation, there exists a possibility to integrate diverse components viz., biological, physical, and chemical in anodic chamber and provides an opportunity to

trigger multiple reactions namely biochemical, physical, physicochemical, electrochemical, oxidation, etc. which cohesively can be termed as bioelectrochemical reactions as a result of substrate metabolic activity and subsequent secondary reactions. The anode chamber of MFC resembles conventional anaerobic bioreactor and mimics the function of a conventional electrochemical cell used for wastewater treatment where the redox reactions help in the degradation of organic matter and toxic/xenobiotic pollutants (Venkata Mohan et al. 2009a; Mohanakrishna et al. 2010a). Potential difference between anodic oxidation and cathodic reduction reactions will have positive influence on the pollutant removal in MFC. The *in situ* biopotential generated helps in the enhancement of degradation of different pollutants in both the anode and cathode chambers. Due to the anodic oxidation potential reactive species like OH^-, O^-, etc., are generated at anode surface which helps to break the complex chemical structures present in wastewater. Sometimes, pollutants themselves act as mediators in electron transfer. For example, elemental sulfur present in the wastewater acts as mediator for electron transfer to the anode and converts to sulfate in the MFC which is easier for degradation (Dutta et al. 2009). Azo dyes also act as mediators for the electron transfer in the MFC and decolorize during reduction (Mu et al. 2009a). Biohazardous toxic compounds such as endocrine disrupting estrogens can also be considered as mediator molecules in MFC (Kiran Kumar et al. 2012).

14.3.2.1 Waste/Wastewater as Electron Donor

Substrate is regarded as one of the most important biological factors in BET/MFC affecting the electron delivery. BET can utilize a wide range of substrates as electron donors including inorganic and organic molecules. However, the efficiency of electron delivery depends on the oxidation state of electron donor and the ratio of the electron donor to the microbe that can oxidize it. Among the simple substrates, glucose and acetate are most widely used anodic fuels with both pure and mixed cultures. However, acetate as electron donor has the advantage of low electron loss over glucose. Any substrate having the possibility of undergoing multiple metabolic pathways will prone to electron loss which can be considered as electron quenching. Glucose is the known best simple substrate that can be oxidized by almost all kinds of microbes but its degradation consist of multiple conversion pathways and hence the electrons will be lost during its conversion. Although the number of electrons generated from glucose is higher than acetate, the energy conversion efficiency is less due to the electron losses. Especially with mixed bacterial suspensions, this problem is significant due to the possible diverse metabolisms. Apart from glucose and acetate several other substrates, viz., sucrose, starch, butyrate, dextran, peptone, ethanol, etc., were also evaluated as anodic fuels (Table 14.2).

BET/MFCs are versatile to utilize wide variety of substrate explored as feed in microbial extracting electric current from a wide range of soluble or dissolved complex organic wastes. The characteristics of the wastewater vary based on the

raw materials used as the source for its generation. Domestic wastewater is considered to be simple and highly biodegradable in nature with low substrate load and hence power generation lasts for only few hours (Venkata Mohan et al. 2009b). Dairy wastewater is rich in nitrogenous compounds such as milk proteins which also may interfere with the power generation. On the contrary, distillery based wastewater is rich in carbon source but due to the presence of complex elements to interference with power generation (Mohankrishna et al. 2010a). The complex nature of pharmaceutical wastewater and low biodegradable nature creates difficulty in their conversion to the reducing equivalents, and moreover the electrons and protons generated will be accepted by the pollutants as intermediary acceptors for their removal instead of generating current. A wide range of wastewater including domestic wastewater (Venkata Mohan et al. 2009b; Jiang et al. 2012), diary wastewater (Saravanan et al. 2010; Venkata Mohan et al. 2010a), potato wastewater (Cusick et al. 2011), pharmaceutical wastewater (Velvizhi and Venkata Mohan 2011), paper industry wastewater (Huang and Logan 2008), swine waste (Min et al. 2005), food waste (Goud et al. 2011; Goud and Venkata Mohan 2011), wheat straw hydrolysate (Zhang et al. 2009a), distillery wastewater (Mohanakrishna et al. 2010a), chocolate Industry wastewater (Patil et al. 2009), vegetable wastewater (Venkata Mohan et al. 2010b), cheese wastewater (Antonopoulou et al. 2009), etc., were studied as electron donors in MFC. Similarly, lignocellulosic biomass (Ren et al. 2007; Wang et al. 2009), dye wastewater (Pant et al. 2008; Sun et al. 2009), landfill leachates (Kjeldsen et al. 2002; Zhang et al. 2008; Greenman et al. 2009; Gálvez et al. 2009), cellulose and chitin (Yazdi et al. 2007), and reed mannagrass (Strik et al. 2008), etc., were also studied in MFC as electron donors. MFC can also be operated in solid phase operation (Venkata Mohan and Chandrasekhar 2011b). Waste generated from different origins, viz., industries, commercial areas, residential areas, etc., can also be potential electron donors in MFC. However, waste having higher biodegradability such as, dairy based wastewater, food wastewater, market based vegetable waste, kitchen based waste, etc., will have good power generation capacity, while the industrial wastewater having low biodegradability will depict lower power output. Still, wastewater could be considered as a potential substrate for MFC due to the dual benefits of converting negative valued waste into bioenergy. However, optimization is still required for up-scaling the process with consorted efforts.

14.3.2.2 Pollutants as Electron Donors/Acceptors

Apart from electron donor, electron acceptor function is also crucial in BET/MFC, especially when waste remediation is considered. Chemotrophic microbes can utilize various pollutants at anode by oxidation discharging the electrons (electron donors) and also can utilize them as electron acceptors in respiration (anaerobic) at cathode which facilitate their remediation. Apart from enhanced substrate removal efficiency, the fuel cell systems also showed considerable reduction in color and total dissolved solids (TDS) of the wastewater in anode chamber (Mohanakrishna

et al. 2010a; Venkata Mohan et al. 2010a, b). Removal of pollutants, such as sulfide (Rabaey et al. 2006), nitrates (Clauwaert et al. 2007; Virdis et al. 2008), perchlorate (Thrash et al. 2007) and chlorinated organic compounds (Aulenta et al. 2007), are also reported in cathode chamber of fuel cell operation during power generation. Apart from oxygen, other compounds viz., nitrates, sulfates, phenol, nitrobenzene, dye molecules (azo dye), etc., will also function as electron acceptors at cathode to accomplish the respiration which facilitates the electron discharge in the circuit (bioelectricity) along with their remediation. However, their function as electron acceptor is based on the thermodynamic hierarchy (Sect. 14.2.3). Some of the compounds viz., sulfur, metals, estrogens, etc., can act as redox mediators for the electron transfer (Kiran Kumar et al. 2012; Chandrasekhar and Venkata Mohan 2012). Nitrates are the best-known electron acceptors after O_2 accounting for denitrification which is an environmentally important microbial process reducing nitrate or nitrite to generate a proton motive force under anaerobic conditions. Denitrifiers have a similar electron-transport system to aerobic respiratory organisms (Kim and Gadd 2008). Similarly, some other microbes and Archea use sulfate and elemental sulfur as their electron acceptor and reduce them. It is also essential to remove nitrogen and sulfur from wastewater treatment plants before discharge to prevent eutrophication. However, microbes which use nitrates as electron acceptors are mostly facultative and which use sulfate/sulfur as electron acceptor are obligate in nature (Kim and Gadd 2008). Nitrates get reduced by both assimilative and dissimilative processes where in assimilative pathway, they are consumed by the microbes as nutrient source for the cellular material, while in dissimilative process they act as electron acceptors (Clauwaert et al. 2007; Kim and Gadd 2008; Virdis et al. 2008; Velvizhi and Venkata Mohan 2011). Some microbes can only use nitrate as electron acceptor but some can use nitrate as both nutrients source as well as electron acceptor. Nitrate gets converted to nitrite by consuming two electrons which further accepts one more electron forming nitric oxide and then get reduced to nitrous oxide by accepting one more electron finally forming nitrogen by accepting one more electron. On the whole the conversion of nitrate to nitrogen, it requires five electrons (Fig. 14.5). Similarly, treatment of inorganic sulfur compounds in MFC also got considerable importance. Sulfate is initially reduced to sulfide and then to hydrogen sulfide when considered as electron acceptor. Sulfide can also be oxidized to elemental sulfur, when considered at anodic oxidation (Fig. 14.5). Sulfide forms as an intermediate during the oxidation of organic compounds and sulfide is oxidized to elemental sulfur. The elemental sulfur deposits on the anode and acts as mediator for the electron transfer instead of anode during the carbon oxidation (Rabaey et al. 2005). Sulfate reduced to sulfide using organics as electron donor by sulfate reducing bacteria to sulfide. More oxidized forms of sulfur such as sulfite, sulfate, and polysulfides can be generated, due to the presence of both sulfides and (microbiologically formed) elemental sulfur (Dutta et al. 2009).

On the other hand, some microbes oxidize (assimilatory reduction) or reduce (dissimilatory reduction) metal ions as electron acceptors or donors. The known metal ions acting as electron acceptors are iron, manganese, selenium, uranium,

chromium, arsenic, vanadium, and cobalt which are considered to be potent pollutants in the environment and their removal is essential prior to waste disposal. Most of the organic compounds degradation under anaerobic conditions is coupled with the ferric iron reduction because of its huge availability on the Earth (Kim and Gadd 2008). MFC can also use the colored dye compounds as alternate electron acceptors which results in their removal. Azo dye and Acid Orange 7 (AO7) are studied for their function as electron acceptors in MFC. The mechanism of their reduction was also fully exploited where they are converted into their reduced intermediates after accepting the reducing equivalents at cathode under anaerobic respiration (Fig. 14.5). Color removal from industrial wastewater, such as distillery and pharmaceutical wastewater, were also reported during anodic oxidation (Venkata Mohan et al. 2009a; Mohanakrishna et al. 2010a; Venkata Mohan et al. 2010a, b; Velvizhi and Venkata Mohan 2011). The toxic halogens and other hydrocarbons are recalcitrant to aerobic remediation but they also can serve as electron acceptors for anaerobic respiration. Apart from these, nitrobenzenes, polyalcohols, and phenols also studied for their treatment either through oxidation or reduction in MFC. Remediation of polyaromatic hydrocarbons was also reported through redox reactions of MFC (Table 14.3).

Pollutant removal during BET operation is possible mainly due to direct (DAO) and indirect anodic oxidation (IAO) mechanisms. The pollutants are adsorbed on the anode surface and get destroyed by the anodic electron transfer reactions in the DAO, whereas the IAO pollutants will be oxidized by the oxidants formed electrochemically on the anode surface under in situ biopotential. DAO facilitates

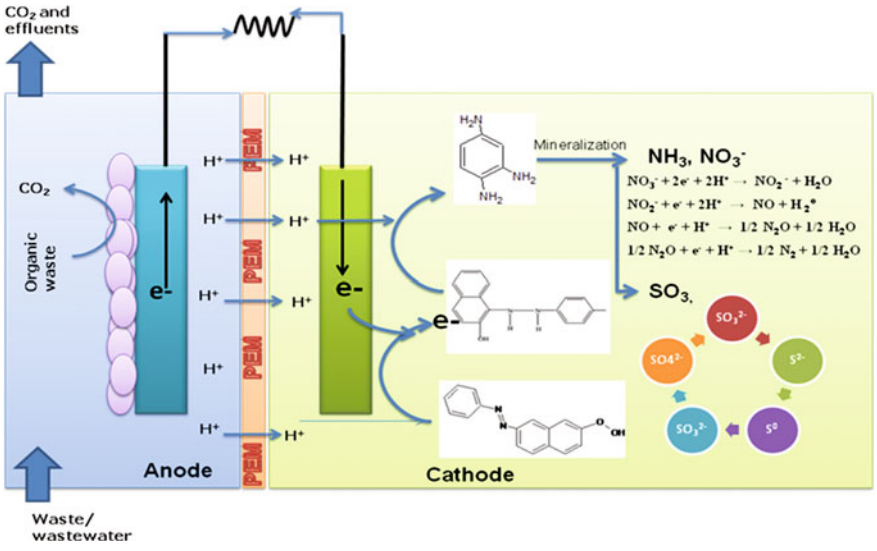

Fig. 14.5 Schematic representation of the anodic oxidation of wastewater as well as cathodic reduction mechanism pertaining to various toxic pollutants, xenobiotics, and nutrients when acting as electron acceptors during BET/MFC operation

Table 14.3 Pollutants functioning as electron donors and acceptors during fuel cell operation

Specific pollutant	Electron acceptor	MFC configuration	Power density	Removal efficiency (% and CE %)	Reference
Pollutant as electron donor					
Phenol	Oxygen	Double chamber	9.1 mW/m^2	90	Luo et al. (2009)
Polyalcohols	Oxygen	Single chamber	2,650 mW/m^2	90	Catal et al. (2008)
Indole	Ferricyanide	Double chamber	51.2 W/m^3	88	Luo et al. (2010)
Estriol	Oxygen	Single chamber	28.32 mW/m^2	54	Kiran Kumar et al. (2012)
Ethenyl estradiol	Oxygen	Single chamber	12.46 mW/m^2	38	Kiran Kumar et al. (2012)
Specific pollutant	Electron donor	MFC configuration	Power Denisty/ Current	Removal efficiency (% and CE %)	Reference
Pollutant as electron acceptor					
Nitrate	Acetate	Double chamber	9.4 mW/m^2	84	Lefebvre et al. (2008)
Sulfide	Acetate	Double chamber	37 W/m^3	87	Dutta et al. (2009)
Perchlorate	Sodium acetate	Double chamber	0.28 mA	97	Butler et al. (2010)
Azo dye	Glucose	Double chamber	110 mW/m^2	77	Mu et al. (2009a)
Nitrobenzene	Sodium acetate	Double chamber	16.5 A/m^3	98	Mu et al. (2009b)
Selenite	Acetate	Double chamber	2,200 mW/m^2	99	Catal et al. (2009)
Nitrophenols	Acetate	Double chamber	–	70	Zhu and Ni (2009)
Pyridine	Glucose	Double chamber	1.7 W/m^3	95	Zhang et al. (2009b)

formation of primary oxidants which further react on the anode yielding secondary oxidants, such as chlorine dioxide and ozone, which might have significant positive effect on the color removal efficiency through the oxidation process. Reactions between water and radicals near the anode could yield molecular oxygen, free chlorine and hydrogen peroxide, hypochlorous acid, etc. which also helps in color/organic oxidation (Mohanakrishna et al. 2010a; Venkata Mohan and Srikanth 2011). Few reports are also available regarding the cathodic function in the effective removal of pollutants. Azo dyes (Mu et al. 2009a), nitrobenzene (Mu et al. 2009b), and nitrate (Lefebvre et al. 2008) are some of the pollutants studied in the cathode chamber of BET. Hypothetically, it can be assumed that in cathode chamber under anaerobic condition, most of the pollutants act as TEA for power generation. Treatment pertaining to anodic chamber was relatively less compared to cathode. Pollutants in the anodic chamber also act as mediators for electron transfer to anode which can increase the power generation efficiency with simultaneous reduction of pollutants. Very less work has been reported in the use of pollutants as mediators. Apart from substrate removal, the fuel cell also showed considerable reduction of toxicity, color, and TDS in wastewater (Venkata Mohan et al. 2010b; Mohanakrishna et al. 2010b; Velvizhi and Venkata Mohan 2011). Application of MFC was also extended to treat solid waste and the toxic aromatic hydrocarbons by using the *in situ* biopotential and considering anode as electron acceptor (Venkata Mohan and Chandrasekhar 2011a, b). Studies related to the mechanism of pollutant reduction and their role in electron transfer or acceptance will give a spectrum of practical feasibility of this technology for the removal of toxic pollutants.

14.3.3 Microbial Electro-Synthesizer

Reduction mechanism at cathode can be effectively used not only for pollutant removal but also for the generation of reduced end products having commercial value. The product formation is based on the electron acceptor and the redox potential of the MFC. For example, ethanol can be formed at cathode by considering acetate as electron acceptor under a redox potential of −0.28 V. Likewise, a diverse range of value-added products can be harnessed from the MFC, especially at cathode in the absence of O_2 as electron acceptor, along with the power generation (Rabaey and Rozendal 2010). Based on the electron accepting conditions at the cathode, different compounds can be synthesized and therefore MFC can also be used for product recovery other than treatment unit. Some of the reactions require less redox potential which can be accomplished by the *in situ* generated biopotential, while some reactions require more redox potential at cathode which cannot be accomplished by the *in situ* potentials (Rabaey and Rozendal 2010). In such cases, the designated redox potential is maintained by applying small external potentials to meet the energy necessary to cross the energy barrier for product formation. Hydrogen production through cathodic reduction

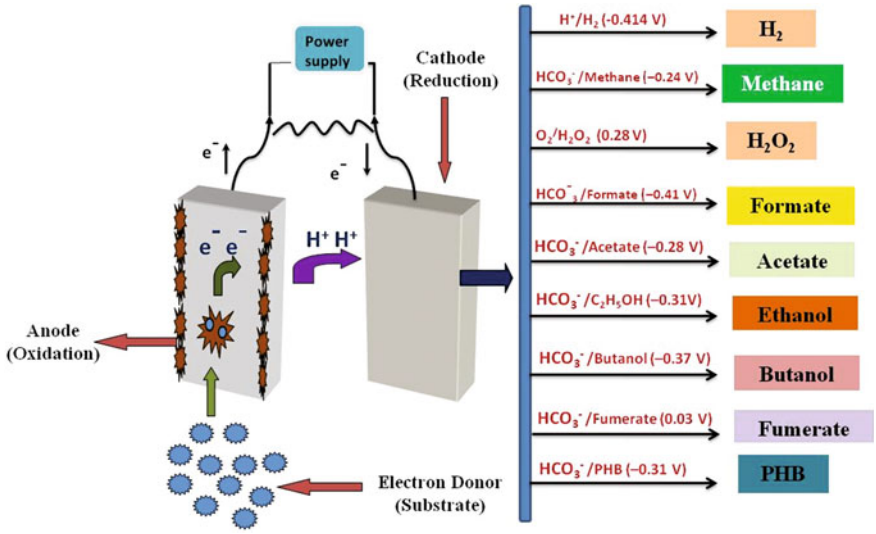

Fig. 14.6 Schematic representation of bioelectrochemical system including its function towards the formation of various reduced end products having commercial importance

reaction has been extensively studied in MFC till date, under the name microbial electrolysis cell (MEC). Other valuable products known to be harnessed at MFC cathode are acetate, ethanol, hydrogen peroxide, butanol, etc. (Fig. 14.6). However, there is scope to generate number of products using the potential driven reduction process at cathode along with power during MFC operation.

14.3.3.1 Microbial Electrolysis for H_2 Recovery

Hydrogen is one of the alternative renewable fuels having high impact in the present bioenergy research. H_2 is considered as green fuel due to the nonemission of green house gases at combustion and have high energy value (122 kJ/Kg) over other existing biofuels. H_2 can be produced through chemical, physical, and biological mechanisms (Venkata Mohan 2010, 2009; Venkata Mohan et al. 2011a). However, H_2 production through biological route, especially through acidogenic fermentation, has attracted recent science due to the lower economics of the process. Moreover, considering wastewater as substrate for the H_2 production is significant due to its dual benefits, viz., H_2 production and waste remediation (Venkata Mohan 2008, 2010). The technology was well established and understood with respect to the operational and regulating factors (Venkata Mohan et al. 2007b, Venkata Mohan et al. 2008g). However, there are some drawbacks for the fermentative H_2 production, viz., low substrate conversion efficiency, accumulation of carbon rich acid intermediates, sudden drop in system pH, etc. (Venkata Mohan et al. 2007b, 2008g; Venkata Mohan and Goud 2012). Some strategies

were developed to overcome these problems such as pretreatment of inoculums (Venkata Mohan et al. 2008g; Srikanth et al. 2010b), secondary integration of photo biological H_2 production (Srikanth et al. 2009; Rashmi Chandra and Venkata Mohan 2011), MFC (Mohanakrishna et al. 2010b), methanogenesis (Venkata Mohan et al. 2008h), polyhydroxy alkanoates production (Reddy and Venkata Mohan 2012), etc. MEC is representing as an alternative H_2 production process by combining the conventional acidogenic process with electrochemical hydrolysis where the activation energy required for the electrolysis is reduced by the microbial metabolism (Call and Logan 2008; Logan et al. 2008 Venkata Mohan and Lenin Babu 2011). Due to the combination of both the processes, the H_2 production from MEC is significantly higher compared to the conventional acidogenic process (Cheng and Logan 2007). Applying additional voltage to the in situ potential generated by the bacterial cell, allows bioenergy generation like hydrogen and methane or various products like hydrogen peroxide at cathode (Liu et al. 2005b; Venkata Mohan and Lenin Babu 2011). Previously, this bioelectrolytic process has been referred to as a biocatalyzed electrolysis cell (BEC) or a bioelectrochemically assisted microbial reactor (BEAMR) (Logan et al. 2005; Ditzig et al. 2007; Tartakovsky et al. 2009) and for the past few years it has been termed as electrohydrogenesis or microbial electrolysis. The performance of MEC has significantly improved within few years after its discovery (Liu et al. 2005b; Logan et al. 2005). MEC is based on the principle that reducing equivalents are generated at anode from the oxidation of organic matter by the electrogenic microbes and get reduced to hydrogen at cathode (Liu et al. 2005b). Recently, few research groups are working towards integrating the wastewater treatment with MEC to produce economically feasible biohydrogen generation. MEC can also utilize the acid-rich effluents generated from hydrogen bioreactor as substrates for H_2 production (Lalaurette et al. 2009). The protons transferred to cathode were reduced to H_2 in presence of electrons coming from the anode under small applied voltages which is required to cross the endothermic barrier to form H_2 gas. The standard redox potential for the reduction of protons to H_2 is -0.414 V which is generally not possible to generate in situ in the system and hence to favor the formation of H_2, an additional voltage is required. Low-energy consumption compared to water electrolysis, high product (H_2) recovery and substrate degradation than dark fermentation process makes MEC as an alternate and potential replacement for the escalating global energy demands. MEC systems are based on a number of components, each of which will play a vital role that can influence the performance efficiency viz., biocatalyst, electrode materials, membranes, applied potential, nature of substrate and its loading rate. Different types of bacterial cultures viz, pure strains, combination of two strains, mixed cultures etc. have been used for the operating MEC (Call et al. 2009; Selembo et al. 2009. Jeremiasse et al. 2010; Venkata Mohan and Lenin Babu 2011). However, H_2 production will vary based on the nature biocatalyst used and the operating conditions. Initially, MEC was operated in dual chamber and later shifted to single chamber where both the electrodes will be placed in the same electrolyte and differentiated based on the external power source connected to the electrodes. Ion selective membranes also

play crucial role in determining the efficiency of MEC but they tend to increase the internal resistance. Similar to the MFC, a multielectrode MEC is required to enhance the biogas generation due to the possible lower cell voltage irrespective of the volume (Rader and Logan 2010). MEC is capable of converting a wide variety of soluble organic matter in wastewater to bioenergy such as H_2 with simultaneous waste treatment (Call and Logan 2008) or CH_4 (Clauwaert and Verstraete 2009). Diverse range of substrates from simple domestic wastewater to complex industrial wastewater can serve as substrate in MEC with good product recovery as well as their treatment. Application of external voltage results in the dominant growth of electrochemically active microbes on anode which can effectively discharge higher number of electrons from the substrate oxidation (Wang et al. 2009; Srikanth et al. 2010a; Venkata Mohan and Lenin Babu 2011). Microbial identification on the anode has shown the dominance of electrogenic microbes on anode during MEC operation (Table 14.4).

14.3.3.2 Other Reduced End Products

Microbial electro synthesis/bioelectrosynthesis is the process of production of chemical compounds through a series of microbially or enzymatically catalyzed reactions in a specially designed electrochemical cell called bioelectrochemical system (BES). Microbially catalyzed synthesis of organic or inorganic compounds in an electrochemical cell where the electricity driven reduction or oxidation of diverse feed stocks. Interaction between biocatalysts and solid electron acceptors (electrodes) forms the basis for microbial electrosynthesis (Rabaey and Rozendal 2010). After H_2, the reduction of CO_2 to reduced end products is other rapidly growing technology (Rabaey and Rozendal 2010). CO_2 in combination with the H_2 and CO (syngas) can be reduced to different products, viz., acetate, ethanol, butanol, etc. However, the conversion is based on the applied potential and the efficiency is based on the gas composition (Rabaey and Rozendal 2010). The disadvantage in CO_2 reduction to organic compounds by considering it as electron acceptor is the requirement of higher number of electrons. For example, the reduction of butyrate to butanol requires only four electrons, while the reduction of CO_2 to butanol requires 24 electrons accounting for higher power requirement for this reaction to happen. However, CO_2 availability is ubiquitous and it is good electron acceptor. Anyhow, its removal from the atmosphere is necessary in the environmental concern to remove carbon footprints (Rabaey and Rozendal 2010). With the aid of small input of electric power, many value-added compounds were formed at the cathode and the major achievements are as follows., the yield of glutamic acid was increased during glucose fermentation (Hongo and Iwahara 1979), yield of butanol was increased during *Clostridium acetobutylicum* fermentation (Kim and Kim 1988), neutral red when electrically reduced serves as the sole electron donor for the growth of *Actinobacillus succinogenes* and for methane production by using mixed population (Park et al. 1999), Acetate formation was shifted to propionate by mediated current supply during glucose fermentation,

Table 14.4 Different electron donors used during MEC operation along with the respective biocatalysts and hydrogen yields

Electron donor	Biocatalyst	MEC configuration	Hydrogen yield	Reference
Acetate	Enriched anode respiring bacteria from MFC	Single chamber; membraneless	4.3 m^3H_2/m^3/day	Lee and Rittmann (2010)
	Anaerobic sludge	Double chamber; with PEM	1.12 mol H_2	Sun et al. (2008)
	Acetic acid fed MFC bacteria	Multi electrode MEC	0.53 m^3H_2/m^3/day	Rader and Logan (2010)
	Shewanella oneidensis	Single chamber; membrane less	0.69 m^3H_2/m^3/day	Hu et al. (2008)
	Geobacter sulfurreducens	Single chamber; membrane less	1.9 m^3H_2/m^3/day	Call et al. (2009)
	Geobacter metallireducens	Single chamber; membrane less	1.3–0.1 m^3	Call et al. (2009)
	Clostridium thermocellum 27,405	Single chamber; cylindrical MEC	Acetic acid-1,400 ml H_2/g COD	Lalaurette et al. (2009)
Succinic acid	*Clostridium thermocellum* 27,405	Single chamber; cylindrical MEC	1,100–130 ml H_2/g COD	Lalaurette et al. (2009)
Formic acid	*Clostridium thermocellum* 27,405	Single chamber; cylindrical MEC	810–260 ml-H_2/g COD	Lalaurette et al. (2009)
Glycerol	Heat treated anaerobic sludge	Single chamber; membrane less	5.39 mmol H_2	Escapa et al. (2009)
Microbial nutrient medium	Mixed culture	Two identical electrochemical cells operated in continuous mode	0.63 m^3H_2/m^3/day	Jeremiasse et al. (2010)
Swine wastewater	Exoelectrogenic bacteria	Single chamber; membrane less	0.9–1.0 m^3H_2/m^3/day	Wagner et al. (2009)
Bovine serum albumin	Exoelectrogenic bacteria	Single chamber; membrane less	21.0 ± 5.0 mmol H_2/g COD	Lu et al. (2010)
Winery wastewater	Exoelectrogenic bacteria	Single chamber; membrane less	0.17–0.01 m^3H_2/m^3/day	Cusick et al. (2010)
Domestic waste water	Exoelectrogenic bacteria	Single chamber; membrane less	0.28–0.04 m^3H_2/m^3/day	Cusick et al. (2010)
Municipal solid waste	Endogenous bacteria of municipal solid waste	Single chamber; membrane less	0.035 m^3H_2/m^3/day	Dictor et al. (2010)
Designed synthetic wastewater	Anaerobic mixed consortia	Single chamber; membrane less	8.42 mol H_2/Kg COD_R-day	Venkata Mohan and Lenin Babu (2011)
Artificial wastewater	Sewage sludge	Double chamber MEC	35 ml H_2	Cheng et al. (2010)

By applying mediated current to the microbial population enhances acetate consumption and ethanol production (Steinbusch et al. 2010), DET from cathode to Geobacter species is established where the biofilm attached involves in the reduction of the fumerate to succinate (Gregory et al. 2004).

14.3.4 Other Applications

Apart from the above-mentioned characteristics, MFC has other faced such as photosynthetic fuel cells, carbon capture cells, sediment based benthic fuel cells, marine applications, and desalination, plant-based (rhizodeposit based) power generation, biocathode and application as biosensor. However, all these applications are still in the state of infancy and needs deeper understanding for their practical implications. Photosynthetic fuel cells (PhFC) are similar to the MFC in operation but instead of chemotrophic mechanism, phototrophic bacteria will act as biocatalyst. PhFC utilizes sunlight as electron donor and the electron will pass through a cascade of proteins generating a proton motive force similar to the MFC and provides feasibility to harness bioelectricity (Chandra et al. 2012). However, algae application as anodic biocatalyst restricts electrogenic efficiency due to its oxygenic photosynthesis where oxygen will be evolved through biophotolysis mechanism unlike photobacteria (anoxygenic photosynthesis) which scavenges all the reducing equivalents generated in the system. Applications of PhFC can be extended to the utilization of acidogenic effluents rich in volatile fatty acids to convert them as either lipids or carbohydrates or other value-added products along with the power generation (Strik et al. 2008). Sediment-based MFC also has a wide applicability due to the possibility of converting their rich organic composition into power. Moreover, the sediment MFC is devoid of PEM which is the costliest material in MFC construction. The sediment-enriched organisms are also highly electrogenic and can discharge the electrons effectively into the exterior environment. Benthic MFC are sediment type which creates the feasibility of harnessing bioelectricity from aquatic ecosystem using natural habitants through employing sediment type fuel cell assemblies by placing electrode in the sediment as anode and connecting it in an electrical circuit to another electrode as cathode in the overlying water layer (Reimers et al. 2001; Bond et al. 2002; Holmes et al. 2004; Venkata Mohan et al. 2009c; Lenin Babu and Venkata Mohan 2012). The electrons flow from anode to cathode through an external circuit, while protons diffuse through the water between the electrodes and develop potential difference which is necessary for current generation. Microbes in the sediment surface layer are also potent reductants which use the metals and other toxic substances (Mn^{2+}, Fe^{2+} and S^{2-}) for electron transfer making them reduced (Tender et al. 2002; Bond and Lovley 2003; Reimers et al. 2005; Ryckelynck et al. 2005; Lowy et al. 2006). The sediment assembly can also be extended to marine applications where the anode is embedded in the anaerobic marine sediment and cathode is overlying in the aerobic sea water (Reimers et al. 2001; Bond et al. 2002; Tender et al. 2002;

Holmes et al. 2004). Application of marine fuel cell could be used to measure both physical and chemical parameters in low-power consuming instrumentation in the marine environment to operate where the operation will be for long term (Shantaram et al. 2005; Lowy et al. 2006). Power generation from the growing plants or from the conversion of organic source at their rhizodeposits to electricity by introducing electrodes was also gaining prominence recently (Cho et al. 2008; Kaku et al. 2008; Strik et al. 2008; Venkata Mohan et al. 2011b). Microorganisms or immobilized enzymes can also act as electron acceptors or initiate the electron acceptor reactions at cathode (Rhoads et al. 2005; He and Angenent 2006; Rosenbaum et al. 2011; Venkata Mohan and Srikanth 2011; Srikanth and Venkata Mohan 2012a, b; Behera et al. 2010). Application of biocathodes for the bioremediation as well as bioelectrosynthesis is gaining prominence in the recent time. Removal of pollutants viz., nitrates, sulfates, metals, etc., through reduction at cathode and conversion of electron acceptors such as CO_2, acetate, etc., to the reduced commercial end products requires the maintenance of certain potential (Rabaey and Rozendal 2010; Srikanth and Venkata Mohan 2012a). Usage of biocathodes will generate some potential which reduces the energy required to cross the activation barrier to form the product and hence biocathode garnered significant importance. However, there are drawbacks with the biocathode application such as electron loss and the alteration of product formation etc. (He and Angenent 2006; Rosenbaum et al. 2011; Srikanth and Venkata Mohan 2012a, b). MFC was also reported as biosensor for the identification of target substrate, BOD, COD, etc., through electroactive biofilms as sensing element and can reflect the real-time microbial activity of bioprocess (Gil et al. 2003; Chang et al. 2004; Spanjers and Van Lier 2006; Di Lorenzo et al. 2009). The multiple functions of MFC can be fully exploited and established in the near future which benefits the society with respect to growing energy demands as well as environmental pollution problems.

14.4 Summary and Future Scope

MFC/BES is one of the alternative ecofriendly devices which facilitate various forms of bioenergy/byproduct generation including waste remediation. The performance of MFC has been studied for different applications using a wide spectrum of substrates as electron donors and acceptors to generate power with their simultaneous remediation. However, there still remain various constraints to make this technology as a viable alternative to the existing technologies. Construction cost of MFC should be reduced as well as its efficiency needs significant increment. The theoretical possible potential is only −1.2 V (from single cell) and hence stacking of number of fuel cells in series with yield feasible power. Membrane-less systems such as sediment type fuel cells should be developed to avoid the membrane cost at large scale. More important is that the application of MFC for the removal of toxic pollutants and xenobiotics should be extensively

studied for their implementation in the existing effluent treatment plants to enhance their treatment efficiency. Detailed studies are required on the recovery of commercially viable reduced end products which increases the economic viability of this application. CO_2 can be considered as viable electron acceptor at cathode and the products such as ethanol and butanol can be recovered, normally used for blending the existing fuels, and will have immediate market value. However, many questions and issues need to be resolved in order to make this technology viable as well as feasible for clean energy production with simultaneous waste remediation.

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Chapter 15
Biomethanation Potential of Biological and Other Wastes

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Abstract Anaerobic technology has been traditionally applied for the treatment of carbon rich wastewater and organic residues. Anaerobic processes can be fully integrated in the biobased economy concept for resource recovery. After a brief introduction about applications of anaerobic processes to industrial wastewater treatment, agriculture feedstock and organic fraction of municipal solid waste, the position of anaerobic processes in biorefinery concepts is presented. Integration of anaerobic digestion with these processes can help in the maximisation of the economic value of the biomass used, while reducing the waste streams produced and mitigating greenhouse gases emissions. Besides the integration of biogas in the existing full-scale bioethanol and biodiesel production processes, the potential applications of biogas in the second generation lignocellulosic, algae and syngas-based biorefinery platforms are discussed.

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15.1 Introduction

We are presently living in the transition from a linear economy (cradle to grave) founded on abundant fossil resources to a circular biobased economy concept (cradle to cradle), where waste and by-products should re-enter the cycle of production and the energy carried should derive from renewable sources. In the circular economy's thinking, biorefinery concepts based on a variety of bioresources, by-products and (bio) wastes are emerging. There is a huge opportunity for anaerobic digestion (AD) as multi-functional process that integrates environmental protection, renewable energy production, nutrients and water recycling (Fig. 15.1). Anaerobic conversion processes should be the core of any treatment process of biodegradable waste or carbon rich wastewater, though complemented with appropriate post-treatment processes either biological or physicochemical (Van Lier and Lettinga 1999). The wide range of feedstocks suitable for biogas production includes animal waste, municipal sludge, industrial wastewater and organic fractions of municipal solid waste (OFMSW), as well as aquatic and terrestrial energy crops whenever available for energy production. One of the main advantages of AD is the possibility of recovering renewable energy in the form of biogas, which is a versatile energy carrier that can be used for electricity production, heating purposes, vehicle and jet fuel and replacement of natural gas by injection of upgraded biogas in the natural gas grid. In addition, biogas may be considered as starting compound for biotechnological production of chemicals. On the other hand, organic waste stabilisation and nutrient redistribution are, besides energy production, objectives of any AD plant (Fig. 15.1).

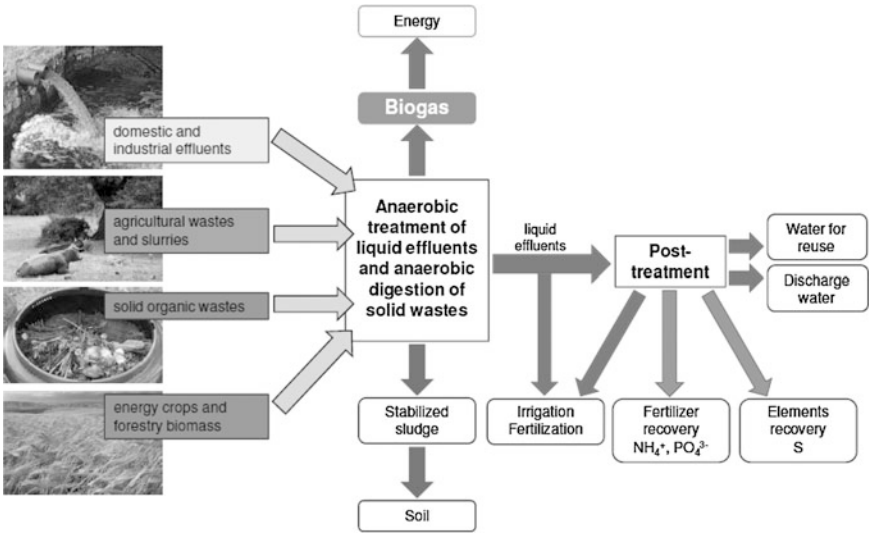

Fig. 15.1 The role of anaerobic processes for sustainable bio-resources recovery (adapted from van Lier and Lettinga 1999)

15.2 Current Applications of Anaerobic Processes

15.2.1 The Biochemical Process of Methane Production

Anaerobic digestion of complex organic substrates proceeds through a series of parallel and sequential steps, with several groups of microorganisms involved. Figure 15.2 depicts the main pathways of the anaerobic degradation of complex substrates under methanogenic conditions. AD starts with the hydrolysis of complex substrates to simpler compounds. Complex substrates, such as carbohydrates, proteins and lipids, are normally present in the form of suspended compounds or colloidal matter and, before transport through the cellular membrane, need to be transformed into smaller molecules. Such process takes place during the hydrolysis step, in which these complex compounds are hydrolysed into their basic building units. This step is aided by extracellular enzymes (hydrolases), which are excreted by fermentative bacteria. Carbohydrates are converted into soluble sugars (saccharides) by cellulases, amylases, xylanases and other hydrolytic enzymes; proteins are degraded via peptides and amino acids by proteases and peptidases; and, lipids are transformed into long chain fatty acids (LCFA) and glycerol by lipases. In the case of complex particulate substrates, hydrolysis of biopolymers can be the rate limiting step in the whole degradation process (Masse et al. 2002; Van Lier et al. 2001; Vavilin et al. 1996). An efficient hydrolysis step is important to make complex substrates accessible for the anaerobic microbial communities, optimising the methanogenic potential of the (waste) water to be treated.

Products formed during the hydrolysis step are further converted inside the bacterial cells in a process known as acidogenesis (or fermentation). Acidogenesis

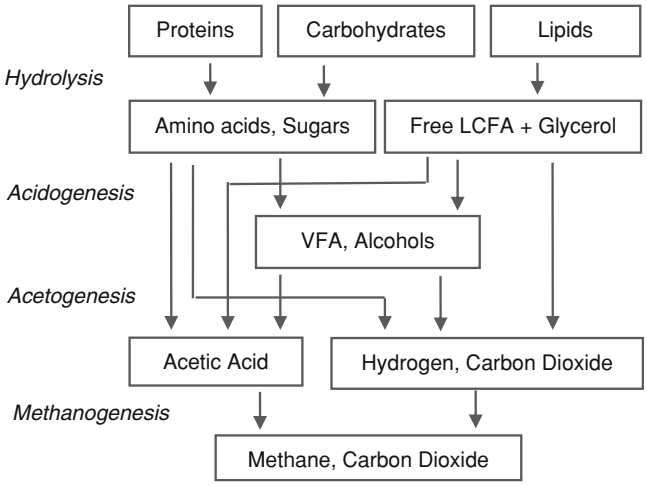

Fig. 15.2 Simplified representation of the anaerobic digestion process (Pereira 2003 adapted from Gujer and Zehnder 1983)

is the first energy yielding step during anaerobic digestion and consists in the degradation of soluble substrates, without the presence of an external electron acceptor. Main substrates for acidogenesis include soluble saccharides, amino acids and glycerol and results in the formation of acetate, propionate, butyrate, carbon dioxide, hydrogen and other organic products, such as lactate and alcohols (Harper and Pohland 1986). Soluble sugars are largely converted into acetate and hydrogen, but formation of propionate, butyrate, lactate and ethanol occurs as well. LCFA degradation requires an external electron acceptor for oxidation, and therefore is covered in the acetogenesis section. Nevertheless, hydrogenation of unsaturated fatty-acids might take place during the acidogenesis step. Normally, the bacteria responsible for the hydrolysis also ferments the resulting monomers (Schink 1997). In general, these bacteria have a short doubling time, and therefore acidogenesis is not regarded as a limiting step in the process of anaerobic digestion (Gujer and Zehnder 1983; Mosey 1983).

Fermentation products (short chain fatty acids and alcohols) and LCFA (resulting from lipid hydrolysis) can be further oxidised to acetate by obligate hydrogen producing acetogens in the acetogenic step. Fatty acids oxidation is coupled to the reduction of hydrogen ions or bicarbonate, functioning as external electron acceptors, to form hydrogen and formate, respectively. Under standard conditions (Temperature of 0 °C and Pressure of 1 atm), these reactions are thermodynamically unfavourable, and the complete conversion of the substrates only proceeds when hydrogen and formate concentration is kept low (Schink and Stams 2006; Stams and Plugge 2009). This is achieved by syntrophic association with hydrogen and formate-utilising microorganisms.

In the presence of inorganic electron acceptors other than protons and CO_2, competition for different substrates may occur. Such is the case of wastewaters containing sulfate, in which sulfate-reducing bacteria can compete with syntrophic acetogenic bacteria for electrons resulting from fatty-acids, and with methanogens for electrons resulting from hydrogen and acetate (Stams et al. 2005).

Methanogenesis is the production of methane and, in various environments, is the final step in the degradation of organic matter. This highly specialised process is carried out by methanogenic archaea, which metabolise the end products of the previous reactions (mainly hydrogen, carbon dioxide, formate, methanol, methylamines, and acetate) to form methane. In anaerobic bioreactors, this process mainly occurs through two pathways: (1) carbon dioxide reduction (hydrogenotrophic methanogenesis (Boone et al. 1989; Schink 1997)), and (2) acetate dissimilation (acetoclastic methanogenesis (Jetten et al. 1992)) (Fig. 15.2). Several authors have reported methanogenesis as being the rate-limiting conversion in the whole anaerobic digestion process in bioreactors (Fang et al. 1995; Huang et al. 2003).

15.2.2 Anaerobic Digestion of Slurries and Biowaste

Anaerobic digestion is already an effective and mature technology to produce renewable energy carriers from organic waste, to reduce odour and pathogen levels in manure and produce a biofertilizer, to reduce greenhouse gas emission from a farmstead and to treat food waste/by-products (Cantrell et al. 2008; Mata-Alvarez et al. 2000; Weiland 2010). According to data from the EurObserv'ER (2010) between 2000 and 2009, the biogas produced in Europe increased about five times. However, there are large differences of biogas technology implementation in Europe. For instance, in Germany, 51.5 ton oil equivalent (TOE) were produced per 1,000 inhabitants as biogas primary energy in 2009, whereas in Portugal only 2.2 TOE/1,000 inhabitants were produced in the same period. The average of the European Union countries was 16.7 TOE/1,000 inhabitants.

In theory, all types of biowaste can be used for biogas production. The composition of the biogas and the biogas productivity depends on the feedstock, on the reactor type and organic loading rate applied, and on the microbial consortium activity. Table 15.1 presents some data on the biogas yields for some types of waste and raw materials. There is a long tradition of anaerobic sewage sludge and animal manure treatment. Presently, agricultural applications are mainly based in co-digestion of manure with available co-substrates such as harvest residues, top and leaves of sugar beets, organic wastes from agriculture related activities, food waste, collected municipal biowaste from households and energy crops (Weiland 2010). The advantages of co-digestion are (Cecchi et al. 1996; Mata-alvarez et al. 2000; Murto et al. 2004; Neves 2009):

- Dilution of toxic substances coming from any of the substrates involved, including, possible detoxification of some xenobiotics, based on co-metabolism process;
- Improved nutrient balance reducing micro and macronutrient deficiency;
- Improving process stability;
- The use of a co-substrate can also help to establish the required moisture contents of the digester feed. Better handling and digestibility can be achieved by mixing solid waste with diluted waste;
- In addition, economic advantages can be significant, derived from the fact of sharing equipment.

Table 15.1 Average biogas yields of several substrates (adapted from Weiland 2010)

	Substrate	m^3 biogas t^{-1} feedstock
Agricultural wastes	Cow manure	25
	Pig manure	30
Agricultural raw materials	Grass	100
	Fodder beets	110
	Wheat corn	630
Non-agricultural wastes	Biowaste	120
	Food residues	240
	Used grease	800

15.2.3 Anaerobic Wastewater Treatment

The application of anaerobic technology for industrial wastewater treatment is also established (Rajeshwari et al. 2000; Angenent et al. 2004). High-rate anaerobic wastewater treatment technology has become a standard for a certain range of industrial wastewaters. Thousands of full-scale installations are in operation worldwide, treating mainly wastewater containing readily degradable organic pollutants such as Volatile Fatty Acids (VFA) and carbohydrates. Reliable technologies, such as the upflow anaerobic sludge blanket (UASB), the expanded granular sludge bed (EGSB) and the internal circulation (IC) reactors, promoted the confidence in AD technology. Recently, the inverted anaerobic sludge blanket (IASB) reactor (Alves et al. 2007) was developed for the treatment of effluents with high content of lipids and the proof of concept demonstrated an efficient treatment capacity for an extremely concentrated slaughterhouse effluent. The first full-scale reference is presently under construction.

15.3 The Role of Biogas in Biorefinery Platforms

15.3.1 Introduction

According to the International Energy Agency (IEA) Bioenergy Task 42, "biorefinery is the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat)".

The integration of AD technology within a biobased economy is a logical and intuitive step now. Biorefineries for the production of chemicals and biofuels from vegetable biomass have been in focus in the recent years (Langeveld et al. 2010). Special attention has been given to the development and optimisation of processes for the production of ethanol and biodiesel, which are presently done at full scale in several countries. Brazil and the United States have well established and growing economy based on ethanol production. Second generation ethanol from lignocellulosic biomass is emerging and will be a mature technology in the near future. Biodiesel production from vegetable oils is representative in countries such as Germany and France. Integration of AD with these processes can help in the maximisation of the economic value of the biomass used, while reducing the waste streams produced and mitigating greenhouse gases emissions. Besides, other products such as compost can be produced and further recycled for agriculture purposes or for vegetable biomass growth.

15.3.2 Biogas Opportunities in Bioethanol Production

First generation bioethanol, derived from starch crops like corn and wheat, or sugar crops like sugar cane and sugar beet, has been rapidly adopted as a primary

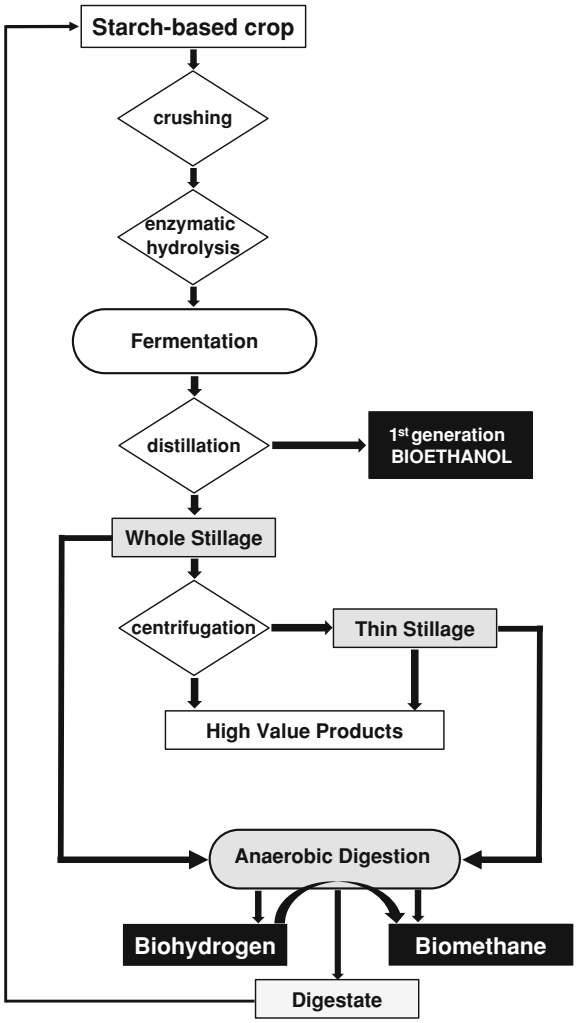

Fig. 15.3 Biorefinery concept for the production of bioethanol from sugar-based crops, including an anaerobic digestion step for the energetic valorisation of the by-products

transportation fuel in the United States and Brazil. Total world production grew from 17.1 billion litres in 2000 to 86.9 billion litres in 2010, with the United States as the top producer with 50 billion litres, mainly corn-derived ethanol, followed by Brazil with 26 billion litres using sugarcane as primary feedstock. The European Union produced 4.4 billion litres of ethanol in 2010, accounting for 5.1 % of world's production (Lichts 2010).

Typical steps on current sugar-based ethanol technology include milling of the sugar cane (or sugar beet crops) to extract the juice, fermentation of sugar to ethanol by yeast and distillation of ethanol (Fig. 15.3). For starch (cereal) based crops, similar procedures are performed, with an additional hydrolysis step to break down the polymers into simple C6 sugars (Fig. 15.4). Both processes produce large amounts of by-products, namely:

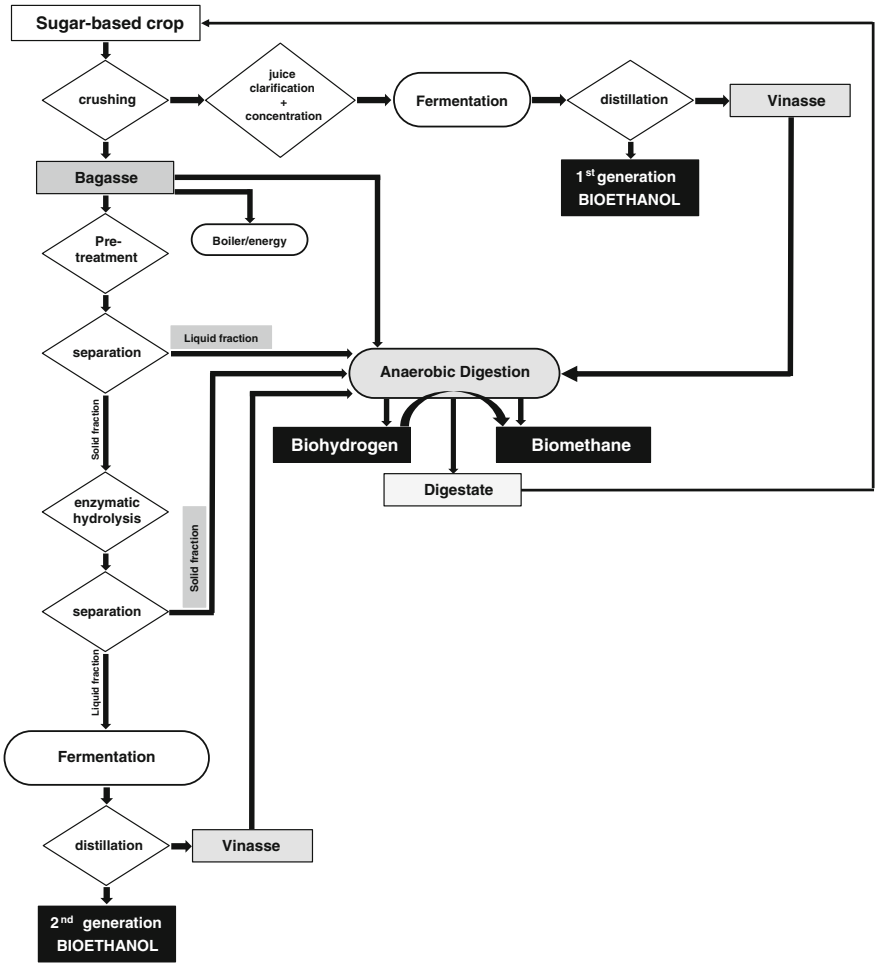

Fig. 15.4 Biorefinery concept for the production of bioethanol from starch-based crops, including an anaerobic digestion step for the energetic valorisation of the by-products

- Bagasse: the biomass left over after sugarcane has been crushed and the juice extracted. Approximately 240 kg are generated per ton of sugarcane (Dias et al. 2009);
- Vinasse: the effluent obtained after ethanol is distilled from the fermented sugar mixture,
- Whole stillage: the effluent obtained after ethanol is distilled from the fermented cereal mixture. Up to 20 litres per litre of ethanol produced depending on the feedstock used (van Haandel and Catunda 1994).

In a traditional corn-to-ethanol process, the whole stillage is centrifuged to produce wet cake (solid fraction) and thin stillage (liquid fraction). A significant

fraction (around 50 %) of the thin stillage is recycled as backset to the second stage of the liquefaction process and the remaining part is evapourated to syrup. The syrup is then mixed with the centrifuged solids to produce distiller's dried grains with soluble (DDGS) that are sold as livestock feed (Eskicioglu et al. 2011). DDGS processing (centrifuging, evaporation and drying) is energy demanding. It can account for approximately 35 % of electricity and 30 % of natural gas consumption of a bioethanol plant (Meredith 2003). Currently, it is still profitable to process whole stillage and sell as animal feed, but as fuel demand increases, the risks of DDGS market saturation increase as well.

Anaerobic digestion has long been considered an alternative approach to handle ethanol by-products, reduce the environmental impact of the generated wastewaters and improve the net energy balance ratio of the process (Figs. 15.3 and 15.4) (Plugge et al. 2009). Research has primarily been focused on thin stillage because it is the largest wastewater contributor. In the 1980s, mesophilic studies on corn thin stillage reported promising performances with methane yields of 250–370 L CH_4 kg^{-1} chemical oxygen demand (COD) removed that could replace about 60 % of the daily energy requirement of the bioethanol plant (Stover et al. 1984). In recent studies, AD of corn thin stillage was evaluated at thermophilic conditions. Whole stillage exits the distillation column at above 55 °C, and thus heating demand to achieve thermophilic digestion is not so high and can provide improved efficiency and economics. Schaefer and Sung (2008) reported methane yields ranging between 600 and 700 L CH_4 kg^{-1} volatile solids (VS) removed during AD of thin corn stillage in thermophilic continuous stirred-tank reactor (CSTR) operated at 30, 20 and 15 days hydraulic retention time (HRT). It was estimated that natural gas consumption at corn derived ethanol plants could be reduced by 43–59 % with this level of methane production. In another study, thin corn stillage was treated in thermophilic sequencing batch reactors (SBR) to produce 254 L CH_4 kg^{-1} TCOD fed with a 10 day HRT (Agler et al. 2008). These authors also estimated that the methane generated would reduce natural gas consumption in conventional dry grind ethanol plants by 51 %, improving the net energy balance ratio of ethanol from 1.26 (conventional) to 1.70. Lee and co-workers (2011) demonstrate that mesophilic anaerobic digestion might provide a more attractive option for enhancing the net energy gain in the existing corn-to-ethanol industry. Thin corn stillage treatment in a mesophilic CSTR at 25 day HRT rendered a methane yield of 271 L CH_4 kg^{-1} COD fed, which if incorporated in a corn-to-ethanol plant could increase the net energy balance ratio to 1.80.

Mesophilic or thermophilic digestion of whole corn stillage has only recently been studied. Biochemical methane potential (BMP) batch assays indicated significant methane potential for whole corn stillage at concentrations ranging from 6.35 to 50.8 g TCOD L^{-1}. Specific methane yields ranging between 401 and 458 L CH_4 kg^{-1} VS added and between 429 and 693 L CH_4 kg^{-1} VS added were obtained at mesophilic and thermophilic condition, respectively (Eskicioglu et al. 2011). However, continuous flow experiments with the full-strength whole corn stillage (254 g TCOD L^{-1}) at organic loading rates of 4.25, 6.30 and

9.05 g TCOD L^{-1} d^{-1} indicated unstable performance under thermophilic conditions and at mesophilic temperatures. Only at 60 day HRT was stable.

In sugarcane-based ethanol plants (Fig. 15.3), the bagasse generated is presently used directly as a solid biofuel to co-generate heat and electricity which is used in the plant and also sold to the electricity grid (Amorim et al. 2011). The vinasse produced (around 12 L for each litre of ethanol distilled (Amorim et al. 2011)), rich in minerals, such as potassium, calcium, magnesium, nitrogen and phosphorus, has been used as a fertilizer in the sugarcane fields. However, the increasing volume of vinasse is saturating the soil and threatening the quality of the ground water. It is not economically feasible to transport the vinasse over longer distances, and therefore a solution to this environmental problem should be found on location. AD is a viable approach to treat cane vinasse (Blonkaja et al. 2003; Peréz-Garcia et al. 2005; Seth et al. 1995; Souza et al. 1992) and contributes for the production of renewable energy.

Bagasse combustion and AD of vinasse are presently implemented at some distilleries at full scale (van Haandel 2005). By using steam turbines fuelled with bagasse combustion, electric power can be generated at a rate of 1 MWh per m^3 of produced alcohol. Anaerobic digestion can be applied to vinasse to produce enough biogas for 0.5 MWh/m^3 of alcohol, bringing total electric power production from subproducts to 1.5 MWh/m^3 of alcohol (van Haandel 2005).

Nowadays, bagasse is also generally recognised as a promising feedstock for cellulosic ethanol production, i.e. second generation (2G) bioethanol (derived from the non-food component of biomass) (Fig. 15.3), and it is expected that biofuel produced in this way will have less impact on the environment. Most processes and technologies for 2G bioethanol are still under development in different research activities and pilot/demo plants but are not yet on the market (IEA 2010). The challenge for biorefineries in the future is to use all side- and by-products from industry processes as well as crop residues.

Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept has been recently evaluated by Rabelo and co-workers (2011). Four different biofuel production scenarios showed that 63–65 % of the energy produced by bagasse combustion could be recovered by combining ethanol production with the combustion of lignin and hydrolysis residues, and AD of pretreatment liquors, whereas only 32–33 % of the energy was recovered by bioethanol production alone (Rabelo et al. 2011). The possibility of using wheat straw for the production of bioethanol (from cellulose), biohydrogen (from hemicellulose) and biogas (from effluents of bioethanol and biohydrogen production) was also evaluated (Kaparaju et al. 2009). Fermentation of cellulose, obtained from hydrothermal pre-treatment of wheat straw and enzymatic hydrolysis, yielded 0.41 g ethanol g^{-1} glucose, while dark fermentation of the hydrolysate produced 178.0 mL H_2 g^{-1} sugars, and the effluents from both bioethanol and biohydrogen processes produced methane with the yields of 0.324 and 0.381 m^3 kg^{-1} VS added, respectively. Six different wheat straws to biofuel production scenarios were further evaluated showing that either use of wheat straw for biogas production or multi-fuel production were energetically most efficient processes

compared to the production of mono-fuel such as bioethanol when fermenting C6 sugars alone (Kaparaju et al. 2009). Other studies focused on the evaluation of whole-crop biorefinery concept. For instance, Luo and co-workers (2011) investigated the utilisation of the whole rapeseed plant (seed and straw) for multiple biofuels production. An ethanol yield of 0.15 g^{-1} ethanol g^{-1} dry straw was obtained after combined alkaline peroxide and steam pre-treatment. Methane alone or hydrogen and methane were produced, in batch, from the individual process by-products (rapeseed cake, glycerol, hydrolysate and stillage) at similar energy yields (11–15 kJ g^{-1} VS). In continuous operation, only the two stage hydrogen and methane fermentation could work stably at an organic loading rate up to 6 g COD L^{-1} d^{-1} with average yields of 45 mL H_2 g^{-1} VS and 347 mL $CH_4 g^{-1}$ VS (Luo et al. 2011). The energy recovery efficiency from rapeseed plant increased from 20 % in the conventional biodiesel process to 60 % in the biorefinery concept, by utilisation of the whole rapeseed plant for biodiesel, bioethanol, biohydrogen and methane production.

15.3.3 Biogas Opportunities in Biodiesel Production

First generation biodiesel is produced from vegetable oils of oleaginous plants (e.g. rapeseed, soybean, sunflower, palm oil, etc.) by transesterification processes or cracking (Nigam and Singh 2011). The competition with agricultural land raised ethical issues and new generations of biodiesel appeared: the second generation from non-edible vegetable oil (e.g. jatropha) and from wastes (e.g. animal fat), and the third generation of biodiesel from algae (Rittmann 2008). Globally, there are more than 350 oil-bearing crops identified as potential sources for biodiesel production (Atabani et al. 2012). From the available techniques for oil conversion in biodiesel, transesterification of oil with alcohol in the presence of a catalyst is the most used and technically feasible (Marchetti et al. 2007).

The costs associated with biodiesel are a limiting factor for their utilisation. In future biorefinery concepts, by-products from the cultivation of energy crops should be used to produce other biofuels and/or added value products. For instance, although biodiesel could be the ultimate economical product in a biorefinery, the by-products from this process can also be utilised for the production of methane and hydrogen in anaerobic digesters, and consequently improving the energy and economic balance of these production systems (Borjesson and Mattiasson 2008). Besides, the excess energy can be sold to the public electricity grid, and the AD digestate may be used as fertilizer for the production of new biomass crops. The main biodiesel derived by-products are:

- Crude glycerol: it occurs in vegetable oils at a level of approximately 10 % (w/w). The make-up of crude glycerol varies depending on the parent feedstock and the biodiesel production process. Crude glycerol generated by the most usual method of homogeneous base-catalysed transesterification, and separated from biodiesel

by settling, contains approximately 50–60 % of glycerol, 12–16 % of alkalies (especially in the form of alkali soaps and hydroxides), 15–18 % of methyl esters, 8–12 % of methanol and 2–3 % of water (Kocsisova and Cvengroš 2006). COD of crude glycerol can exceed 1,000 g L^{-1} and the pH goes over 9.

- Biodiesel processing wastewater: water is used at the end of the biodiesel production chain to remove impurities such as excess of oil and methanol, residual catalyst, soap and glycerol. A large amount of wastewater is generated in this process, from 0.2 to 1.2 L per litre of biodiesel produced. This wastewater has a high pH value of approximately 9 and a COD content of hundreds of grams per litre, which is particularly attributed to methanol, glycerol and oil and grease (Phukingngam et al. 2011).
- Crop waste after oil extraction (cake): it refers to the remaining biomass (aquatic and terrestrial energy crop) after the oil extraction for biodiesel production. This waste still hold some lipids. Traces of solvent, salts and pigments are other examples of elements that may be found in the waste.

Wastes and by-products from the biodiesel industry still contain high energetic potential. To optimize the energetic balance of both aquatic and terrestrial crops used for biodiesel production, an anaerobic digestion process can be included in a biorefinery structure to convert the wastes and by-products in methane and hydrogen (Fig. 15.5).

Glycerol is the main by-product of biodiesel production (by the transesterification process). The crude glycerol possesses very low value because of the impurities contained. As the demand and production of biodiesel grow exponentially, the huge amounts of glycerol produced and subsequent destination is a problematic issue associated with biodiesel manufacturing. Usually, crude glycerol is refined and channelled to markets in the pharmaceutical and cosmetic industries (Demirbas 2009). When refined to a chemically pure substance, it would be a very valuable by-product. Purifying it to that stage is costly and generally out of the range of economic feasibility for the majority of small/medium biodiesel facilities. Currently, some biodiesel producing companies from the European Union are facing problems in getting rid of excess glycerol, as disposal is also quite expensive (Luo et al. 2011). Studies have been conducted to investigate alternative glycerol utilisation routes such as production of ethanol, 1, 3-propanediol and other high value products (Silva et al. 2009). Also, the AD of crude glycerol to produce methane and hydrogen is being explored to make biodiesel more competitive. However, high contents of COD and possible accumulation of fatty acids, the presence of methanol, inorganic salts, unreacted mono-, di-and triglycerides and methyl esters and the lack of nitrogen represents severe disadvantages for AD since these characteristics can inhibit the process.

The co-digestion of crude glycerol with a complementary substrate is the most used technique to overcome these problems, by decreasing the C:N ratio or by diluting the waste. For example, the co-digestion of potato processing wastewater with glycerol increased the methane per litre of wastewater treated by a factor of 1.5 (Ma et al. 2008). Adding glycerol to manure can increase significantly the

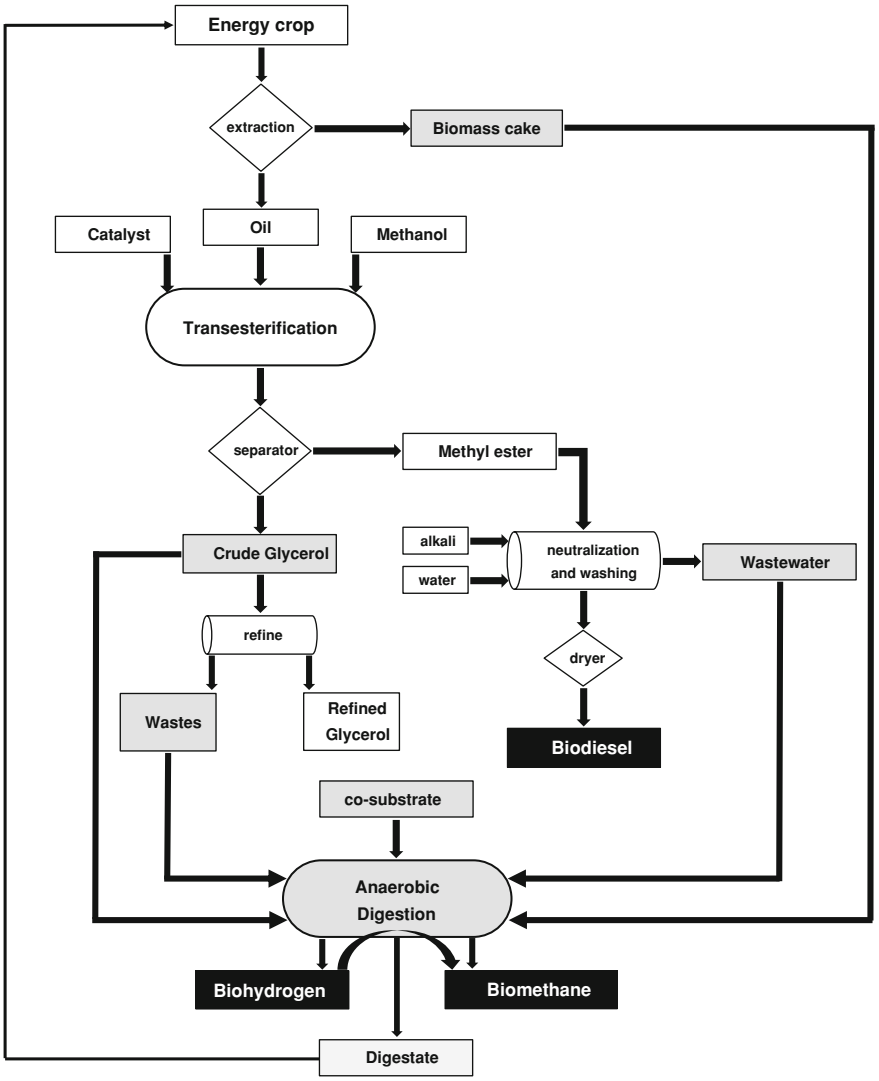

Fig. 15.5 Biorefinery concept for production of biodiesel, including an anaerobic digestion step for the energetic valorisation of wastes and by-products

methane production. Under mesophilic conditions, the addition of 4 % glycerol to screened manure increased biogas production by up to 400 %, and at thermophilic conditions, using sonicated mixtures of ground cattle manure with 6 % added glycerol, 0.35 m^3 CH_4 kg^{-1} COD removed were obtained (Castrillón et al. 2011). Amon and co-workers (2006) showed that the addition of crude glycerol (6 %) to a mixture of maize silage, pig manure and rapeseed meal, increased methane production from 570 to 680 L CH_4 kg^{-1} VS. Mesophilic anaerobic treatment of crude

glycerol as an only organic substrate is feasible, although the specific inhibition effects and requirements resulting from the nature and composition of the substrate can cause difficulties (Kolesarova et al. 2011). Glycerol should be carefully added to an anaerobic co-digestion facility because if certain threshold values are exceeded, severe damages can be done to the process and partial or complete inhibition can be caused. Different limits were reported, from 1 to 6 % of glycerol (Amon et al. 2006; Fountoulakis et al. 2010; Holm-Nielsen et al. 2008). At those limits no VFA accumulation was observed as signal of organic overloading. The different limits may be explained by the different characteristics of the co-substrate, mainly related with their nitrogen content and respective C:N ratio imposed in the anaerobic reactor.

Besides the traditional vegetable oil and animal fat, other substrates have been used to produce biodiesel, such as used-cooking oils. Also, biodiesel from microalgae is gaining market relevance, although mainly in research and development stage (see Sect. 15.3.4). According to the EN14214 (2008), the biodiesel obtained by the catalysed transesterification process requires purification, which generates large amounts of highly polluted wastewater. This wastewater has pH values in the range of 9.2–10.8, COD from 168 to 300 g L^{-1} and fat content of 18–22 g L^{-1} (Jaruwat et al. 2010). A combination of acidification—electrocoagulation with a subsequent AD step to efficiently purify wastewater derived from biodiesel manufacturing was developed (Siles et al. 2011). The anaerobic biodegradability of acidified—electrocoagulated wastewater was found to be 98 %, while the methane yield coefficient reached 297 L CH_4 kg^{-1} COD removed (1 atm, 0 °C). Also, the anaerobic co-digestion of glycerol and wastewater derived from biodiesel manufacturing, in which COD was found to be 1,054 and 428 g L^{-1}, respectively, was studied in batch laboratory-scale reactors at mesophilic temperature (Siles et al. 2010). Wastewater biodegradability was found to be near 100 %, while the methane yield coefficient was 310 L CH_4 kg^{-1} COD removed (1 atm, 25 °C).

The AD of the energy crop fraction not rich in oil and the remaining fraction after oil extraction are potentially good candidates for valorisation in an AD process. The high nitrogen content of lipid-free cake may be problematic for the process if ammonia exceeds inhibitory values (0.1–1.1 g N L^{-1}). Though the rapeseed cake can be efficiently degraded with a methane yield of 378 L CH_4 kg^{-1} VS, corresponding to 82 % of the theoretical value (Luo et al. 2011). Gunaseelan (2009) examined two integrated biorefineries schemes for the energetic valorisation of *Jatropha curcus*: (1) convert plant pruning, fruit hulls and de-oiled seed cake to methane concomitantly with oil to biodiesel, and (2) convert the seeds, plant pruning and fruit hulls entirely to methane. According to the author, the first scheme would produce 90 GJ ha^{-1} y^{-1} (with 54 GJ from the oil) and the second alternative would produce 97 GJ ha^{-1} y^{-1}. These results were obtained based on batch assays; therefore, conclusions should be drawn carefully.

As conclusion is possible to state that anaerobic co-digestion technology could be readily integrated into existing biodiesel facilities, thus establishing true biorefineries and revolutionising the biodiesel industry by dramatically improving its economics (Yazdani and Gonzalez 2007). Besides, AD could help circumvent the

disadvantages of chemical catalysis such as low product specificity, use of high pressure and/or temperatures, inability to use crude glycerol with high levels of contaminants, etc. For instance, for 1 ha rapeseed plant per year, 1,230 kg biodiesel and 627 kg ethanol could be obtained, but also, 27.4 kg hydrogen and 1,626 kg methane can be achieved by anaerobic co-digestion of the by-products (Luo et al. 2011).

15.3.4 Biogas Opportunities from Algae

Currently, biodiesel from vegetable oils and bioethanol from starch or sugar crops are the most technically feasible and commercialised alternative renewable biofuels. Algae should be seen as a promising source for bioenergy production in the future, since it has several advantages over other energy crops, including high yields and growth rates, the capacity to capture CO_2, and do not compete with food crops for arable land (Table 15.2). Two major drawbacks are still associated with the production and transformation of algae to bioenergy, i.e. the quantity of nutrients required and the high costs associated with dewatering. The inclusion of

Table 15.2 Advantages and disadvantages of algae biomass as energy crop

Advantages	Disadvantages
High photon conversion efficiency (high biomass yields per hectare)	Costs of cultivation
Produced all year round	Supply of CO_2 for high efficiency production
High growth rates	Harvesting process
Numerous species	High sodium concentration in marine species
Load on freshwater source is reduced (can utilise salt and wastewater streams)	Presence of sand
Do not compromise food production (improved land use efficiency)	High content of nitrogen and phosphate
Help in bio-fixation of waste CO_2 (CO_2-neutral fuel production)	Low C/N ratio
Assimilate nutrients and produce dissolved oxygen	
Does not require herbicides or pesticides application	
Valuable co-products such as proteins and residual biomass (fertilizer)	
Biochemical composition can be mutated to increase the yield	
Low lignin content	
Releases low amounts of H_2S	
Produces non-toxic and highly biodegradable biofuels	
Double credits under new EU directives	

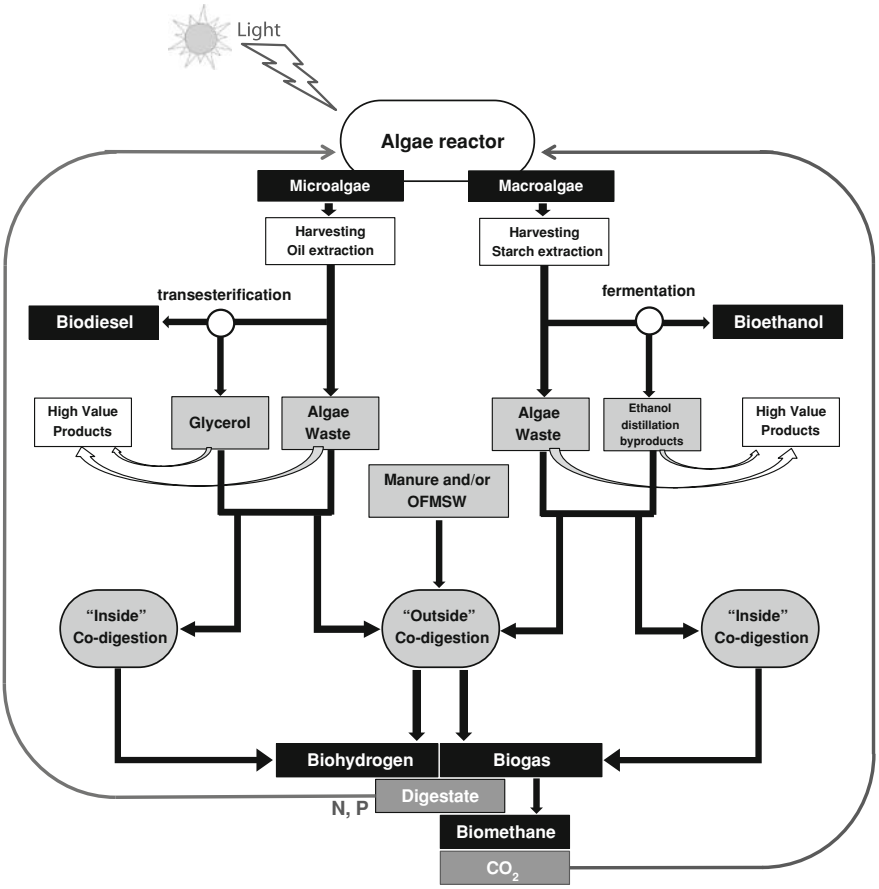

Fig. 15.6 Algae-based biorefinery showing the several biofuels produced and the potential role of anaerobic co-digestion

an AD process, in a biorefinery concept (Fig. 15.6), may help in overcoming both problems since it may provide the necessary nutrients (and CO_2) for the biomass cultivation, and supply the energy needed for a positive economical balance.

Algae are already used for the production of several high value products, including pigments, antioxidants, fatty acids, vitamins, pharmaceutical products and protein-rich feed for both animal and human consumption. Regarding the biofuels market, algae are the only feedstock potentially capable of completely replace the fossil fuel dependency. In the last three or four decades, many public and private investors, like ExxonMobil, have become interested and millions of dollars have been invested worldwide. Research in algae-biomass for biodiesel, bioethanol, biogas and hydrogen is a hot topic nowadays, although there is not yet production of biofuel from algae at a commercial scale (Demirbas 2009; Rusten and Sahu 2011).

Both micro (photosynthetic cells mostly unicellular) and macroalgae (multicellular, fast growing, marine and freshwater plant-like) have the necessary characteristics to be used as biomass for biofuel production. Microalgae can have up to 80 % of the dry weight in lipids, being therefore a potential good energy crop for biodiesel production. The yields of oil and fuels can be much higher (10–100 times) than terrestrial energy crops. However, unlike terrestrial energy crops, extensive drying is required before the biofuels production as the presence of water will inhibit several downstream processes, such as lipid extraction and transesterification.

The extraction of lipids from microalgae generates a by-product, mainly composed of proteins and polysaccharides, which can go up to 60 % of the total biomass. There is an increased demand for protein-rich substances available for human food and animal feed. However, in a more integrated approach, microalgae residues can be fermented to produce bioethanol and/or biogas and have further high-value products extracted in a biorefinery type concept (Fig. 15.6). Also, a two-step AD biorefinery may be a good alternative, with the consecutive production of hydrogen and methane. Mussgnug and co-workers (2010) observed an increase of 23 % in the methane yield from *Chlamydomonas reinhardtii* after H_2 production, compared with the AD of fresh microalgae.

There is no substantial knowledge about the production of bioethanol from microalgae and/or microalgae wastes but there are several research studies exploring their methane potential (Table 15.3). The methane yield is very dependent on the algae species, values of 187 and up to 387 L CH_4 kg^{-1} VS were obtained with *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*, respectively. Regarding the biohydrogen production, 66 L H_2 kg^{-1} VS were obtained with the residues of *Scenedesmus* after transesterification and pre-treatment with NaOH at 100 °C (Yang et al. 2011).

Macroalgae are more suitable for bioethanol or biogas production, due to the high carbohydrates content that can go up to 60 %, depending on the species, the season and place of cultivation. In contrast, the low content in lipids makes the biodiesel production unfeasible. Currently, no commercially credible assessment of the economic feasibility of macroalgae cultivation for biofuel production has been published. However, several research studies report the BMP of macroalgae (Table 15.3). Species from the genera *Gracilaria*, *Laminaria*, *Ulva* and *Sargassum* are the most studied and with highest methane potential. Currently, the vast majority of macroalgae are collected for human consumption and for hydrocolloid production.

The use of macroalgae resources is improbable to support a stand-alone biogas or bioethanol process. However, it is possible to introduce these processes into an existing facility where other biomass raw materials can be processed, such as co-digestion with manure or the OFMSW (Fig. 15.6). Couple algae production and wastewater treatment seems a very promising approach to two big markets, fuels production and wastewater treatment. In fact, wastewaters derived from municipal, agricultural and industrial activities potentially provide cost-effective and sustainable means of algae-biomass growth for subsequent biofuels production

Table 15.3 Macro and microalgae species and respective biochemical methane potential (BMP)

	$\%CH_4$	L CH_4 kg^{-1} VS	Conditions	Pre-treatment	Reference
Microalgae					
Arthrospira platensis	61	293	38 °C/batch	–	Mussgnug et al. (2010)
Chlamydomonas reinhardtii	66	387	38 °C/batch	–	Mussgnug et al. (2010)
Chlamydomonas reinhardtii	–	310	38 °C/batch	24 h @ 105 °C	Mussgnug et al. (2010)
Chlamydomonas reinhardtii	–	476	38 °C/batch	H_2 production	Mussgnug et al. (2010)
Chlorella sp.	–	245	35 °C/CSTR (HRT = 15d)	Dried, transesterification, add glycerol	Ehimen et al. (2011)
Chlorella sp.	68	302	35 °C/CSTR (HRT = 15d)	Dried, transesterification	Ehimen et al. (2011)
Chlorella kessleri	65	218	38 °C/batch		Mussgnug et al. (2010)
Chlorella kessleri	–	159	38 °C/batch	24 h @ 105 °C	Mussgnug et al. (2010)
Chlorella vulgaris	–	240	35 °C/CSTR (HRT = 28d)	–	Ras et al. (2011)
Dunaliella salina	64	323	38 °C/batch	–	Mussgnug et al. (2010)
Euglena gracilis	67	325	38 °C/batch	–	Mussgnug et al. (2010)
Scenedesmus obliquus	62	178	38 °C/batch	–	Mussgnug et al. (2010)
Macroalgae					
Enteromorpha sp.	–	154 ± 7	37 °C/batch	–	Costa et al. (2012)
Gelidium amanssii	–	239	35 °C/batch	Ethanol production (fermentation residue)	Park et al. (2012)
Gelidium amanssii	–	283	35 °C/batch	Ethanol production (saccharification residue)	Park et al. (2012)
Gracilaria sp.	–	280–400	35 °C/batch	–	Bird et al. (1990)
Gracilaria sp.	–	182 ± 23	37 °C/batch	–	Costa et al. (2012)
Laminaria sp.	–	260–280	–	–	Chynoweth (2005)

(continued)

Table 15.3 (continued)

	$\%CH_4$	L CH_4 kg^{-1} VS	Conditions	Pre-treatment	Reference
Laminaria digitata	–	500	–	–	Morand and Briand (1999)
Laminaria digitata	–	219	35 °C/batch	–	Adams et al. (2011)
Macrocystis	–	390–410	–	–	Chynoweth (2005)
Ulva sp.	59	110	35 °C/batch	–	Briand and Morand (1997)
Ulva sp.	55	94	35 °C/batch	Washed	Briand and Morand (1997)
Ulva sp.	49	145	35 °C/batch	Dried	Briand and Morand (1997)
Ulva sp.	52	177	35 °C/batch	Ground dried	Briand and Morand (1997)
Ulva sp.	54	203	35 °C/CSTR (HRT = 15d)	Grounded	Briand and Morand (1997)
Ulva sp. (hydrolysis juice)	–	313–330	BFBR (HRT = 2.5–5d)	Pre-hydrolysed	Morand and Briand (1999)
Ulva sp.	–	127	35 °C/batch	–	Otsuka and Yoshino (2004)
Ulva sp.	–	180	35 °C/batch	Washed, dried, grounded	Otsuka and Yoshino (2004)
Ulva sp.	–	148	35 °C/batch	Centrifuged and grounded	Peu et al. (2011)
Ulva sp.	–	196 ± 9	37 °C/batch	–	Costa et al. (2012)
Sargassum fluitans	–	143–182	–	–	Gunaseelan (1997)
Sargassum pteropleuron	–	119–171	–	–	Gunaseelan (1997)
Sargassum sp.	–	260–380	–	–	Chynoweth (2005)
Sargassum spp.	–	120–190	35 °C/batch	–	Bird et al. (1990)
Seaweed	44	120	37 °C/batch	–	Nkemka and Murto (2010)
Seaweed (leachate)	62	120	37 °C/batch	Pre-hydrolysed	Nkemka and Murto (2010)

(Pittman et al. 2011). AD may be used to convert the algae-biomass in bioenergy (biogas and hydrogen), and algae may act as a remediation agent by removing nutrients and sequestering CO_2, making an in situ biomethane upgrade. It was already reported that the anaerobic co-digestion of *Ulva* sp. with waste activated sludge seems to have a positive synergetic effect on the sludge biodegradability rate, with an increase up to 26 % (Costa et al. 2012).

The biogas produced in the AD step may serve as the primary source of energy for the production and conversion of the algae-biomass. Moreover, the CO_2 generated from the combustion of biogas or from the purification to biomethane, and the nutrient-rich digestate formed during the AD, can be recycled in a closed-loop to produce algae-biomass (Fig. 15.6). Two main bottlenecks can be identified in the AD of algae biomass. First, the biochemical composition and the nature of the cell wall may decrease their biodegradability. Then, the high cellular protein content, impaired after the oil extraction in the case of microalgae, imposes a low C/N ratio (around 6:1), far from the ideal for anaerobic digestion, and may potentially result in the production of toxic ammonia concentrations (Sialve et al. 2009). The co-digestion of microalgae residues with a nutrient-deficient co-substrate, such as glycerol, the major by-product generated in the biodiesel industry, has the potential to improve the overall energy recovered as methane. It was reported that a C/N ratio of 12.4 increased the methane production by more than 50 % when co-digesting the microalgae residues with glycerol, compared with the methane production obtained by digesting the residues alone (Ehimen et al. 2011). It is important to state that glycerol is a versatile chemical with more than 1,000 known commercial applications; however, this market has becoming saturated due to the strong growth in biodiesel production.

In conclusion, we can say that microalgae have high oil content but are difficult to cultivate and harvest and macroalgae present low-cost cultivation and harvesting possibilities but are low in lipids. In a biorefinery all routes should be explored, either to produce biofuels or high-value products. Therefore, it seems very attractive the integration of an AD step in an algae-based biorefinery since it seems the logical answer for the two major drawbacks previously detected, generates energy that can balance the unfavourable energetic bill, and can provide the nutrients and carbon dioxide needed for the biomass growth. Concomitantly, algae may be seen as a bioremediation agent to remove nutrients and capture CO_2 in a wastewater treatment plant.

15.3.5 Biogas from Syngas

Anaerobic digestion is suitable for converting virtually all organic materials to methane. However, some more recalcitrant substrates, such as lignocellulosic biomass or other dry wastes (plastic and rubber, etc.), demand (thermo) chemical pre-treatments, which are often costly and do not always substantially improve methane production. Gasification of all kind of compact biomass/wastes, followed by a biological process for the conversion of the resulting syngas (mixture of CO,

CO_2 and H_2) to methane, would be a feasible and promising alternative for the valorisation of recalcitrant materials.

Coal gasification has been traditionally used to produce syngas, which can be further used in thermochemical catalytic processes to produce fuels, such as methane (Fig. 15.7a). However, syngas bioconversion (Fig. 15.7b) has several advantages over catalytic processes: it can operate at milder temperatures and pressures, a fix CO/H_2 ratio is not required, there is less susceptibility to the impurities in the gas, and it does not require any costly pre-treatment of the feed gas or costly metal catalysts (Abubackar et al. 2011; Henstra et al. 2007). Syngas direct conversion to methane can be accomplished by various methanogens, such as *Methanosarcina* and *Methanothermobacter* species (Eqs. 15.1 and 15.2) (Daniels et al. 1977; O'Brien et al. 1984; Rother and Metcalf 2004).

$$4CO + 2H_2O \rightarrow 3CO_2 + CH_4 \tag{15.1}$$

$$CO + 3H_2 \rightarrow CH_4 + H_2O \tag{15.2}$$

Alternatively, a two-step process could be designed in which:

1. CO is firstly converted into acetate by acetogenic carboxydotrophic bacteria (Eqs. 15.3 and 15.4) or to H_2 by bacteria able to perform the water shift reaction (Eq. 15.5):

$$4CO + 4H_2O \rightarrow CH_3COO^- + 2HCO_3^- + 3H^+ \tag{15.3}$$

$$2CO + 2H_2 \rightarrow CH_3COO^- + H^+ \tag{15.4}$$

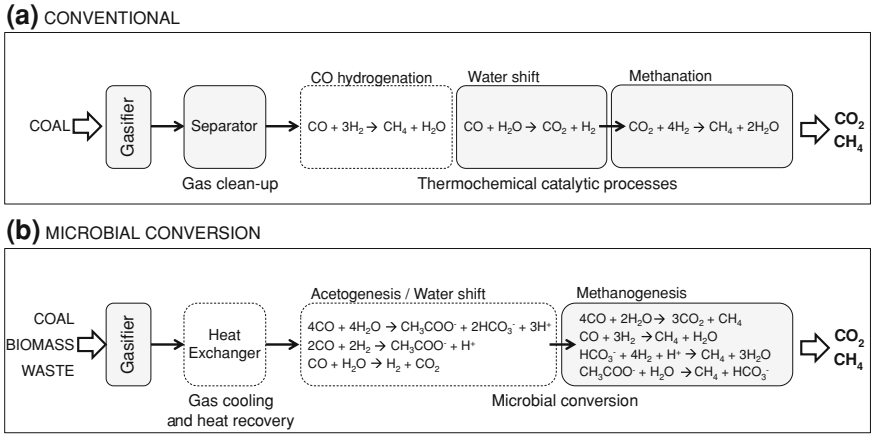

Fig. 15.7 Conventional (thermochemical) **a** and microbiological **b** routes for methane production from syngas deriving from coal, biomass or recalcitrant wastes (adapted from Basu et al. 1993)

$$CO + H_2O \rightarrow H_2 + CO_2 \quad (15.5)$$

2. Acetate and H_2 are further converted into methane by acetoclastic (Eq. 15.6) and hydrogenotrophic (Eq. 15.7) methanogens:

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad (15.6)$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \quad (15.7)$$

Hydrogenotrophic methanogens can utilise H_2 and CO_2 initially present in syngas for producing methane as well.

The capability of CO conversion to acetate has been identified in several bacteria from different taxa, e.g. *Clostridium*, *Peptostreptococcus*, *Moorella* and *Desulfotomaculum* species (Henstra et al. 2007). H_2 production via water shift reaction has been shown to occur in anaerobic bacteria, as for example *Rhodospirillum rubrum* (Kerby et al. 1995), *Rhodopseudomonas palustris* (Jung et al. 1999), *Carboxydothermus hydrogenoformans* (Svetlitchnyi et al. 2001), *Carboxydibrachium pacificum* (Sokolova et al. 2001), *Carboxydocella thermoautotrophica* (Sokolova et al. 2002), *Thermincola carboxydiphila* (Sokolova et al. 2005), etc.

Thus far, most of the studies on syngas anaerobic conversion have focused on the utilisation of pure cultures of microorganisms and strongly directed to ethanol production (Abrini et al. 1994; Cotter et al. 2009; Kundiyana et al. 2011). Fu and Mazzella (1990) described the potential of using pure/defined-cultures to convert CO and H_2 into methane. These authors developed a two-stage process for continuous syngas conversion: in the first stage, a small amount of syngas feed was used by *Peptostreptococcus productus* to produce acetate, which would be used in the second stage bioreactor for stimulating culture growth. The second stage employed a co-culture of *Rhodospirillum rubrum* and *Methanobacterium formicicum* for combined water shift and methanation of the remaining syngas feed.

Syngas conversion to methane by anaerobic mixed cultures is practically unexplored, and few reports are available on this subject (Guiot et al. 2011; Sipma et al. 2003). Sipma and co-workers (2003) tested seven anaerobic sludges from wastewater treatment reactors for their ability to convert CO at 30 and 55 °C. All the tested sludges could convert CO in the assays at mesophilic temperature, with a CO depletion rate between 0.14 and 0.62 mmol CO day^{-1}. Conversion of CO at 55 °C was achieved by five of the tested sludges and CO depletion rates varied between 0.73 and 1.32 mmol CO day^{-1}. Methane and/or acetate and methane and/or H_2 were the main products deriving from CO conversion during incubation at mesophilic and thermophilic conditions, respectively. Continuous CO conversion to methane, using a closed-loop 30 L gas-lift reactor, has been shown by Guiot et al. (2011). A maximum CO conversion of 75 % was obtained for a CO partial pressure of 0.6 atm and a gas recirculation ratio of 1:20. Under these conditions, methane yield (CH_4/CO) was approximately 95 % and other metabolites accumulated only at trace concentrations.

15.4 Future Prospects

Anaerobic digestion is an established technology with thousands of known applications worldwide. In a circular biobased economy concept (cradle to cradle), the reuse of all waste streams to produce valuable products and/or fuels should be mandatory. AD represents a relatively cheap technology, that integrated in biodiesel and/or bioethanol facilities, in a biorefinery concept, can represent a significant milestone in the economic viability of those technologies by using their (waste) water and by-products to generate biofuels (biomethane and/or biohydrogen) and a biofertilizer.

Several topics still need optimisation to definitively make AD of energy crops and biofuels production by-products economically feasible. Development of efficient and economically viable pre-treatments to improve the biodegradation of more recalcitrant feedstocks is urgent to increase the yield of AD processes. Biogas produced in AD plants is primarily composed of methane and CO_2, but it contains traces of other gases (NH_3, H_2S, etc.). To be used as a vehicle fuel or injected in the natural gas grid it has to be upgraded and compressed. Diverse technologies have been developed during the past years (water scrubbing, carbon molecular sieves, membranes, etc.). These techniques are costly and independent from the AD process, therefore suitable and costly technologies still to be developed.

Algae are the only crop capable of replacing the fossil fuel dependency, even though all the potential of algae-based biorefineries, it is still in the beginning of its development and many research and development is needed to achieve the desired efficiency and competitiveness. Genetic and metabolic engineering is likely to play an important role in improving microalgae strains to increase the lipids content and the easiness of extraction. The possibility to release valuable biochemical molecules using enzymatic hydrolysis from microalgae without dewatering the culture could have a major impact on the energetic needs for algae biofuels production. In fact, drying the algal biomass consumes about 69 % of the input energy (Jones and Mayfield 2011; Sander and Murthy 2010). Algae are a remediation agent that can be used in wastewater treatment. Couple algae-biomass production, nutrients removal, CO_2 sequestration and biogas production may represent an important milestone in the bioenergy goals, since the market of wastewater treatment is immense. However, an appropriate technology for biomass harvesting must be developed to bridge these technologies.

Concerning the syngas platform biorefinery, a significant challenge for the effective utilisation of syngas biologically is clearly the modest gas-liquid mass-transfer rates of the conventional gas–liquid contacting technologies (e.g. stirred tank reactors, airlift reactors or bubble columns) and the low solubility of the major syngas components in the aqueous culture medium (Bredwell et al. 1999). In fact, microbial syngas conversion depends strongly on the mass transfer of syngas to water (van Kasteren et al. 2005). One way of addressing this issue, and a future challenge in syngas fermentation, is the improvement of the volumetric mass transfer coefficient (kLa) for syngas-components and the development of appropriate bioreactor design.

In the future, biogas production will be based on a wide range of aquatic and terrestrial energy crops that will grow with sustainable and versatile methods. Organic waste, by-products from the food, agriculture and biorefinery industry will be naturally included in the several AD plants available worldwide.

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Chapter 16
Production of Bioethanol from Biomass: An Overview

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Abstract This chapter analyzes the main research trends on production of fuel ethanol from lignocellulosic materials. The main features of different pretreatment and detoxification methods are presented. The importance of process integration to simplify the overall process and improve the conversion of biomass into ethanol is discussed. Strategies for microbial strain development are disclosed in the framework of such integrated processes like simultaneous saccharification and co-fermentation and consolidated bioprocessing. The main challenges to fully develop the biomass-to-ethanol process are highlighted. Finally, the need of integrating the research efforts on molecular techniques and process integration is recognized.

16.1 Lignocellulosic Biomass as Feedstock

The biomass is organic matter made by living organisms that contain energy stored from the sun. The radiant energy from sunlight is absorbed by plants. This energy is converted into chemical energy in the form of glucose, starch or cellulose, through photosynthesis. The energy contained in the biomass (bioenergy) can be released and used by means of its combustion. Thus, the woody biomass is employed by many rural communities all around the world for heating and cooking. The biomass can also be burned in boilers to produce heat and electricity (solid biofuels). In addition, it can be transformed into liquid biofuels that, in turn,

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are used by the transport sector. Many organic materials can be converted into gaseous biofuels by anaerobic digestion. In this way, the biomass has become the fourth largest energy source after coal, oil and natural gas. It is the most important renewable energy option at present. In terms of energy, the annual global primary production of biomass is equivalent to the 4,500 EJ (1 EJ = 10^{18} J) of solar energy captured each year. The global energy consumption is 490 EJ today, but the current use of biomass for energy is only 50 EJ, mainly in the form of traditional noncommercial woody biomass (Ladanai and Vinterbäck 2009).

The lignocellulosic biomass represents a source of sugars with a great availability on Earth. Many of the materials with a high lignocellulosic content are wastes from different economic activities, in particular, agricultural residues. Thus, their economic utilization for biofuel production implies the employ of plant material not usable for human food. The lignocellulosic biomass has the potential of being a valuable feedstock for production not only of liquid and solid biofuels, but also of a relatively wide spectrum of chemicals and materials. In fact, in the framework of the future biorefineries, the biomass can become the long-term source of hydrocarbon chains and building blocks needed for the humankind to meet its huge requirements of basic organic compounds, synthetic polymers, pharmaceuticals, housing products, and among many others.

In the specific case of the liquid biofuels, the lignocellulosic biomass has acquired great relevance due to its significant content of different fermentable sugars that can be processed into ethanol or even into other fuel alcohols like butanol. These so-called second generation biofuels do not employ the fermentable sugars obtained from resources utilized for food production as sugarcane, sugar beet, corn, or other cereals. Thus, the "food vs. fuel" dilemma can be solved in an environmentally and socially sustainable way leading to the potential global utilization of the vast biomass resources available in almost each country of the world. This ideal situation contrasts with the control exercised by governments and corporations on fossil resources, which are not distributed evenly in the crust. In addition, the biomass is a renewable resource that can be used to sustainably supply bioethanol over the long term. Moreover, it is recognized that the utilization of fuel ethanol produced from lignocellulosics allows the net reduction of greenhouse gas emissions and can contribute to the diversification of rural economies in particular cases (e.g. dedicated energy crops).

However, the main drawback of the lignocellulosic biomass is its recalcitrance that imposes several challenges to the scientific and engineering global community. Due to this recalcitrance, the valuable sugars contained in the biomass are not easily accessible making necessary the employ of chemical and biochemical agents and physical chemical procedures to breakdown the complex lignocellulosic structure. In addition, the most used industrial ethanologenic microorganisms are not capable of assimilating in an efficient way all the sugars released during the processing of biomass. This decreases the conversion and product yield of the overall process and discourages high scale commercial projects for bioethanol production from lignocellulosic materials. Fortunately, the ongoing research and development as well as the existing demonstrative facilities allow being optimistic

about the possibility to use this versatile resource for fuel ethanol production on a large scale as a realistic alternative to fossil fuels.

The lignocellulosic complex is the most abundant biological material on Earth. Its production is estimated at about 200×10^9 ton per year, only 3 % of which is used in nonfood areas, such as the paper and pulp industries (Zhang 2008). An important fraction of economically significant crops corresponds to lignocellulosic materials. For instance, in European countries, 35 % of the harvested over ground biomass of the wheat crop is straw and 45 % is grain (Claassen et al. 1999). The lignocellulosic biomass is made up of very complex biopolymers nonusable for food. The main components of lignocellulosic biomass are cellulose, hemicellulose, and lignin in addition to a small amount of extractives, acids, and minerals (Cardona et al. 2010b).

The cellulose, a β-glucan, is a polymer composed of glucose molecules linked by β (1,4) bonds. It contains between 7,000 and 15,000 glucose units. Due to its linear nature and to the interactions by hydrogen bonds between the OH groups of a same chain or of different chains, cellulose forms very stable crystalline microfibers difficult to break. In general, the cellulose composes 40–60 % of dry matter of lignocellulosic biomass (Hamelinck et al. 2003). The hemicellulose composes 20–40 % of lignocellulosic biomass and consists of very short branched chains of monosaccharide units (200 in average). In their order, the monosaccharides present in hemicellulose are xylose and arabinose (both pentoses), and galactose, glucose and mannose (these latter sugars are hexoses). Other carbohydrate-related compounds like glucuronic, methyl glucuronic and galacturonic acids are also present in hemicellulose structure. Furthermore, the hemicellulose contains, in a lower proportion, acetyl groups esterified to some OH groups of its different sugars. Due to the predominance of xylose, the hemicellulose can be considered as a xylan. Considering its branched structure, the hemicellulose does not form crystalline structures but amorphous ones. Thus, this biopolymer is more soluble in water and has a higher susceptibility to the hydrolysis (Hamelinck et al. 2003). The cellulose and hemicellulose are the source of fermentable sugars for different process microorganisms to convert them into ethanol.

The lignin comprises from 10 to 25 % of lignocellulosic biomass. This component is a very complex phenolic polymer composed of phenyl propane units linked by C–C and C–O–C bonds forming a three-dimensional amorphous structure (Lee 1997). The structural units of lignin are based on the cinnamyl alcohols. Thus, *p*-hydroxyphenyl units are derived from the *p*-coumaryl alcohol, the guaiacyl units are derived from the coniferyl alcohol, and the syringyl units are derived from the sinapyl alcohol. The lignin has hydrophobic character and is a sort of cement between the cells. The interaction and combination between the hemicellulose and lignin provide a covering shell to the cellulose making its degradation more difficult.

The different lignocellulosic materials can be classified into the following groups according to their origin: agricultural wastes (straws, corn stover), agroindustrial residues (sugarcane bagasse, olive stone), hardwood (poplar, oak, birch, and aspen), softwood (pine, fir, cedar, and spruce), cellulosic wastes (newspaper,

waste office paper, and paper sludge), herbaceous biomass (switchgrass, alfalfa hay, and coastal Bermuda grass), and municipal solid waste (wasted paper, cardboard, fruit and vegetable peels, garden residues, and wood items). The most representative materials regarding their potential as feedstock for fuel ethanol production among the agricultural and agro-industrial residues are sugarcane bagasse, corn stover, and wheat straw.

Sugarcane bagasse is generated in sugar mills after extracting the juice from the cane stalks. It is used as a solid biofuel for production of steam and electricity required by the same sugar-production process, remaining frequently an electricity surplus to be sold to the grid (Cardona and Sánchez 2007). About 540 million tons per year of sugarcane bagasse are produced (Cardona et al. 2010a). The yield of bagasse from each ton of sugarcane ranges from 280 kg (Moreira 2000) to 312 kg (Kim and Dale 2004). In turn, the ethanol yield from bagasse reaches 140 L/ton, which implies a global potential production of ethanol from cane bagasse of 58.2 million L/year, an amount greater than all the ethanol produced worldwide in 2007 (Cardona et al. 2010b; Kim and Dale 2004). One feature of cane bagasse related to the other lignocellulosic materials is its low ash content (about 2.4 %) that implies a better performance during fermentation.

Corn stover comprises the stems, leaves, and cobs resulting from corn harvest and is considered as a very promising feedstock for ethanol production in USA, since it is the most abundant agricultural residue in that country: 196 million ton (Graham et al. 2007). A factor to be considered in the case of agricultural residues is that, unlike sugarcane bagasse, their total utilization can lead to soil erosion and reduction of its organic matter content, so the sustainable fraction of collectable crop residues should be defined. Conservation tillage practices for crop residue removal require that 30 % or more of the soil surface be covered with crop residues after planting to reduce soil erosion by water (Kim and Dale 2004). Cereals straws comprise the dry stalks of a cereal plant, crushed or not, after the grain or seed has been removed and have a high content of hemicellulose related to cellulose. Wheat straw presents a high availability in the world (about 529 million ton/year). Wheat straw is an attractive low cost feedstock for production of fuel ethanol because of abundance, renewability and low lignin content. Wheat straw contains lower amounts of lignin and higher levels of cellulose and hemicellulose compared to corn stover (Buranov and Mazza 2008).

Wood lignocellulosic biomass has been considered as a potential feedstock for bioethanol not only in the case of the wood itself, but also in the case of its derivatives (sawdust, shavings, and the collected biomass resulting from forestry activities such as branches, stalks, trunk pieces and trees from forest thinning). Softwood has a higher content of lignin making it more difficult to process into fuel ethanol compared to hardwood. Herbaceous biomass, in turn, represents the lignocellulosic materials from grasses and related plants that have neither woody stems nor woody roots. These plants present reduced lignin content, grow very fast and have reduced nutritional requirements, so they are excellent candidates for their exploitation as crops dedicated to bioenergy production. In this sense, switchgrass has been proposed repeatedly with this purpose.

16.2 Biomass-to-Ethanol Process

Conversion of lignocellulosic raw materials into fuel ethanol is a complex process. It requires several steps, some of which are not completely developed at the present day. Compared to the ethanol production from sucrose-containing materials like sugarcane and sugar beet, the biomass processing implies a greater amount of unit processes and involves a higher amount of substances to be dealt with. For instance, after the breakdown of the lignocellulosic complex, many compounds are released along with several types of carbohydrates (fermentable and not fermentable) present in the lignocellulosic matrix. In addition, some degradation products are formed that can be toxic for fermenting microorganisms. For this reason, it is necessary to include additional steps such as detoxification and neutralization of the pretreated biomass. Moreover, the main component to be transformed into ethanol, the cellulose, should be hydrolyzed prior to or simultaneously with the fermentation step. This hydrolysis step is also distinctive of the production of fuel ethanol from starchy materials like corn. The comparison of ethanol production processes from the three main types of feedstocks is presented in Table 16.1.

Regarding the possibilities to implement several ways of process integration, the biomass-to-ethanol process offers some significant opportunities, which is derived of its inherent complexity. As a mean to decrease the productions costs and improve the efficiency of the different processing steps, the accomplishment of two or more procedures in one single vessel or in a coupled way has become one of the main research directions for production of ethanol from biomass. The goal is to decrease the cost of producing one liter of ethanol from lignocellulosic materials compared to the employ of sucrose-containing feedstocks or starchy materials (see Table 16.1).

The conversion of lignocellulosic biomass into fuel ethanol requires several process steps: pretreatment, detoxification, cellulose hydrolysis, fermentation, ethanol separation and dehydration, and effluent treatment. In addition, the production of some co-products may require some additional steps in the framework of the biorefinery concept.

16.2.1 Pretreatment of Lignocellulosic Biomass

The pretreatment plays a key role in the overall process for ethanol production from biomass. If this step is not successfully accomplished, the conversion of feedstocks will become very low and the costs will be unjustifiably high. The pretreatment procedures themselves and their efficiency directly affect the amount, availability, and quality of the fermentable sugars that will be assimilated by the microorganisms during the subsequent fermentation, the step where the ethanol is formed. At the same time, this process step is energy consuming and may require the addition of chemicals that need to be accounted in the final ethanol cost and environmental performance of the global process. In fact, the pretreatment is one

Table 16.1 Comparison of ethanol production processes

Aspect	Sugar to ethanol	Starch to ethanol	Biomass to ethanol
Availability of raw materials	High	Medium	Very high
Utilization of food resources	Yes	Yes	No
Conditioning of feedstock	Milling, simple pH adjustment	Wet or dry milling	Milling
Pretreatment	Not required	Not required	Required; partial or total degradation of hemicellulose
Detoxification	Not required	Not required	Some degradation products after pretreatment should be removed
Hydrolysis of carbohydrate polymers	Not required	Amylases are used	Cellulases or acids are used
Fermentation	Batch and continuous regimes; fermentation of glucose and fructose	Batch and continuous regimes; glucose fermentation	Mainly batch processes; hexose fermentation, pentose fermentation (optional)
Process microorganism	*S. cerevisiae*, *Z. mobilis*	*S. cerevisiae*, *Z. mobilis*, *K. marxianus*; amylolytic recombinant yeasts	*S. cerevisiae*, *Z. mobilis*, *C. thermocellum*; recombinant *S. cerevisiae*, *Z. mobilis*, *E. coli*
Possibilities of reaction–reaction integration	Not needed	SSF, CBP	SSF, SSCF, CBP
Possibilities of reaction–separation integration	Yes	Yes	Yes
Possibilities of energy integration	Yes	Yes	Yes
Possibilities of co-generation	Yes; sugarcane bagasse can be burned to obtain thermal and electric energy	No	Yes; solid residue containing lignin can be employed for co-generation
Main co-products	Concentrated stillage for fertilization; press mud for animal feed	DDGS (dry milling); CCDS (wet milling)	Furfural, bioproducts from xylose fermentation, and lignin
Existing large-scale commercial production facilities	Yes, mostly in some tropical countries (Brazil, India, Colombia)	Yes, mostly in North America and Europe	No
Production costs, US$/L anhydrous ethanol (Sánchez and Cardona 2008)	0.198–0.215	0.233–0.338	0.396
Ouput/input energy ratio (Sánchez and Cardona 2008)	8.0 (sugarcane); 1.9 (sugar beet)	1.34–1.53	6.0

CBP consolidated bioprocessing, *CCDS* corn condensed distiller's solubles, *DDGS* dried distiller's grains with solubles, *SSCF* simultaneous saccharification and co-fermentation, *SSF* simultaneous saccharification and fermentation

of the most expensive steps: the unit costs of pretreatment can reach about US$0.08/L EtOH (Mosier et al. 2005).

As mentioned above, the lignocellulosic biomass is a complex where the lignin and hemicellulose represent a sort of seal covering the cellulose, the biopolymer with the highest potential to release the fermentable glucose. Therefore, the pretreatment of the lignocellulosic materials has the following goals:

- Breakdown of the cellulose-hemicellulose matrix.
- Reduction of the crystallinity degree of cellulose and increase of the fraction of amorphous cellulose.
- Hydrolysis of hemicellulose and formation of hexoses and pentoses derived from it.
- Release and partial degradation of lignin.
- Increase of the biomass porosity.
- Reduction of the formation of by-products inhibiting the subsequent steps of cellulose hydrolysis and fermentation.
- Elimination of the need of reducing the biomass particle size.

During the last 50 years, many methods to pretreat the lignocellulosic biomass for ethanol production have been proposed. Some of these methods have reached a certain degree of maturity (e.g. dilute-acid pretreatment), while it is expected that the research on other less aggressive methods can contribute to the improvement of the overall process in terms of both economic and environmental performance. These methods can be divided into physical, chemical, physical–chemical, and biological. In a previous review (Sánchez and Cardona 2008) and in the well-known work of Sun and Cheng (2002), the details of several of these procedures were disclosed. The main features of the pretreatment methods are presented in Table 16.2.

Most reports on ethanol production from biomass involve the use of dilute-acid pretreatment, steam explosion, ammonia fiber explosion (AFEX), alkaline pretreatment, and organosolv process. The first two methods share certain similarity related to the employ of moderate or high pressure under acidic conditions (steam explosion releases acetic acid or may be improved by adding sulfuric acid) making the cellulose more accessible to further enzymatic hydrolysis and allowing the formation of pentoses and hexoses from hemicellulose. It is considered that steam explosion is the most effective method for hardwood while dilute-acid hydrolysis shows higher recovery of hemicellulose-derived sugars, although it is most expensive due to the need of neutralizing the resulting streams from the process (Lynd 1996; Sánchez and Cardona 2008). Both methods release potential toxic substances for fermenting microorganisms. AFEX is one of the leading pretreatment technologies, which significantly enhances enzymatic digestibility without physically stripping out hemicellulose and lignin from the biomass, unlike other pretreatments (Gao et al. 2010). Although AFEX has lower levels of inhibitor generation and lignin loss, it requires the addition of xylanases to degrade the remaining hemicellulose (Lau et al. 2009). The latter two pretreatment methods mentioned above are chemical procedures that do not need high pressures and exhibit a high decrease of the crystallinity degree of cellulose and significant

Table 16.2 Main features of different pretreatment methods of the lignocellulosic biomass

Method	Fundament	Further cellulose conversion	Hemicellulose degradation	Lignin degradation/ solubilization	Inhibitors formation	Remarks	References
Physical methods:							
Mechanical comminution	Size reduction of biomass particles	50–60 %	–	No	No	Very high energy costs; reduces cellulose crystallinity	Alvo and Belkacemi (1997)
Pyrolysis	Thermal treatment without oxygen	–	Yes	No	Mostly phenols, benzene, furan, furfuryl derivatives, and oxygenat. compounds	>300 °C; formation of volatile products and char	Khiyami et al. (2005)
Physical chemical methods:							
Steam explosion	Hydrothermal treatment using saturated steam with subsequent decompression	Up to 90 %	80–100 %	No	Yes, mostly furfural	160–290 °C, $p = 0.69$–4.85 MPa; high solids load (>50 %); acids released from biomass catalyze the process; addition of H_2SO_4, SO_2 or CO_2 improves the effic. of further enzyme hydrolysis; hardwood	Ballesteros et al. (2004), Hamelinck et al. (2005)
Liquid hot water (LHW)	Pressurized hot water	>90 %	80–100 %	20–50 % lignin solubiliz.	None or low	170–230 °C, $p > 5$ MPa; low solids load (< 20 %)	Laser et al. (2002)
Ammonia fiber explosion (AFEX)	Ammonia treatment under pressure with subsequent decompression	50–90 %	0–60 %	10–20 % lignin solubiliz.	None or low	90–130 °C, $p = 1.12$–4.48 MPa	Dale et al. (1996), Gao et al. (2010)
CO_2 explosion	CO_2 treatment under pressure with subsequent decompression	>75 %	Yes	No	No	Up to 200 °C, $p = 5.62$ MPa	Agbor et al. (2011), Menon and Rao (2012), Sun and Cheng (2002)

(continued)

Table 16.2 (continued)

Method	Fundament	Further cellulose conversion	Hemicellulose degradation	Lignin degradation/ solubilization	Inhibitors formation	Remarks	References
Chemical methods:							
Ozonolysis	Treatment using ozone	>57 %	–	Yes	No	20–25 °C, $p = 0.1$ MPa; hardwood and softwood	Sun and Cheng (2002)
Dilute-acid hydrolysis	Treatment using dilute acids under moderate pressure and high temperature	–	80–100 %	No	Yes	120–200 °C, $p = 1$ MPa; 0.75–5 % H_2SO_4, HCl or HNO_3; up to 40 % solids load; hardwood, herbaceous biomass	Hamelinck et al. (2005)
Concentrated-acid hydrolysis	Treatment by concentrated acids and high temperature	–	~100 %	–	Yes; glucose degradation products	170–190 °C, $p = 0.1$ MPa; 10–30 % H_2SO_4; high solids load; acid recovery needed; MSW	Cuzens and Miller (1997)
Alkaline hydrolysis	Treatment using alkalis at moderate temperature	>65 %	>50 %	24–55 % lignin removal	Low	60–120 °C, $p = 0.1$ MPa; NaOH, $Ca(OH)_2$; hardwood	Kaar and Holtzapple (2000)
Microwave/radio-frequency assisted alkaline pretreatment	Dielectric heating of biomass presoaked in alkaline solutions by microwave or radio frequency	50–60 % (MW)/ 68 % (RF)	–	~100 % (MW)/ 75 % (RF) lignin removal	–	190 °C (MW)/90 °C (RF), $p = 0.1$ MPa; 0.1–0.2 g alkali/g biom.; 2.45 GHz (MW)/ 27.12 MHz (RF); 10–50 % solids load	Hu and Wen (2008), Hu et al. (2008)
Oxidative delignification	Use of peroxidase and H_2O_2	95 %	~100 %	50 % lignin solubiliz.	–	20 °C, $p = 0.1$ MPa; 2 % H_2O_2	Sun and Cheng (2002)
Wet oxidation	Treatment using oxygen under pressure and water	–	–	Yes	Yes	195 °C, $pO_2 = 1.2$ MPa; small amounts of Na_2CO_3 or H_2SO_4; high solids load	Varga et al. (2004)

(continued)

Table 16.2 (continued)

Method	Fundament	Further cellulose conversion	Hemicellulose degradation	Lignin degradation/ solubilization	Inhibitors formation	Remarks	References
Organosolv process	Treatment using organic solvents	–	~100 %	~100 % lignin solubiliz.	Yes	100–250 °C, $p = 0.1$ MPa; methanol, ethanol, acetone, ethylene glycol; solvent recovery needed; hardwood and softwood	Agbor et al. (2011), Pan et al. (2005)
SPORL	Treatment by dilute solutions of sodium bisulfite and sulfuric acid (liquor)	–	64–100 %	Up to 40 % lignin removal	Yes; basically furfural and HMF	180 °C, $p = 0.1$ MPa; 3:1 ratio of liquor to biomass	Zhu et al. (2010)
Pretreatment with ionic liquids	Dissolution of biomass by selecting proper ionic liquids, then addition of an anti-solvent to precipitate pretreated biomass without lignin	75–90 %	Yes	32–94 % lignin solubiliz.	Low	120 °C, $p = 0.1$ MPa; cations: alkyl methylimidazolium, alkyl methyl-pyridinium; anions: acetate, alkyl phosphate	Tan and Lee (2012), Weerachanchai et al. (2012)
Biological methods:							
Fungal pretreatment	Solid-state cultivation of brown-, white- and soft-rot fungi on biomass particles	–	–	Yes	No	30 °C, $p = 0.1$ MPa; slow process (3–5 weeks); fungi produces cellulases, xylanases and ligninases	Tengerdy and Szakacs (2003), Wang et al. (2012)
Biorganosolv pretreatment	Fungi decompose the lignin network followed by ethanol treatment	–	90–100 %	Partial lignin degradat.	No	30 °C (cultivat.), 140–200 °C (ethanolysis), $p = 0.1$ MPa	Itoh et al. (2003)

HMF hydroxylmethyl furfural, *MSW* municipal solid waste, *MW* microwave, *RF* radio frequency, *SPORL* Sulfite pretreatment to overcome recalcitrance of lignocellulose

efficiencies in the degradation of lignin (especially in the case of organosolv process). The main attractive feature of the alkaline pretreatment is its simplicity but with less efficiency in terms of further cellulose conversion. The employ of organic solvents is very suitable for lignocellulosic materials with high lignin content (e.g. softwood), but has the main disadvantage that they have to be recovered, which adds additional costs to the overall process. Undoubtedly, the separation, solubilization, or degradation of lignin is quite desirable, since it can alter or block the binding of cellulases to the cellulose in the following processing steps. Taking into account that one of the challenges of the biomass-to-ethanol process is the increase of the specific activity of these costly enzymes, methods that allow the fractionation of biomass (separation of cellulose and lignin, hydrolysis of hemicellulose) are very attractive, but the level of development of such procedures is still immature to justify a cost-effective operation. One alternative is the combination of two (or more) pretreatment methods in such a way that, during the first pretreatment procedure, the lignocellulosic matrix breaks down, the fraction of amorphous cellulose increases and hemicellulose degrades to recover valuable sugars, and then the lignin can be separated or degraded through the second pretreatment method. For instance, Shahbazi et al. (2005) proposed a sequential fractionation scheme involving steam explosion and alkaline delignification where several products can be obtained during the pretreatment of softwood: extractives, cellulose, lignin, and solubilized hemicellulose.

The pretreatment with liquid hot water (LHW) is one of the most promising and attainable at mid term method (10–15 years). Laser et al. (2002) point out that this method is comparable to dilute-acid pretreatment but without addition of acids or generation of neutralization wastes. LHW presents elevated recovery rates of pentoses and do not produce inhibitors under optimal conditions. However, the allowable solids load is much less than that for steam explosion that is usually greater than 50 %.

Recently, some relatively novel pretreatment methods are being developed. The utilization of ionic liquids offers some significant advantages related to their ability of solubilizing the lignocellulosic biomass at moderate temperature and under atmospheric pressure. The properties of the ionic liquids (extractive capabilities, low melting point, nonvolatility, high polarity, environmental friendliness, and high possibilities of being functionalized to act as acids, bases or ligands, among others) have made them to be very suitable to extract lignin and dissolve the carbohydrates present in the biomass (mostly cellulose) decreasing its crystallinity (Weerachanchai et al. 2012). During pretreatment with ionic liquids, they act as solvent to dissolve cellulose or lignin or both biopolymers disrupting the shield formed by lignin and hemicellulose. Ionic liquids can also alter cellulose crystalline structure when the dissolved cellulose is regenerated for subsequent reutilizations. This is accomplished by precipitating out from ionic liquids through the addition of anti-solvent such as water, methanol, ethanol and acetone (Tan and Lee 2012). Thus, the resulting pretreated biomass is essentially more susceptible to the enzymatic hydrolysis overcoming the barrier of the biomass recalcitrance.

Among the novel pretreatment methods proposed, the combination of microwave or radio frequency with alkaline pretreatment should be highlighted. In this case, the dielectric heating (microwave or radio frequency) uses the ability of some compounds to transform electromagnetic energy into heat. When the biomass is treated by dielectric heating, the more polar part will absorb more energy leading to a "hot spot" within nonhomogeneous materials. This type of heating results in an "explosion" effect among the particles and improves the disruption of the recalcitrant structures of the biomass (Hu et al. 2008). Other efforts have been directed to the application of electrolyzed water at low pH, which has a strong oxidizing effect, or at high pH, which has a reducing potential. The employ of electrolyzed water may allow further cellulose conversion of up to 95 % if combined with other methods like a mild alkaline pretreatment in order to remove the lignin and the hemicellulose with a minimum formation of inhibitors (Wang et al. 2010). Other new pretreatment methods like the sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) have shown a promising performance as well (see Table 16.2).

The fungal pretreatment is a low-cost option to improve the digestibility and fermentability of the lignocellulosic biomass, although its main drawbacks are the length of the process and the fact that the fungi consume part of the carbohydrates that, otherwise, could be converted into ethanol during the fermentation step. In particular, the white-rot fungi like *Coriolus versicolor* have the ability to degrade the lignin present in the biomass favoring the subsequent cellulose hydrolysis and glucose fermentation. If the biological pretreatment is followed with other procedure like LHW, the efficiency of the overall process can be significantly enhanced. Wang et al. (2012) demonstrated this approach obtaining, in addition, a high hemicellulose removal and significant increases in glucose yield during the following hydrolysis step using commercial cellulases. If the fungal pretreatment is accomplished following other mild pretreatment method, the length of the overall process can be significantly reduced. For instance, Yu et al. (2009) combined a chemical pretreatment (2 % H_2O_2 during 48 h) with the delignification using solid-state cultures of *Pleorotus ostreatus* and reached a reduction of the biological pretreatment from 60 to 18 d. The authors indicate that the enhancing of the efficiency could possibly attribute to the structure disruption of the biomass during the first pretreatment step. It should be noted that white-rot fungi produces cellulases as well, along with other valuable bioproducts like bioactive polysaccharides (Montoya et al. 2011a, b).

16.2.2 Detoxification of Biomass Hydrolyzates

After the pretreatment step, the lignocellulosic complex is broken down in a high degree, the cellulose reduces its crystallinity degree, the lignin and the cellulose are separated from each other, and the hemicellulose is mainly degraded. The resulting biomass slurry is separated into a solid fraction containing cellulose and lignin, and

a liquid fraction that contains the soluble compounds including the products of the hemicellulose hydrolysis. One indicator of the pretreatment efficiency is the recovery of sugars, i.e., the amount of released xylose, arabinose, mannose and other sugars derived from the hemicellulose. The sugars contained in this hemicellulose hydrolyzate are valuable considering that there exist some yeasts and other microorganisms, which are able to assimilate them in order to synthesize ethanol. Ethanol produced in this way is added to the ethanol produced during the conventional fermentation of the glucose released during the cellulose hydrolysis in order to improve the efficiency of the overall biomass-to-ethanol process. However, some degradation products formed as consequence of the action of chemicals, high temperature or pressure during pretreatment remain in the hemicellulose hydrolyzate along with extractives from the biomass itself. For instance, the furfural is formed as a degradation product of the xylose released under temperatures higher than 120 °C. In addition, the lignin can be partially degraded in dependence on the pretreatment method. This partial degradation is responsible for the phenolic compounds present in the hemicellulose hydrolyzate. If this hydrolyzate is to be used as a culture medium for ethanologenic fermentation (during the pentose fermentation as represented in Fig. 16.1), all these compounds are potentially toxic for the pentose-assimilating microorganisms. Alternatively, the whole slurry obtained after pretreatment can be directed to the cellulose hydrolysis step in order to obtain glucose. Thus, all the sugars from the hemicellulose degradation and cellulose hydrolysis (biomass hydrolyzate) are then fermented into ethanol by special microorganisms assimilating both hexoses (mostly glucose) and pentoses (mostly xylose) as will be discussed below. Nevertheless, the toxic compounds are present in this biomass hydrolyzate as well decreasing the efficiency of the fermentation step. For this reason, a detoxification step is required in both processing alternatives.

Several detoxification methods have been proposed in the framework of fuel ethanol production from lignocellulosic biomass. As in the case of the pretreatment, these methods can be divided into physical, chemical, and biological, whose main features are presented in Table 16.3. Alkaline detoxification is the most employed detoxification method, especially when $Ca(OH)_2$ is used (overliming). The addition of alkali up to very high pH values leads to the formation of a significant amount of precipitate composed by calcium salts, which entrain the inhibitory compounds or causes them to settle. In addition, many inhibitors are unstable at pH higher than 9. Alkaline treatment is considered one of the best detoxification procedures since a high percentage of substances such as furan aldehydes and phenolic compounds can be removed by this method, improving the fermentability of the resulting liquid medium especially when biomass hydrolyzates pretreated by dilute acid are employed (Persson et al. 2002). However, this method implies the implementation of a neutralization step after the lime is added and a subsequent filtration procedure to remove the precipitate. This negatively affects the economy of the global process.

The fermenting microorganisms such as yeast has a natural ability to reduce the furan aldehydes to the corresponding less inhibitory alcohols. This biological conversion of inhibitors into less inhibitory compounds directly in the fermenter is

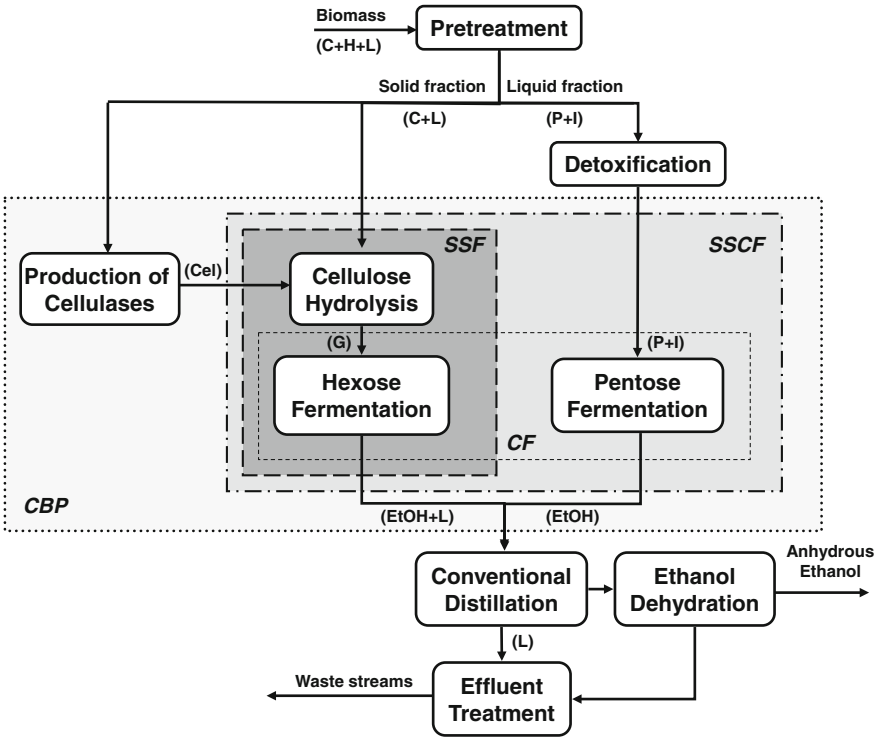

Fig. 16.1 Schematic diagram of the overall process for fuel ethanol production from lignocellulosic biomass. The possibilities for reaction–reaction integration are shown. *CF* co-fermentation, *SSF* simultaneous saccharification and fermentation, *SSCF* simultaneous saccharification and co-fermentation, *CBP* consolidated bioprocessing. Main components of the streams: *C* cellulose, *H* hemicellulose, *L* lignin, *Cel* cellulases, *G* glucose, *P* pentoses, *I* inhibitors, *EtOH* ethanol. From Cardona and Sánchez (2007)

named in situ detoxification. However, reduction of furfural by *Saccharomyces cerevisiae* has been associated with effects such as increased consumption of NADH, decreased growth, decreased ethanol productivity, and accumulation of toxic levels of acetaldehyde (Alriksson et al. 2011). Thus, the biological in situ detoxification of furfural may therefore represent a partially inhibited fermentation. In particular, it has been suggested that the primary cause of inhibition by furfural was the process of its reduction rather than a direct inhibitory action for the case of ethanologenic *Escherichia coli* (Miller et al. 2009). Precisely, a novel detoxification method proposed by Alriksson et al. (2011) implies the addition of reducing agents such as sodium dithionite or sodium sulfite directly to the fermentation vessel in order to accomplish the in situ reduction of inhibitors like furfural. In this way, the efficiency of the fermentation can be significantly enhanced and the ethanol yield can reach values above the reference fermentation based on a medium containing glucose and mannose without inhibitors.

Table 16.3 Main features of different detoxification methods of the hemicellulose hydrolyzate resulting from the pretreatment step

Method	Fundament	Remarks	Relative yield[a]	References
Physical methods				
Evaporation	Volatile inhibitors are removed	Reduction of acetic acidand phenolic compounds in nonvolatile fraction	93 %	Palmqvist and Hahn-Hägerdal (2000)
Extraction	Removal of inhibitors by using organic solvents	Diethyl ether, ethyl acetate; 3:1 org. phase to aqueous phase ratio	93 %	Cantarella et al. (2004), Palmqvist and Hahn-Hägerdal (2000)
Adsorption	Inhibitors are retained in the adsorbent bed	Activated carbon, amberlite hydrophobic adsorbent; some loss of sugars	90 %	Lee et al. (2011), Weil et al. (2002)
Chemical methods				
Neutralization	Modification of solubility of toxic compounds, then removal of them by membrane filtration or adsorption	$Ca(OH)_2$, CaO, NaOH, pH = 6	–	Cantarella et al. (2004), Yu and Zhang (2003)
Overliming	Removal of toxic compounds by entrainment in the precipitate of calcium salts (e.g. gypsum)	$Ca(OH)_2$, pH = 9–10.5, 50 °C; mostly removal of furfural, HMF, acetic acid, and part of phenolic compounds; low sugar loss	Comparable to that of ref. fermn.	Lin et al. (2012), Palmqvist and Hahn-Hägerdal (2000), Saha et al. (2005)
Ionic exchange	Resins adsorbs the inhibitors, regeneration with ammonia	Amberlyst A20, Poly(4-vinyl pyridine); removal of acetic acid, furfural and phenolic compounds	Comparable to that of ref. fermn.	Wooley et al. (1999b), Xie et al. (2005)
Detoxification with reducing agents	Reducing agents improves the conversion of furan aldehydes by the fermenting microorganisms	Sodium dithionite or sulfite; implementation of in situ detoxification during fermentation	>100 %	Alriksson et al. (2011)
Biological methods				
Enzymatic detoxification	Degradation (oxidation) of phenolic compounds by ligninases	Laccase, lignin peroxidase, 30 °C	–	Moreno et al. (2012)
Microbial detoxification	Bacteria degrade toxic compounds without utilizing sugars	50 °C, *Ureibacillus thermosphaericus*	Comparable to that of overliming	Okuda et al. (2008)

[a] Comparison of the ethanol yield for fermentation of the detoxified hydrolyzate related to that of the reference fermentation (100 %) based on a glucose-based medium without inhibitors
HMF hydroxymethyl furfural, *ref. fermn* reference fermentation

16.2.3 Cellulose Hydrolysis

The cellulose is the main source of fermentable sugars present in the lignocellulosic biomass for ethanologenic fermentation. After pretreatment and detoxification steps, the cellulose has been separated from the lignocellulosic complex and increased its amorphous fraction. Thus, this biopolymer is ready for its hydrolysis into glucose units or even for its direct conversion into ethanol. Unfortunately, the two microorganisms with the greatest probability to be used in a commercial process for bioethanol production at high scale nowadays (*S. cerevisiae* or *Zymomonas mobilis* are not able to assimilate the cellulose. For this reason, almost all the process configurations proposed for production of ethanol from lignocellulosics comprise a cellulose hydrolysis step. This process can be carried out by strong acids or cellulolytic enzymes. The former method implies the use of dilute or concentrated sulfuric or hydrochloric acids. The hydrolysis of cellulose through dilute acids is performed at 200–240 °C at 1.5 % acid concentration and entails the partial degradation of glucose into hydroxymethyl furfural (HMF) and other nondesirable products. The hydrolysis using concentrated acids (e.g. 30–70 % H_2SO_4) exhibits higher glucose yields (about 90 %) in relatively short times (10–12 h), but the acid recovery is required (Sánchez and Cardona 2008). This significantly increases the cost of the hydrolysis step.

The employ of cellulases has demonstrated better results for the subsequent fermentation since no degradation products are formed from glucose. However, the process is slower than the acid-catalyzed hydrolysis. Actually, complex cellulolytic preparations are used within the process for fuel ethanol production from biomass. These preparations contain several types of cellulases each one with different mechanisms of action on the cellulose. In general, the commercial cellulolytic products are obtained from *Trichoderma reesei* by submerged aerobic fermentation using glucose or lignocellulosic materials as feedstocks. This fungus releases a mixture of cellulases, among which at least two cellobiohydrolases, five endoglucanases, β-glucosidases and hemicellulases can be found (Zhang and Lynd 2004). Cellobiohydrolases break down $\beta(1,4)$ linkages from nonreducing or reducing ends of the cellulose chain releasing cellobiose or even glucose, whereas endoglucanases hydrolyze these same linkages randomly inside the chain. The action of cellobiohydrolases causes a gradual decrease in the polymerization degree while endoglucanases cause the rupture of cellulose in smaller chains reducing rapidly the polymerization degree. Endoglucanases especially act on amorphous cellulose, whereas cellobiohydrolases are capable to act on crystalline cellulose as well (Lynd et al. 2002). The β-glucosidases are responsible of hydrolyzing cellobiose formed by the action of cellobiohydrolases into two molecules of glucose. The combined action of these enzymes is synergic leading to the conversion of cellulose into glucose. The cellulases should be adsorbed onto the surface of substrate particles before hydrolysis of insoluble cellulose takes place. Three-dimensional structure of these particles in combination with their size and shape determines if β-glycosidic bonds are or are not accessible to enzymatic

attack (Zhang and Lynd 2004). This makes hydrolysis process to be slower related to the enzymatic degradation of other biopolymers. For comparison, the hydrolysis rate of starch by amylases is 100 times faster than the hydrolysis rate of cellulose by cellulases under industrial processing conditions (Cardona et al. 2010b). The most important factors to be taken into account for hydrolysis of cellulose contained in lignocellulosic materials are the reaction time, temperature, pH, enzyme dosage, and substrate load.

The utilization of cellulases directly affects the global costs of biomass-to-ethanol process. The cellulases available for ethanol industry account for 36–45 % of the costs of bioethanol produced from lignocellulosic materials. According to different evaluations (Mielenz 2001; Reith et al. 2002; Sheehan and Himmel 1999), a 30 % reduction in capital costs and 10-fold decrease in the cost of current cellulases are required for this process to be competitive related to ethanol produced from starchy materials. These analyses evidence the need of improving the cellulase performance in the following aspects: Increase of thermal stability, improvement of the binding to cellulose, increase of specific activity, and reduction of the non-specific binding to lignin. In particular, the increase of specific activity of cellulases may reduce the costs significantly. It is estimated that a 10-fold increase in specific activity could lead to US¢15.85 savings per liter of ethanol produced. Among the strategies to enhance the specific activity are the increase in the efficiency of active sites through protein engineering or random mutagenesis, augment of thermal tolerance, improvement in the degradation of the crystalline structure of cellulose, enhancement of the synergism among the cellulases from different sources, and reduction of non-specific bindings (Cardona et al. 2010b; Sheehan and Himmel 1999).

In general, the costs of cellulases are considered high. The cost of the currently available cellulase preparations is very high that limits the commercial implementation of ethanol production from biomass. This cost can reach US$16/100,000 filter paper units (FPU, a way for measuring cellulase activity). Percival Zhang et al. (2006) point out that Genencor International and Novozymes Biotech have developed submerged fermentation processes allowing the reduction of the cellulase cost from US$5.40/gal ethanol to about US$0.20/gal ethanol. Reduction in the cost of cellulases can be achieved only by concerted efforts, which address several aspects of enzyme production from the raw material used for production to microbial strain improvement. Use of cheaper raw materials and cost-effective fermentation strategies like solid-state fermentation can improve the economics of cellulase production (Sukumaran et al. 2009). According to preliminary evaluations of the National Renewable Energy Laboratory of the United States (NREL) cited by Tengerdy and Szakacs (2003), the cost of in situ cellulase production by submerged culture is US$0.38/100,000 FPU. Hence, cellulase costs comprise 20 % of ethanol production costs assuming them in US$1.5/gallon. On the other hand, and as cited above, commercial cellulase cost is prohibitive for this process. In contrast, these authors indicate that the cost for producing cellulases by solid-state fermentation of corn stover would be US$0.15/100,000 FPU that would correspond to US$0.12/gal EtOH, i.e. near 8 % of total costs.

Reduction in the cost of fuel ethanol may also be achieved by efficient technologies for saccharification (hydrolysis), which includes the use of more effective enzyme cocktails and hydrolysis conditions. *Trichoderma* fungi currently employed for commercial cellulase production produce very less quantities of β-glucosidase compared to the other cellulases. As cellobiohydrolases are inhibited by cellobiose, β-glucosidase from other source needs to be added in order to complement the action of the cellulases from this fungus. Moreover, the product of the reaction catalyzed by the β-glucosidase, the glucose, inhibits it. Due to this, the efficiency of enzymatic hydrolysis cannot be improved much more by increasing enzyme loading, so most of the enzyme added for saccharification remains unutilized. In this way, commercial preparations are supplemented with β-glucosidases from sources like *Aspergillus niger*. In the seek of a cost-effective process, the production of *T. reesei* cellulases and *A. niger* β-glucosidase by solid-state fermentation is being developed (Sukumaran et al. 2009).

In general, the main research directions for cellulase engineering of noncomplexed cellulase systems (fungal cellulases) are aimed at the rational design (protein engineering) for each cellulase, based on knowledge of the cellulase structure and the catalytic mechanism. In addition, the research efforts are also aimed at the directed evolution for each cellulase, in which the improved enzymes or ones with new properties were selected or screened after random mutagenesis and/or molecular recombination. The greatest advantage of directed evolution is that it is independent of knowledge of enzyme structure and of the interactions between enzyme and substrate. Finally, the research trends should be also directed to the reconstitution of cellulase mixtures (cocktails) active on insoluble cellulosic substrates, yielding an improved hydrolysis rate or higher cellulose digestibility (Percival Zhang et al. 2006).

16.2.4 Fermentation Step

During the fermentation step, all the sugars derived from the pretreatment and cellulose hydrolysis steps are converted into ethanol by using one or different fermenting microorganisms. There exist several options to perform this conversion depending on the type of sugars assimilated by the process microorganisms and on the technological configuration of the process.

16.2.4.1 Fermentation of Cellulose Hydrolyzates

Once the cellulose has been hydrolyzed, the resulting glucose-containing stream should be processed into ethanol. For this, conventional fermentation using yeast is mostly used. In this sense, the process is similar to that employed for fermenting the glucose solutions obtained after the enzymatic hydrolysis of the starch when starchy materials are used as feedstock. The ethanolic fermentation is one of the most

studied biological processes. Nevertheless, the need of increasing the efficiency of ethanol production including the usage of alternative feedstocks has led to the development of new fermentation methods with better techno-economic and environmental indicators. Traditionally, the most used microorganism for ethanolic fermentation is the yeast *S. cerevisiae*. However, for ethanol production from lignocellulosic biomass, there is a wider variety of process microorganisms employed (e.g. *Zymomonas* bacteria, xylose-assimilating yeasts, or thermophilic clostridia). *S. cerevisiae* converts hexoses in pyruvate through glycolysis, which is decarboxylated to obtain acetaldehyde that is finally reduced to ethanol generating two moles of ATP by each mol of consumed hexose under anaerobic conditions. In addition, this microorganism also has the ability to convert hexoses in CO_2 by aerobic respiration favoring the production of yeast cells. Therefore, aeration is an important factor for both cell growth and ethanol production. Although these yeasts have the ability to grow under anaerobic conditions, small amounts of oxygen are needed for synthesis of such substances like fatty acids and sterols. Inhibition of cell growth by ethanol decreases at microaerobic conditions related to fully anaerobic cultivation. *S. cerevisiae* has demonstrated its elevated resistance to the presence of inhibitors in the lignocellulosic hydrolyzate. In the case of the more productive continuous regime, one way to enhance this resistance is the increase in the cell retention to prevent wash-out and maintain high yeast cell density.

Other yeast species have been proposed for fermentation of cellulose hydrolyzates. In particular, the thermotolerant yeast *Kluyveromyces marxianus* has demonstrated their capability for fermenting glucose at relatively high temperatures of 40–45 °C (Singh et al. 1998). The bacterium *Z. mobilis* is one promising ethanologenic microorganism considering its ethanol yield higher than that of *S. cerevisiae* and its growth rate. The spectrum of assimilable substrate by this bacterium is quite similar to the yeast: glucose, fructose, sucrose and maltose.

The fermentation of cellulose hydrolyzates obtained after the enzymatic hydrolysis of the washed solid fraction of the pretreated biomass generally does not imply special difficulties since the inhibitor concentrations are very low. Nevertheless, compared to starch and sugarcane fermentations, the sugar concentrations after hydrolysis are often low with values approaching typically not more than 70 g/L (Brethauer and Wyman 2010). This is explained by the difficulties associated to the handling of suspensions with solids concentrations greater than 10 % by weight to the bioreactors for enzymatic hydrolysis of cellulose and to the inhibition of cellulases with the glucose formed. Thus, a concentration of the hydrolyzate stream might be needed to achieve higher concentrations, but the operating costs would be increased as well.

The fermentation of the hydrolyzates can carried out batchwise or continuously. In the latter case, the recycling of part of the effluent stream could be required due to the need of maintaining a high cell concentration. However, the recycling leads to the accumulation of inhibitors within the fermenter. Thus, a detoxification process is required if the whole slurry from pretreatment step enters the cellulose hydrolysis and fermentation steps.

16.2.4.2 Pentose Fermentation

The detoxified hemicellulose hydrolyzate resulting from the pretreatment step contains an important amount of pentoses and hexoses. The xylose, a pentose, is the most relevant sugar of this hydrolyzate. To take advantage of this stream, xylose-assimilating yeasts can be employed to produce ethanol, but in this case, the biomass utilization rates are lower related to microorganisms that only assimilate hexoses. Ogier et al. (1999) have compiled information about the main fermentative indexes for the most promising pentose-assimilating yeasts: *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus*. Most pentose-fermenting yeasts are mesophiles. One of the main challenges in pentose fermentation lies in the fact that the productivities of pentose utilizing microorganisms are less than those of hexose-fermenting ones. On the other hand, there are a few cases where the immobilization of these yeasts increases the ethanol productivity (Chandrakant and Bisaria 1998), unlike the case of hexose-fermenting yeasts or *Z. mobilis*. Generally, the assimilation rate of pentoses by natural pentose-fermenting microorganisms is slower than that for hexoses. For example, although *P. stipitis* yeast can naturally ferment pentose sugars to ethanol, hexose sugars are used preferentially, and pentose uptake is competitively inhibited by hexoses. Thus, pentose fermentation is only possible at very low glucose concentrations. In addition, microaerophilic conditions are required, which are difficult to maintain in large-scale systems, and even then yields are low (Brethauer and Wyman 2010).

The dimorphic filamentous fungus *Mucor indicus* is a promising alternative to *S. cerevisiae* as it is capable of xylose fermentation, is safe for humans, and produces ethanol from hexoses with comparable yields and productivities. Brethauer and Wyman (2010) have indicated the possibility of implementing continuous processes for ethanol production from biomass using this microorganism. Other microorganisms are able to assimilate both hexoses and pentoses. In particular, thermophilic clostridia have the ability to utilize xylose and synthesize ethanol as well. Ogier et al. (1999) also have compiled information about the fermentative indexes for the xylose-assimilating thermophilic bacteria *Clostridium thermosaccharolyticum*, *Thermoanaerobacter ethanolicus* and *Bacillus stearothermophilus*. Lynd et al. (2001) report low concentrations (in the order of 25 g/L) of ethanol obtained by *C. thermosaccharolyticum* cultivated in xylose-based media during batch and continuous cultures. These authors studied the influence of different factors limiting the substrate utilization for continuous cultures at progressively higher feed xylose concentrations.

16.2.4.3 Co-fermentation of Lignocellulosic Hydrolyzates

The co-fermentation process is aimed at the complete assimilation by the microbial cells of all the sugars resulting from lignocellulosic degradation and consists in the employ of a mixture of two or more compatible microorganisms that assimilate both the hexoses and pentoses present in the medium. This means that

the fermentation is carried out by a mixed culture. However, the use of mixed cultures faces the problem consisting in that microorganisms utilizing only hexoses grow faster than pentose-utilizing microorganisms leading to a more elevated conversion of hexoses into ethanol (Cardona and Sánchez 2007). To solve this problem, the employ of respiratory deficient mutants of the hexose-fermenting microorganisms has been proposed. In this way, the fermentation and growth activities of the pentose-fermenting microorganisms are increased considering that they grow very low when are cultivated along with rapid hexose-fermenting yeasts. Considering the indicators for the process using only the glucose-assimilating bacterium *Z. mobilis* grown on the biomass hydrolyzate, the productivities of the mixed culture are less than those of the bacterium, but the yields are comparable, which offers a space for further research (Delgenes et al. 1996). One of the additional problems arisen in this kind of configurations is that pentose-fermenting yeasts present a greater inhibition by ethanol that limits the use of concentrated substrates in the system (Cardona et al. 2010b).

Other variant of co-fermentation consists in the utilization of a single microorganism capable of assimilating both hexoses and pentoses in an optimal way allowing high conversion and ethanol yield. Although in the nature these microorganisms exist, their efficiency and ethanol conversion rates are reduced for implementation of an industrial process. Hence, the addition of an enzyme transforming the xylose into xylulose (xylose-isomerase) to the culture medium has been proposed. In this way, microorganisms exhibiting high rates of conversion to ethanol and elevated yields (like *S. cerevisiae*) can assimilate the xylulose involving it in the metabolic pathways leading to the ethanol. On the other hand, a high efficiency in the conversion to ethanol can be reached through the genetic modification of yeasts or bacteria already adapted to the ethanolic fermentation. The microorganisms most commonly modified for this purpose are *S. cerevisiae* and *Z. mobilis*, to which genes encoding the assimilation of pentoses have been introduced. The other approach for genetic modification is the introduction of genes encoding the metabolic pathways for ethanol production to microorganisms that are capable of fermenting both hexoses and pentoses in their native form. The "design" of ethanologenic bacteria like *E. coli* or *Klebsiella oxytoca* is an example of such type of approach. The employ of these recombinant microorganisms allows the implementation of the co-fermentation process intended to the more complete utilization of the sugars contained in the hydrolyzates of lignocellulosic biomass (Cardona and Sánchez 2007).

16.2.5 Ethanol Recovery and Dehydration

The recovery of ethanol produced by different technological configurations and from diverse types of feedstocks is accomplished in a very similar way. The ethanol content in the culture broth resulting from fermentation processes oscillates between 2.5 and 10 % (by weight). The utilization of fuel ethanol as a

gasoline oxygenate requires a high-purity ethanol, so it is necessary to concentrate the ethyl alcohol up to 99.5 % obtaining the anhydrous ethanol, which is the suitable form used for ethanol-gasoline blends. The first step of the ethanol recovery scheme is the concentration of ethanol contained in culture broths. This process is carried out in a distillation (concentration) column achieving ethanol content about 50 %. This product is removed from the column by a side stream. The overhead vapors contain mostly CO_2 (about 84 %), a significant amount of ethanol (12 %), and a small amount of water. The following step is the rectification of this concentrated stream in order to obtain a product with 90–92 % ethanol composition, which is near to the azeotropic mixture of ethanol and water (95.6 %). To achieve 99.5 % or more of ethanol purity from streams containing 90–92 % ethanol, it is necessary to employ nonconventional separation operations like pressure-swing distillation, azeotropic distillation, extractive distillation, adsorption, and pervaporation. All these operations have found industrial application in the fuel ethanol industry (Cardona et al. 2010b).

The adsorption is one of the most important unit operations currently employed in the biofuel industry for ethanol dehydration. In this operation, the ethanol–water mixture passes through an apparatus usually cylindrical that contains a bed with an adsorbent material. Due to the difference in the affinity of molecules of water and ethanol with respect to the adsorbent, the former remains entrapped in the bed while the ethyl alcohol passes through this same bed increasing its concentration in the stream leaving the apparatus. Adsorption of water employing the so-called molecular sieves to dehydrate ethanol has been the technology that has acquired more development in the last years in the fuel ethanol industry. In fact, this technology has been replacing the azeotropic distillation (Cardona et al. 2009).

The adsorption operation requires that, once the adsorbent bed is saturated with the water that needs to be removed, its desorption should be accomplished to make possible the reutilization of the adsorbent material (regeneration cycle). For regeneration of the sieves, hot gas is needed. This rapidly deteriorates them especially if the bed is fed with a liquid stream during the previous water adsorption cycle. To counter this deterioration, the pressure-swing adsorption (PSA) technology was developed. This technology involves the use of two adsorption beds. While one bed produces vapors of anhydrous ethanol superheated under pressure, the other one is regenerated under vacuum conditions by recirculating a small portion of superheated ethanol vapors through the saturated sieves. The system feeding is carried out using the overhead vapors from the rectification column. The ethanolic vapors obtained in the regeneration cycle and that can contain 28 % water are recirculated to the rectification column (Montoya et al. 2005; Wooley et al. 1999b). In this way, the molecular sieves life can be prolonged for several years that, in turn implies very low costs related to the replacement of adsorbent material, and therefore reduced operating costs (Guan and Hu 2003; Madson and Monceaux 1995). The process flowsheet for ethanol recovery and dehydration in the case of the adsorption with molecular sieves is depicted in Fig. 16.2.

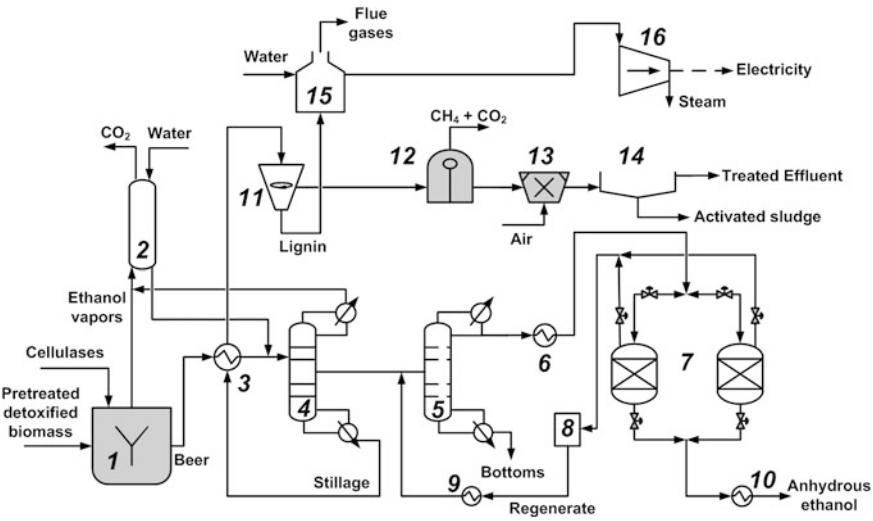

Fig. 16.2 Process flowsheet of product concentration, dehydration by molecular sieves and effluent treatment steps for ethanol production from lignocellulosic biomass; *1* SSCF bioreactor, *2* scrubber for recovery of ethanol vapors, *3* preheater, *4* concentration column, *5* rectification column, *6* 9 heat exchangers, *7* molecular sieves, *8* regenerate tank, *10* product cooler, *11* centrifuge, *12* anaerobic digester, *13* activated sludge tank, *14* clarifier, 15 boiler/burner, *16* turbogenerator

16.2.6 Effluent Treatment

Fuel ethanol production from lignocellulosic biomass generates solid wastes, atmospheric emissions, and liquid effluents. The atmospheric emissions mostly correspond to the gas outlet stream from fermenters or integrated bioreactors that is washed with water in the scrubbers in order to recover the volatilized ethanol (see Fig. 16.2). The gases exiting from the scrubbers contain mainly carbon dioxide that is released into the atmosphere. The CO_2 can be employed for production of dry ice or beverages. However, if these gases are not utilized, they should be considered in the calculation of the environmental impact of ethanol producing facilities (Cardona et al. 2010b). In this regard, it should be emphasized that the bioethanol practically presents net emissions of CO_2 near to zero considering that the plant biomass already fixed the CO_2 during its growth and only this carbon dioxide is released during the combustion of fuel ethanol in the engines. In contrast, the burning of fossil fuels releases into the atmosphere additional amounts of carbon dioxide that was fixed by the plant biomass millions years ago.

The solid wastes formed during production of fuel ethanol are strongly linked to the raw material from which it is produced. The production of lignocellulosic ethanol generates lignin as the most important solid residue. This polymer can be isolated during the pretreatment step if some pretreatment methods like the pretreatment with solvents (organosolv process) or oxidative delignification are

employed (this fact is considered in Fig. 16.3 et seq.). Nevertheless, most pretreatment methods allow that the lignin remains in the solid fraction resulting from this processing step along with the cellulose. After enzymatic hydrolysis using cellulases, the lignin remains in the liquid suspension until the end of the process where it can be recovered from the stillage stream as shown in Fig. 16.2. The lignin has a high-energy value (25.4 MJ/kg), and therefore it is used as a solid biofuel for feeding boilers or cogeneration.

The stillage is the major effluent of all flowsheets for ethanol production involving the submerged fermentation of streams containing sugars or carbohydrate polymers. The stillage represents the residual liquid material obtained after distillation of ethanol from the fermented wort (wine) and contains both solid and soluble matter. Precisely, the elevated organic load of the stillage is responsible of the high polluting properties of this burden, so this stream should undergo treatment to reduce this load and minimize the environmental impact during its discharge to the water streams. Due to the elevated organic matter content of stillage, methods for its treatment and economic utilization should be implemented in the industry. Among the most used methods for stillage treatment, the irrigation, recycling, evaporation, incineration, anaerobic digestion, and composting should be highlighted. Some of the new treatment methods allow the production of value-added products as discussed elsewhere (Cardona et al. 2010b). By applying process systems engineering tools like process simulation, Cardona et al. (2006) performed the evaluation of several option for treatment of the stillage generated during ethanol production from biomass. The results obtained show that the best option comprises the centrifugation of stillage, incineration of the solids obtained in a cogeneration unit for production of power and steam, and anaerobic digestion of the thin stillage resulting from centrifugation followed by aerobic biological treatment. This configuration is schematically depicted in Fig. 16.2. The proposed treatment of the thin stillage coincides with the treatment scheme suggested by Merrick and Company (1998).

16.3 Process Integration for Ethanol Production from Biomass

The process integration offers a great potential to process industry through the improvement of chemical and biotechnological processes. This potential is especially recognized if considering the many ways in which process integration contributes to the enhancement of energy efficiency of many industrial processes as detailed in a previous work (Cardona et al. 2008). Process integration, as a mean for process intensification, is a successful approach for designing improved technological configurations for fuel ethanol production in which production costs can be reduced. This fact is remarkably important taking into account that the main objective of using liquid biofuels like bioethanol is the progressive displacement of

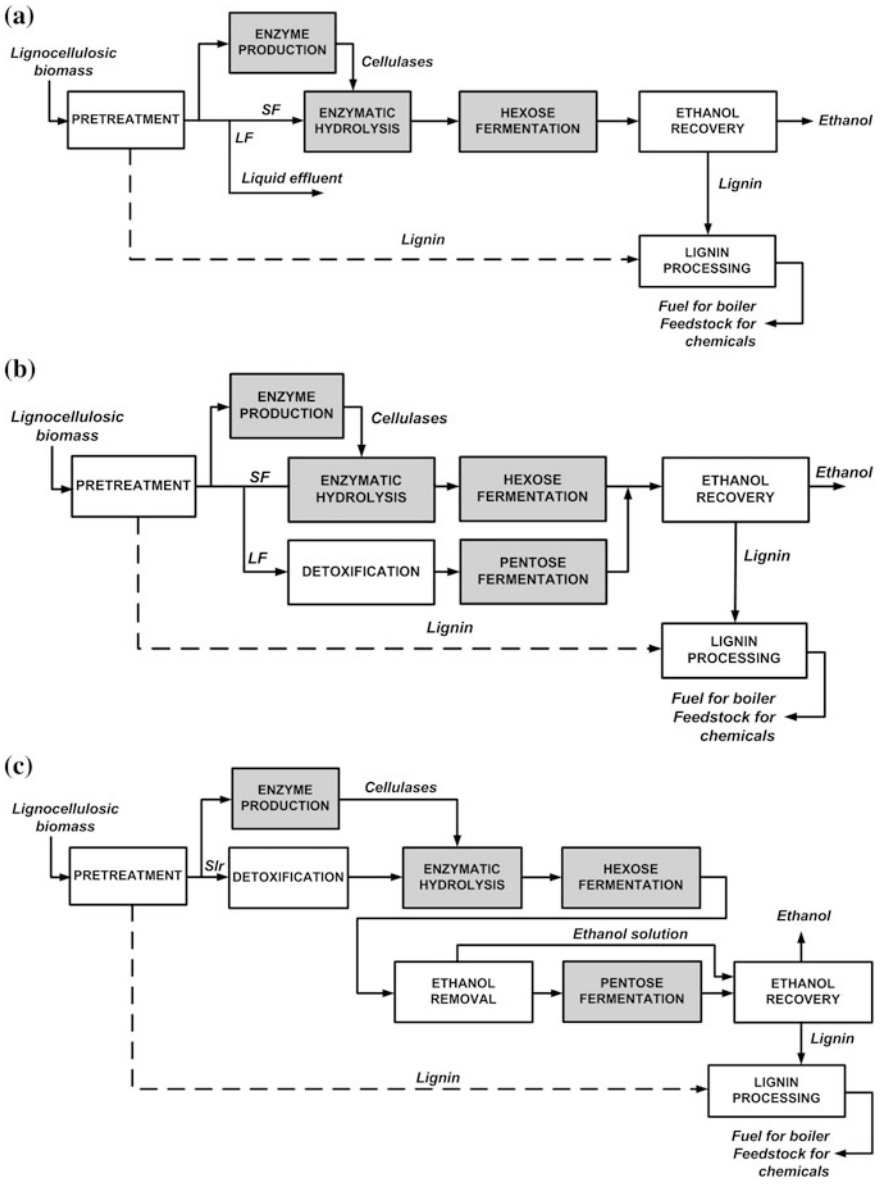

Fig. 16.3 Technological options for fuel ethanol production from lignocellulosic biomass by separate hydrolysis and fermentation (*SHF*). **a** SHF without utilization of hemicellulose-derived pentoses, **b** SHF with hexose and pentose fermentation performed in parallel, **c** SHF with pentose fermentation performed after hexose fermentation; *LF* liquid fraction, *SF* solid fraction, *Slr* slurry, the *gray-shaded boxes* represent enzymatic or microbial processes; the *dashed lines* represent an optional source of lignin when a pretreatment method allowing the fractionation of biomass is used (e.g. organosolv process)

fossil fuels that implies the sustainable exploitation of the huge biomass resources of our planet and the utilization of clean and renewable energy sources.

16.3.1 Reaction–Reaction Integration

Process integration is gaining more and more interest due to the advantages related to its application in the case of bioethanol production: reduction of energy costs, decrease in the size and number of process units, intensification of the biological and downstream processes, improvement of environmental performance of the overall process, and among others. For instance, the combination in a same single unit of the enzymatic hydrolysis and the microbial transformation leads to the reduction of the negative effect due to the inhibition of the enzymes caused by the products of the reaction catalyzed by them. This corresponds to an integration of the reaction–reaction type. There exist different possibilities for reaction–reaction integration during production of ethanol from lignocellulosic biomass (see Fig. 16.1). This type of integration mainly includes the combination of the enzymatic reactions for hydrolysis of cellulose with the microbial conversion of formed sugars into ethyl alcohol.

To expose the possibilities of process integration in the framework of biomass-to-ethanol process, it is necessary to indicate the main features of the basic non-integrated configuration, the separate hydrolysis and fermentation. Then, different integrations options are briefly described.

16.3.1.1 Separate Hydrolysis and Fermentation

The initial configuration proposed for bioethanol production comprises the cellulose hydrolysis followed by the fermentation of the glucose released during the enzymatic process. This configuration is called separate hydrolysis and fermentation (SHF) and has a sequential character. The main feature of SHF is that each step can be performed at its optimal operating conditions (for instance, 50 °C and pH of 4.5 for cellulose hydrolysis and 32 °C and pH of 4–5 for yeast fermentation). SHF is the technology with most possibilities to be implemented at demonstration semi-industrial facilities or commercial scale. In fact, most of the existing demonstration plant for bioethanol production from lignocellulosics employs this technology. For instance, Abengoa Bioenergy has developed a SHF technology on a pilot scale in York (Nebraska, USA), which has been tested in the demonstration plant located in Salamanca (Spain). This plant has operated over 5,000 h continuously, achieving a production of 5 million liters of ethanol per year from cereal straw and herbaceous biomass. It is considered the largest demonstration plant in the existence to produce second-generation bioethanol in the world. This company has announced the beginning of construction of the first

commercial plant that will produce 100 million L/year of bioethanol from corn stover, wheat straw, and switchgrass in Hugoton (Kansas, USA) (Abengoa 2011).

The main options for producing ethanol from biomass by SHF are depicted in Fig. 16.3. It should be noted that detoxification is not needed if the pretreated biomass is thoroughly washed with water in order to obtain a solid stream containing cellulose (and lignin) to be converted into glucose by the cellulases. Although several companies are developing new and more effective cellulases for ethanol industry, their cost is still elevated so most designs proposed involve the production of the cellulase required by the process in the same ethanol production facilities (in situ cellulase production). For this, part of the pretreated biomass is diverted to a submerged aerobic fermentation where fungal cells utilize it to synthesize extracellular cellulases. However, this scheme does not make use of the sugar released during the pretreatment that undoubtedly reduce the overall conversion of the process and final ethanol yields.

In order to increase the conversion of biomass into ethanol, the sugars derived from hemicellulose degradation should be utilized. For this, xylose-fermenting microorganisms like *C. shehatae* or *P. stipitis* are employed to produce ethanol from the hemicellulose hydrolyzate (this process is indicated as pentose fermentation in Fig. 16.3) along with the conventional yeast used for fermentation of the glucose derived from cellulose hydrolysis (hexose fermentation). In this case, the pentose fermentation can be carried out in a separate unit in parallel or after the glucose fermentation. In the latter scheme, ethanol should be removed from the broth resulting from hexose fermentation to decrease the end-product inhibition effect of the growth rate for xylose-assimilating yeasts, which are more sensitive to ethanol than glucose-assimilating yeasts or bacteria. In addition, a detoxification step is required as the whole slurry from pretreatment enters the cellulose hydrolysis and the two fermentation steps. This slurry contains toxic compounds that can inhibit not only the growth of fermenting microorganisms, but also the enzymes employed for glucose production from cellulose even if it is diluted with water.

Depending on the pretreatment method, the lignin can be recovered in this step (e.g. by the organosolv process) or remain in the stillage of the distillation column used for the preliminary ethanol recovery. Thus, the lignin can be separated from the stillage by centrifugation and burnt in boiler for generating the steam required by the overall process. Alternatively, the lignin may be employed as a feedstock for production of valuable chemicals or adsorbents like activated charcoal.

16.3.1.2 Separate Hydrolysis and Co-fermentation

The first integration approach is to perform the transformation of both pentoses and hexoses in one single fermenting unit (see Fig. 16.4). This process can be called separate hydrolysis and co-fermentation (SHCF), and has the advantage of reduced capital costs since no additional vessel is required for pentose fermentation. SHCF can be considered an example of reaction–reaction integration since

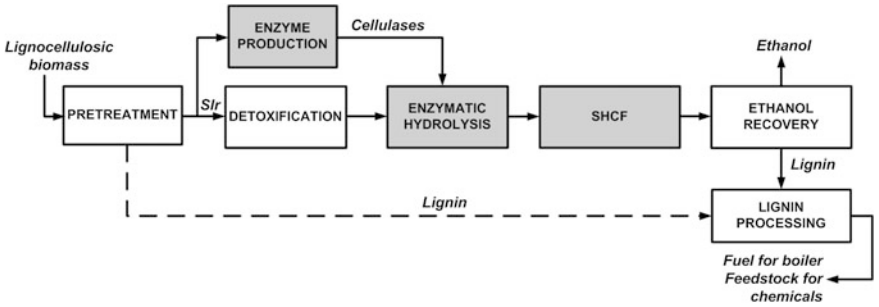

Fig. 16.4 Schematic diagram of fuel ethanol production from lignocellulosic biomass by separate hydrolysis and co-fermentation (SHCF); *Slr* slurry, the *gray-shaded boxes* represent enzymatic or microbial processes; the *dashed lines* represent an optional source of lignin when a pretreatment method allowing the fractionation of biomass is used (e.g. organosolv process)

two biochemical processes (fermentation of glucose and fermentation of xylose) are combined and simultaneously performed in the same single unit. Although xylose-utilizing yeasts can carry out the co-fermentation of glucose and xylose, their ethanol yields are low. As mentioned above, mixed cultures can be used but problems associated with the optimal conditions for fermentation of two different microorganisms are arisen. For this reason, the current research trends are aimed at developing microorganisms with the ability of assimilating both types of sugars with increased ethanol yields by using recombinant DNA technology. There are many works reporting the development of specific strains of ethanol producing microorganisms with these desired traits. Thus, recombinant *S. cerevisiae* (Hong et al. 2003; Zaldivar et al. 2005) or *Zymomonas mobilis* (Leksawasdi et al. 2001) strains have been developed. Alternatively, bacteria naturally assimilating these two sugars like *E. coli* are transformed in order to obtain an ethanologenic microorganism (Dien et al. 1998; Ingram et al. 1999; Vinuselvi and Lee 2012).

16.3.1.3 Simultaneous Saccharification and Fermentation

One of the most important advances in bioethanol industry is the development and implementation of processes in which the hydrolysis of the glucan (starch, cellulose) and the conversion of glucose into ethanol are carried out in a simultaneous way in the same single unit. This process is known as simultaneous saccharification and fermentation (SSF) and has been successfully implemented in the production of ethanol from corn, especially in wet-milling plants. The concept of SSF process was firstly described by Takagi et al. (1977). Takagi, Suzuki and Gauss had previously patented the SSF technology for bioethanol production (Gauss et al. 1976) by which the yeasts metabolize simultaneously the glucose into ethanol in situ during the enzymatic saccharification of the cellulose. This patent expired in 1993 and it has been utilized for small-scale demonstrations (Ingram and Doran 1995), but until now, no commercial plants have been built at industrial level.

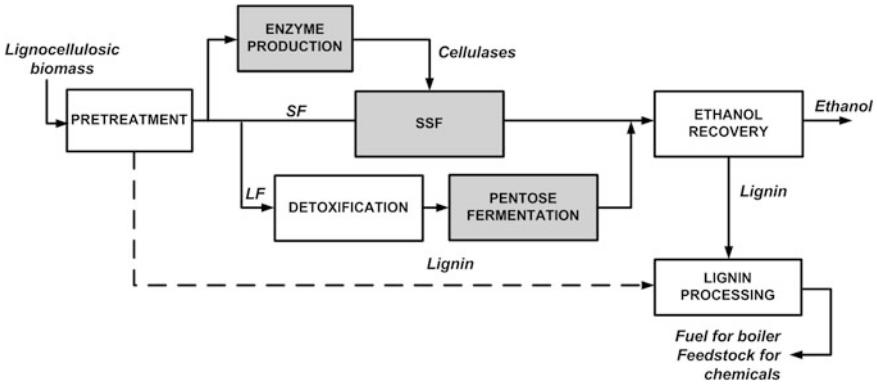

Fig. 16.5 Schematic diagram of fuel ethanol production from lignocellulosic biomass by simultaneous saccharification and fermentation (*SSF*); *LF* liquid fraction, *SF* solid fraction, the *gray-shaded boxes* represent enzymatic or microbial processes; the *dashed lines* represent an optional source of lignin when a pretreatment method allowing the fractionation of biomass is used (e.g. organosolv process)

Conversion of cellulose into ethanol by SSF implies that several enzymes with cellulolytic activity (basically endoglucanases, cellobiohydrolases and β-glucosidase) are added to the suspension obtained by mixing water with the solid fraction resulting from the pretreatment step and that contains cellulose and lignin (Fig. 16.5). In the same way, glucose-fermenting microorganisms (yeasts) are added to this mixture in the bioreactor where SSF is accomplished for immediately converting the glucose formed into ethanol. Taking into account that sugars (glucose, cellobiose) are much more inhibitory for conversion process than ethanol is, SSF can reach higher rates, yields and ethanol concentrations compared to SHF (Wyman et al. 1992). The increased ethanol concentration in the culture broth allows the reduction of energy costs during distillation. In addition, SSF offers an easier operation and a lower equipment requirement than the sequential process since no hydrolysis reactors are needed. Moreover, the presence of ethanol in the broth makes it less vulnerable to the action of undesired microorganisms (Wyman 1994). Nevertheless, SSF has the inconvenient that the optimal conditions for hydrolysis and fermentation are different, which implies a difficult control and optimization of process parameters. In addition, larger amounts of exogenous enzymes are required (Cardona and Sánchez 2007).

A significant amount of reports on batch SSF has been published recognizing, in this way, that this integrated process is one of the most promising. Nevertheless, only limited information has been published about continuous SSF. The continuous regime for SSF entails experimental challenges related to the homogenous delivery of solid substrate, the extended run times needed, and the complexities of the continuous systems as stated by Brethauer and Wyman (2010). These authors point out that batch systems are hampered by mixing problems at high solid substrate loadings, which can be avoided by operation of a CSTR (continuous

stirred-tank reactor) with high conversion of insolubles to ethanol. Furthermore, in a batch reactor, the high amounts of β-glucosidase added are only required at the beginning of the reaction when cellobiose production is highest. They suggest that β-glucosidase loading can be reduced in a continuous system because cellobiose production slows with conversion.

As mentioned above, one of the main disadvantages of SSF processes using lignocellulosic biomass lies in the different optimum conditions of enzymatic hydrolysis of cellulose and fermentation. Varga et al. (2004) proposed a nonisothermal regime for batch SSF process applied to wet oxidized corn stover; In the first step of SSF, small amounts of cellulases were added at 50 °C in order to obtain better mixing conditions. In the second step, more cellulases were added along with the yeast *S. cerevisiae* at 30 °C. In this way, the final solid concentration in the hydrolyzate could be increased up to 17 % dry matter concentration achieving 78 % ethanol yield. In general, increased cultivation temperature accelerates metabolic processes and lowers the refrigeration requirements. Yeasts as *K. marxianus* have been tested as potential ethanol producers at temperatures higher than 40 °C. Ballesteros et al. (2001) carried out several fed-batch SSF tests at 42 °C during 72 h using *K. marxianus* in the case of by-products of olive oil extraction achieving 76 % ethanol yields of theoretical for olive pulp. If, when thermotolerant yeasts are used, the microbial cells can also assimilate pentoses, the SSF process can become more promising. Yeasts as *Candida acidothermophilum*, *C. brassicae*, *S. uvarum* and *Hansenula polymorpha* can be used for these purposes. In this case, the addition of a larger amount of nutrients to the medium is required. The difficulty lies in the fact that higher temperatures enhance the inhibitory effect of ethanol. Therefore, the isolation and selection of microorganisms that could be adapted in a better way to these hard conditions should be continued.

One of the most relevant factors in the alcoholic fermentation is the possibility of infection by acidolactic bacteria. This problem has been reported for both batch (Stenberg et al. 2000) and continuous regimes (Schell et al. 2004) of SSF processes. These bacteria can consume the sugars not utilized by the main ethanologenic microorganisms used during SSF and represent a challenge for the design of effective biomass-to-ethanol processes. Therefore, the utilization of microorganisms capable of assimilating all the sugars present in the feed stream of the SSF is crucial to avoid the proliferation of undesired contaminating microbes. This suggests the need of a higher degree of reaction–reaction integration.

16.3.1.4 Simultaneous Saccharification and Co-fermentation

A higher degree of integration can be achieved by including the fermentation of hemicellulose-derived pentoses in the same single unit where SSF is carried out. This process is called simultaneous saccharification and co-fermentation (SSCF). In this way, only two vessels (one for enzyme production and the other for the SSCF process) are employed for conversion of the pretreated lignocellulosic

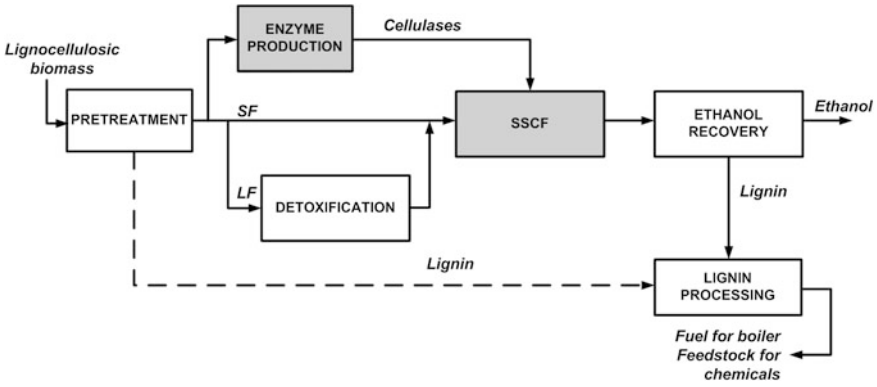

Fig. 16.6 Schematic diagram of fuel ethanol production from lignocellulosic biomass by simultaneous saccharification and co-fermentation (*SSCF*), *LF* liquid fraction, *SF* solid fraction, the *gray-shaded boxes* represent enzymatic or microbial processes; the *dashed lines* represent an optional source of lignin when a pretreatment method allowing the fractionation of biomass is used (e.g. organosolv process)

biomass into ethanol in one integrated process as that depicted in Fig. 16.6. During the SSCF, the hydrolysis of cellulose by using cellulases added to the bioreactor, the fermentation of the glucose released from the enzymatic process, and the fermentation of pentoses present in the feed stream are simultaneously accomplished in the same single unit. Besides the effectiveness of the employed cellulases, the key factor in SSCF is the utilization of an efficient ethanol-producing microorganism with the ability of assimilating not only hexoses (mainly glucose), but also the pentoses (mainly xylose) released during the pretreatment step as a result of hemicellulose degradation. For this, genetically engineered microorganisms like *S. cerevisiae* (Jin et al. 2010), *Z. mobilis* (McMillan et al. 1999) and *E.coli* (Kang et al. 2010) has been developed and favorably proven in SSCF processes for ethanol production from lignocellulosic materials.

In an initial stage, the co-fermentation of mixed cultures was studied (Cardona and Sánchez 2007). For example, the co-culture of *P. stipitis* and *Brettanomyces clausennii* has been employed for the SSCF of aspen at a trade-off temperature of 38 °C yielding 369 L EtOH per ton of aspen during batch process, as reported by Olsson and Hahn-Hägerdal (1996). However, actual SSCF process using one process microorganism has been demonstrated in the case of ethanol production from yellow poplar through a bench-scale integrated process that included the dilute-acid pretreatment of feedstock, conditioning of hydrolyzate for fermentation, and a batch SSCF (McMillan et al. 1999). In this case, the recombinant bacterium *Z. mobilis* assimilating xylose was used. SSCF is the process on which is based on the technology designed as a model process by the NREL for production of fuel ethanol from aspen wood chips (Wooley et al. 1999b) and corn stover (Aden et al. 2002). It is projected that SSCF can be carried out in continuous regime with a residence time for the whole system of cascade fermenters of 7 d at

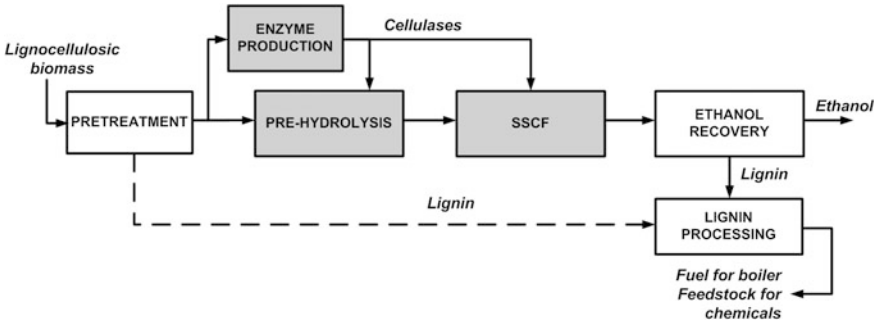

Fig. 16.7 Schematic diagram of fuel ethanol production from lignocellulosic biomass by a two-step simultaneous saccharification and co-fermentation (*SSCF*); the *gray-shaded boxes* represent enzymatic or microbial processes; the *dashed lines* represent an optional source of lignin when a pretreatment method allowing the fractionation of biomass is used (e.g. organosolv process)

30 °C (Cardona and Sánchez 2007). As in the case of SSF of biomass, the development of microbial strains able to grow at elevated temperatures can improve the techno-economic indicators of the process. Thus, ethanol-producing microorganisms capable to assimilate both types of sugars at temperatures higher than 50 °C could reduce the cellulase costs by a half taking into account that a 20 °C increase during saccharification can lead to double cellulose hydrolysis rate (Wooley et al. 1999a).

The configuration shown in Fig. 16.6 implies the separation of the slurry of pretreated biomass, washing of the solid fraction, and detoxification of the liquid hemicellulose hydrolyzate in order to prepare and condition the feed stream to the SSCF bioreactor. All these operations add costs and complexity to the overall process. For this reason, new trends in the development of SSCF processes are aimed at the development of microorganisms resistant to the toxic compounds formed during the pretreatment step. In this way, the whole slurry could be directly fed to the SSCF bioreactor without the need of any separation or washing step and avoiding the costly detoxification. Geddes et al. (2011) point out that newly developed strains such *E. coli* MM160 and *S. cerevisiae* 424A can be used for these purposes. However, the final ethanol concentrations and yields are still low for a large-scale commercial operation, although the results obtained are better than those for most SSF processes. These problems may be explained by the high solids content of the stream entering the SSCF bioreactor, which can achieve 10–20 %. One way to tackle this challenge is to implement a two-step process where a pre-hydrolysis is carried out before the actual SSCF. In this way, a sort of liquefaction analogous to the corn-to-ethanol process is carried out to reduce the solids level and enhance the efficiency of the enzymatic hydrolysis. After this step, the stream containing about 10–15 % solids undergoes SSCF (see Fig. 16.7). It is envisioned that such pre-hydrolysis can be effectively performed in a CSTR with a residence time between 1 and 6 h (Geddes et al. 2011). Other difficulty that can be overcome by a two-step SSCF process is the low xylose uptake rate related to

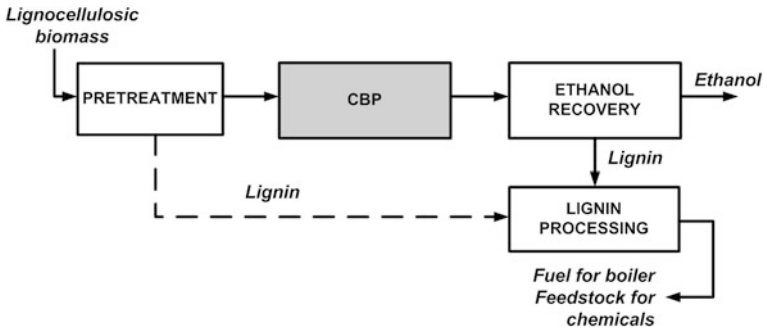

Fig. 16.8 Schematic diagram of fuel ethanol production from lignocellulosic biomass by consolidated bioprocessing (*CBP*); the *gray-shaded box* represents a microbial process; the *dashed lines* represent an optional source of lignin when a pretreatment method allowing the fractionation of biomass is used (e.g. organosolv process)

glucose. For instance, Jin et al. (2010) proposed a two-step SSCF process on AFEX-treated switchgrass using commercial enzymes and the inhibitor-resistant *S. cerevisiae* 424A, which gives higher ethanol yield with improved xylose consumption compared to the corresponding SSCF process. The process included the hydrolysis of the hemicellulose remaining in the pretreated biomass with hemicellulases first to release xylose, which was then fermented by recombinant *S. cerevisiae* 424A, followed by adding cellulases to hydrolyze the cellulose into glucose and continue the fermentation.

16.3.1.5 Consolidated Bioprocessing

The highest degree of integration is represented by a configuration where the fermentable sugars and polysaccharides (mostly cellulose) resulting from the pretreatment of lignocellulosic biomass are directly converted into ethanol by a microorganism (or consortium of microorganisms) in one single unit (see Fig. 16.8). This process is known as consolidated bioprocessing (CBP) and leads to the maximum reduction of process units during the biomass-to-ethanol process. Schematically, CBP consists in the unification of all the enzymatic and microbial processes represented by gray-shaded boxes in Figs. 16.3–16.7. This configuration implies that no capital or operation expenditures are required for enzyme production within the process. In this way, there is no need of adding the costly cellulolytic enzymes to the biomass to obtain significant amounts of glucose. In addition, the deviation of part of the feedstock to the production of cellulases is not further required since the CBP concept entails that a single microorganism produces the needed enzymatic complexes to degrade the cellulose and even the hemicellulose.

It is considered that the costs of ethanol production from biomass can be significantly reduced by improving the conversion of lignocellulosics. Lynd (1996)

had projected that the reduction of production costs due to an advanced configuration involving the CBP is three times greater than the reduction related to the scale economy of the process and ten times greater than the reduction associated with a lower cost of the feedstock. This decrease would be accomplished thanks to the reduction of more than eight times in the costs of biological conversion (Lynd et al. 1996). Afterwards, Lynd et al. (2005) reported the comparative simulation of SSCF and CBP processes assuming aggressive performance parameters intended to be representative of mature technologies. Their results indicate that production costs of ethanol for SSCF can reach US¢ 4.99 per liter including the costs of dedicated cellulase production, whereas CBP can give total costs of only US¢ 1.11 per liter demonstrating the future effectiveness of this process configuration.

To develop an effective CBP process to produce fuel ethanol from biomass, a suitable microorganism has to be selected. The candidate CBP microorganism should exhibit the following features:

- Be resistant to the inhibitors generated during the pretreatment.
- Produce cellulases or cellulolytic complexes to convert the cellulose into glucose.
- Produce hemicellulases to obtain a higher amount of pentoses and other hemicellulose-derived sugars.
- Convert the hexoses (glucose, mannose and galactose) into ethanol.
- Convert the pentoses (xylose and arabinose) into ethanol.
- Show reduced end-product inhibition by the ethanol formed.
- Reduce the amount of fermentation by-products like lactate or acetic acid.
- Show high growth rates.
- Produce high ethanol titres.
- Be stable under industrial conditions.

As can be inferred from previous sections, such a microorganism capable of transforming biomass into ethanol with high yield and efficiency does not exist in the nature. However, some naturally occurring microorganisms exhibit most of the above-mentioned traits but with low ethanol yields. The thermophilic bacterium *Clostridium thermocellum* is one of these microorganisms, since it is able to degrade cellulose and convert the glucose obtained into ethanol. *C. thermosaccharolyticum*, in turn, has the ability to utilize pentoses. As both bacteria can be cultured in the same vessel under the same fermentation conditions, their mixed cultivation represents a CBP process to produce ethanol from biomass. Other thermophilic clostridia can be cultured along with *C. thermocellum* in order to depolymerize lignocellulosic carbohydrate polymers and utilize pentoses as *C. thermolacticum* obtaining significant ethanol yields (Xu and Tschirner 2011). In fact, the production of ethanol from biomass by using *C. thermocellum* is a first step toward CBP as has been demonstrated experimentally (Shao et al. 2011; South et al. 1993). Nevertheless, the employ of clostridia has some important drawbacks as their low ethanol tolerance and reduced ethanol yields due to the formation of acetic acid and salts of other organic acids like lactates (Baskaran et al. 1995; McMillan 1997; Wyman 1994). This makes that the final ethanol

concentrations be low (0.8–60 g/L) compared to the traditionally used yeasts with long cultivation times of 3–12 days (Szczodrak and Fiedurek 1996).

Unlike fungi employed for cellulase production in the framework of SHF, SSF, and SSCF processes, thermophilic bacteria like *C. thermocellum* do not secrete individual cellulolytic enzymes to the culture medium to degrade the cellulose. These microbes have a complexed cellulase system named cellulosome that represents a multi-enzyme consortium that efficiently binds to heterogeneous insoluble cellulose-containing substrates being anchored to the bacterial cells, i.e. the cellulose hydrolysis implies the formation of a cellulose-enzyme-microbe complex. The cellulosome shows a higher efficiency in cellulose hydrolysis compared to the non-complexed cellulase systems from fungi like *T. reesei*. Lynd et al. (1999, 2002) point out that most research efforts on cellulose hydrolysis are being carried out within the context of the enzymatically oriented intellectual paradigm, which focuses on cellulose hydrolysis as primarily an enzymatic rather than microbial phenomenon. In this context, the development of SHF, SSF, and SSCF processes that utilizes fungal cellulases is in the framework of this paradigm. In contrast, the development of CBP processes for bioethanol production corresponds to the microbial oriented paradigm, which considers the cellulose hydrolysis as a microbial phenomenon. As there exists no natural microorganism exhibiting the whole combination of features required for efficient CBP-based ethanol production, intensive research on genetic modification of different microbial strains is being carried out in past two decades. In the framework of the CBP, two strategies have been employed: native cellulolytic strategy and recombinant cellulolytic strategy. The main aspects of these strategies are presented in Table 16.4. Undoubtedly, ongoing research efforts on genetic and metabolic engineering will make possible the development of effective and stable strains of microorganisms to convert lignocellulosic biomass into ethanol. This fact will surely imply a qualitative improvement in the industrial production of fuel ethanol in the future.

16.3.2 Reaction–Separation Integration

Reaction–reaction integration allows the increase of process efficiency through the improvement of reaction processes. However, separation is the step where major costs are generated in process industry. Therefore, reaction–separation integration could have a significant impact on the overall biomass-to-ethanol process. The reaction–separation integration is particularly an attractive alternative for the intensification of alcoholic fermentation processes. When ethanol is removed from the culture broth, its inhibition effect on growth rate is diminished or neutralized that leads to a substantial improvement in the performance of ethanol-producing microorganisms. This improved performance may permit the increase of substrate conversion into ethanol. In particular, higher conversions make possible the utilization of concentrated culture media (with sugars content greater than 150 g/L) resulting in increased process productivities. From energy viewpoint, this type of

Table 16.4 Strategies for developing genetically modified microorganisms to be used in the framework of the CBP-based ethanol production from lignocellulosic biomass

Strategy	Native cellulolytic	Recombinant cellulolytic
Fundament	Modify microorganisms having a high native cellulolytic activity to improve ethanol production	Modify microorganisms having high ethanol yields making them cellulase producers
Challenges	Increase of ethanol yield, titres and tolerance Reduction or elimination of fermentation by-products	Complexity of the insertion of cellulolytic genes into the hosts Improve cellulose and hemicellulose degradation rates
Cellulase system	Complexed (cellulosome)	Non-complexed/minicellulosomes
Host microorganism	*C. thermocellum*, *C. cellulolyticum*	*S. cerevisiae*, *Z. mobilis*; ethanologenic *E. coli*, *K. oxytoca*
Enzymes expressed	Pyruvate decarboxylase from *Z. mobilis dd*Alcohol dehydrogenase from *Z. mobilis*	Endo/exoglucanase from *Bacillus* sp. *β*-glucosidase from *B. circulans* Cellobiohydrolase from *Thermoascus aurantiacus* Endoglucanases from *T. reesei*
Ethanol titre	Up to 26 g/L	1–40 g/L
References	Guedon et al. (2002), Lynd et al. (2005)	Cho and Yoo (1999), Hong et al. (2003), Zhou and Ingram (2001)

integration leads to the increase of ethanol concentration in the culture broth. This fact has a direct effect on distillation costs since more concentrated streams feeding the columns imply lower steam demands for the reboilers and therefore, lower energy costs.

This type of integration can be accomplished by coupling the separation unit to the fermenter (conjugated process) or combining the cultivation and the separation in the same unit (simultaneous process). For instance, vacuum chamber, stripping columns or pervaporation modules can be coupled to fermentation vessels. Alternatively, the membrane modules can be submerged in the culture broth or an extractive agent can be directly added to the cultivation medium during the fermentation process. These options for reaction–separation integration are presented in Table 16.5. Furthermore, one unit where reaction–reaction integration is applied (e.g. SSF) may be coupled to a separation unit. This represents a process with an integration of the reaction–reaction–separation type allowing a higher degree of integration.

16.3.3 Separation–Separation Integration

The separation–separation integration is particularly relevant for ethanol recovery and dehydration. This type of integration is accomplished by coupling two or more separation units with different mass-transfer foundations. For instance, the extractive distillation employed for ethanol dehydration combines the distillation

Table 16.5 Different options of reaction–separation integration for production of fuel ethanol

Option	Fundament	Remarks	References
Vacuum	Coupling the fermentation tank to a vacuum chamber allows ethanol removal due to its higher volatility	50 mm Hg; cell recycling; productivity 23–82 g/(L × h); high energy costs	Costa et al. (2001), Cysewski and Wilke (1977)
Gas stripping	Ethanol removal by absorption employing a stripping gas	Stripping gas: CO_2; fermenter coupled to a stripping column; productivity 8–16 g/(L × h)	Dale and Moelhman (2001), Taylor et al. (1996, 2000)
Membrane	Broth is passed across selective membrane to remove ethanol from water	Different types of membranes (e.g. silicate or polypropylene membranes); membrane distillation or pervaporation modules coupled to fermenter; productivity 2–48 g/(L × h); membrane fouling	Brandberg et al. (2005), Lee et al. (2000), (Nakamura et al. 2001), Sánchez et al. (2005)
Extractive fermentation	Addition of selective solvent to the broth for ethanol removal followed by decantation and recycling of aqueous phase to fermenter	Solvents: fatty alcohols like *n*-dodecanol, mixture of biocompatible solvents; in situ extraction or separate extractor coupled to fermenter; possibility of SSEF and HFMEF; productivity 1–55 g/(L × h); solvent regeneration is required	Kang et al. (1990), Moritz and Duff (1996), Sánchez et al. (2006)

HFMEF hollow-fiber membrane extractive fermenter, *SSEF* simultaneous saccharification and extractive fermentation

with the liquid–liquid extraction in order to "break" the ethanol–water azeotrope in multi-column systems. Variations to this process have been proposed like the utilization of salts (NaCl, KCl, KI, or $CaCl_2$) as extractive agents in a process called saline extractive distillation that involves conventional distillation and crystallization by vacuum evaporation and spray drying for salt recovery (Llano-Restrepo and Aguilar-Arias 2003; Pinto et al. 2000). On the other hand, the utilization of pervaporation modules for ethanol dehydration through their coupling to the previous distillation step is other example of separation–separation

integration (Tsuyomoto et al. 1997). Different evaluations indicate that this dehydration scheme has lower operation costs than azeotropic or extractive distillation (Szitkai et al. 2002; Tsuyomoto et al. 1997) but higher than adsorption using molecular sieves (Sánchez and Cardona 2012).

16.4 Conclusion

The advantages of the biofuels in general, and bioethanol in particular, are evident considering the progressive depletion of the fossil fuel reserves and the changes of the global climate generated by their use. Bioethanol is an environmentally clean source of energy with a high output-input energy ratio and represents an important commodity in the world energy market. Its production from sucrose-containing materials has attained a considerable degree of maturity but can directly affect the food safety. Ethanol from starchy materials has already affected the food safety and its environmental benefits are not clear. In contrast, biomass ethanol (a second generation biofuel) does not compete for food resources, its energy balance is favorable and the worldwide availability of lignocellulosic materials allows that almost all the countries can produce it. However, the production costs of ethanol from lignocellulosic biomass are still high. Many research groups and centers in different countries are making progress in the task of lowering production costs of lignocellulosic ethanol. Nevertheless, the challenges to overcome are still formidable.

The current pretreatment methods should be optimized considering the future commercial operation of ethanol production facilities employing biomass as feedstock. Some pretreatment technologies seem to have reached their best indicators (e.g., dilute acid pretreatment) and others show incremental improvements. New promising methods have to be tested at pilot scale and demonstration facilities (SPORL, ionic liquids) but they represent disruptive innovation techniques. The main goal is oriented to the reduction of inhibitor generation, biomass fractionation, improved sugars recovery, and enhancement of cellulose digestibility.

The cost of cellulolytic enzymes remains high despite the great progress of the enzyme-producing companies. Although the technologies developed for in situ production of enzymes have achieved some cost reductions, the relatively low specific activity of those enzymes and the binding of cellulases to other polymers like lignin represent serious challenges to be undertaken through genetic and metabolic engineering. The change of paradigm oriented to consider the cellulose hydrolysis as a microbial process will entail significant improvements toward the simplification of the overall process by highly integrated technologies such as CBP. However, the newly developed microbial strains for CBP are far from their industrial cost-effective utilization. In this way, such integrated processes like SSF and SSCF will be the preferred option in the mid-term. One important issue in the strain development is the utilization of microorganisms resistant to inhibitor. This will allow an important process simplification since the utilization of the whole

slurry of pretreated lignocellulosic biomass and the elimination of the detoxification procedures will have beneficial economic and environmental effects on the overall biomass-to-ethanol process. On the other hand, the application of reaction–separation integration has the potential to improve the fermentation performance by diminishing the end-product inhibition effect on growth rate. In this sense, new membranes technologies are being developed and other upgraded techniques for ethanol removal from culture broth as extractive fermentation could potentially represent important advances.

Undoubtedly, the multidisciplinary research efforts that combine new molecular approaches for strain development and process integration in the framework of process systems engineering, will allow expansion and commercial implementation of innovative technologies to exploit the vast resources of lignocellulosic materials available to mankind in a cost-effective and environmentally sustainable way.

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Chapter 17
Biobutanol Production from Biomass

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Abstract There is a demand for the increased production and usage of biofuels from both environmental and a political point of view. Biobutanol has great potential to become a novel replacement fuel for gasoline and diesel or an additive compound of these fuels in the future. This chapter reveals the superior fuel properties of butanol over ethanol, including better energy content, usability, safety, and easier distribution of the fuel. Traditional biochemical (acetone-butanol-ethanol fermentation) and chemical production processes of butanol are reviewed. In addition, the novel production routes of biobutanol are highlighted. Challenges in the implementation of feasible biochemical production processes of butanol in industrial scale include cost of raw materials, low product yield from fermentation, and expensive processing techniques. Process development techniques and different methods in order to increase the yield of fermentative butanol production by means of for instance new processing technologies and metabolic engineering are discussed.

17.1 Introduction

The interest for production and usage of biofuels is promoted by limited fossil fuel resources, environmental concerns and tightening legislation regarding the use of fossil fuels as well as release of carbon dioxide (CO_2) and other emissions in the air. The share of biofuels in transportation fuel markets is expected to grow rapidly during the next decades. Bioethanol and biodiesel are currently the dominant

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biobased fuels in transportation. However, new alternative renewable fuels are also needed to fulfill the increasing fuel demands in the future. With many advantageous properties and numerous utilization ways, biobutanol has a great potential to become a novel renewable biofuel alternative for the fuel and chemical market.

Although there are several alternatives for the production of butanol from biomass-based materials, butanol is produced nowadays mainly via catalytic processes starting from fossil fuels. This review chapter points out the shortages of biomass-based processes and focuses on the target points for process developments needed in order to gain a feasible and competitive biobutanol production process.

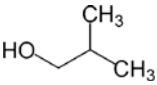
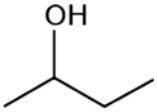
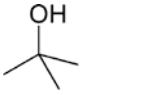
17.2 Properties and Usage of Butanol

Butanol (C_4H_9OH), also called as butyl alcohol, or biobutanol if produced from biomass, has four structural isomers with the common names of *n*-butanol, isobutanol, *sec*-butanol, and *tert*-butanol (Table 17.1). Butanol is used mainly as an intermediate compound for butyl acrylate and methacrylate esters, which are needed for lacquers, paints, and surface coatings. Butyl acetates and glycol ethers are also common derivatives produced from butanol (Kirschner 2006). In addition, butanol is used as a direct solvent and as raw material in the manufacturing of textiles and plasticizers, amino resins and butylamines (Burridge 2009). Chemical and physical properties such as boiling point, octane number, and viscosity of butanol can vary between different isomers, but the main applications in use as solvents, industrial cleaners, or biofuels are the same for all isomers.

Besides the use of butanol as a solvent and a platform chemical, superior fuel properties enable its use as direct transportation fuel or fuel additive:

- **Distribution.** As less corrosive and having lower water solubility than ethanol, butanol can be distributed via existing pipelines and distribution stations.
- **Blending ability with gasoline and diesel**. Compared to ethanol, blending is possible in higher concentrations without any vehicle retrofitting. Thereby the share of renewable components in the final fuel mixture could be increased.
- **Energy content, octane values and air to fuel ratio.** Values are closer to gasoline compared to ethanol. Fuel economy (km/L) is better than with ethanol.
- **Usability.** Compared to ethanol, the lower heat of vaporization enables the easier starting of a motor in cold weather and decreases the ignition problems. In addition, tendencies toward cavitation and vapor lock problems are lower in the case of butanol and the need for special fuel blends during summer and winter time can be reduced.
- **Safety.** Butanol is safer to use and handle compared to ethanol due to the lower vapor pressure and higher flash point. In addition, butanol generates lower amounts of volatile organic compound (VOC) emissions in internal combustion engines.

Table 17.1 Molecular structure, production and use of butanol isomers (Hahn et al. 2012; Jin et al. 2011)

Isomer	Molecular structure	Production methods	Main use
n-butanol 1-butanol		fermentation Reppe synthesis Oxo synthesis crotonaldehyde hydrogenation	Solvent, thinner, or platform chemical in surface coatings, plastic and textile industry, gasoline additive
isobutanol 2-methyl-1-propanol		fermentation Reppe synthesis Oxo synthesis catalytic hydrogenation of carbon monoxide homologization reaction	Solvent, diluent, and additive for nitrocellulose and synthetic resins. Component of cleaners and printing inks, gasoline additive
sec-butanol 2-butanol		acid-catalyzed hydration of *n*-butene	Solvent, component of brake fluids, cleaning agents, perfumes, and fruit essence. Mainly hydrogenated to 2-butanone and used as a solvent
tert-butanol 2-methyl-2-propanol		2-methylpropene hydration a by-product of methyl *tert*-butyl ether and propylene oxide production	Solvent, used e.g. in preparation of peroxides, oil-soluble resins, and antioxidants.

Butanol can be mixed in various blending ratios with gasoline, ethanol, or diesel. Studies in relation to pure and mixed butanol combustion in engines using techniques of spark ignition (SI), direct injection (DI), compression ignition (CI), or cooperative fuel research (CFR) have been performed intensively during the past few years (Dernotte et al. 2010; Szwaja and Naber 2010; Wallner and Frazee 2010; Cairns et al. 2009; Wallner et al. 2009). Jin et al. (2011) have reviewed the combustion studies done by several research groups. They concluded that engine power is not reduced in unmodified SI engines when the butanol content in gasoline blends is below 20 vol %. The emissions of soot, carbon monoxide (CO), and nitrogen oxides (NO_x) are reduced in the butanol-diesel blends compared with pure diesel combustion (Rakopoulos et al. 2010). However, depending on the used engine type, blending ratios and operating conditions, total hydrocarbon, CO and NO_x emissions may also increase in some cases with SI engines. More experimental research and development of kinetic models are needed in order to optimize the combustion process (Jin et al. 2011).

Butanol can be used also as raw material for the production of oxygenated diesel fuels. For example, dibutyl ether (DBE) can be produced by partial dehydration of butanol using sulfonated mesoporous silica and organosilica as catalysts (Sow et al. 2005). In addition, saturated hydrocarbon fuels can be produced e.g. by catalytic dehydration of *n*-butanol to 1-and 2-butene at low temperatures between

200 and 350 °C (Lu et al. 1995). Butene can be further oligomerizated with cation exchange resins or catalysts such as amorphous silico-aluminas, polyphosphoric acid, or zeolites with large pores to produce jet and diesel fuels (reviewed e.g. by Harvey and Meylemans 2011). Further, Wright et al. (2008) used methylaluminoxane (MAO) activated metallocene catalysts for the high selectivity conversion of 1-butene to oligomers, which can be further processed to jet fuels.

17.3 History of Butanol Production

Figure 17.1 represents the highlights and turning points of the butanol production. Pasteur reported in 1861 about the formation of butanol in the microbial fermentation (Gabriel 1928). In the early twentieth century, acetone, amyl alcohol, and butanol were produced by fermentation of starch-based biomasses for further production of synthetic rubber (Fitz 1878; Gabriel and Crawford 1930): Fernbach isolated and patented a bacterial culture producing butanol from potato starch in 1911 and, soon after that, in 1912, Fernbach's former assistant Weizmann isolated a strain using starch as a substrate with a higher product yield (Gabriel 1928; Jones and Woods 1986). The isolated bacterium strain was later named as *Clostridium acetobutylicum* and it has been the most studied and used microorganism for butanol production. Both the Fernbach and the Weizmann processes have been used in industrial scale production plants for the production of acetone and butanol. During the First World War (1914–1918) acetone was the main product of the fermentation processes, required in the production of cordite i.e., smokeless gunpowder. Butanol turned to be the main product in 1920 as it was found to have good solvent properties for the manufacturing of quick drying lacquers needed in fast expanding automobile industry (Gabriel 1928; Gabriel and Crawford 1930). Over the following decades, biobutanol was produced widely in several industrial plants all over the world (García et al. 2011). In spite of continuous research and process development activities, many of the plants were closed down in the 1960s because of the risen costs of substrates. Petrochemical synthesis processes started to dominate the manufacturing of butanol as they are more cost efficient.

Research related to the biochemical fermentation process continued, however, mainly in academia and in some pilot plants (Nimcevic and Gapes 2000). New feed stocks, development of pretreatment methods, improvement of fermentation practices and downstream processes have been actively studied during the past few decades and several reviews are published recently (Jin et al. 2011; García et al. 2011; Kumar and Gayen 2011; Ezeji et al. 2010; Ezeji et al. 2007). China has restarted the acetone-butanol-ethanol (ABE) production in several production plants already in operation (Ni and Sun 2009). In addition, several companies e.g. BP, DuPont, Cobalt Biofuels, Green Biologics, Gevo, Metabolic Explorer, and TetraVitae Bioscience are developing biobutanol production processes intensively and new production facilities are expected to be opened in the near future (Jin et al. 2011).

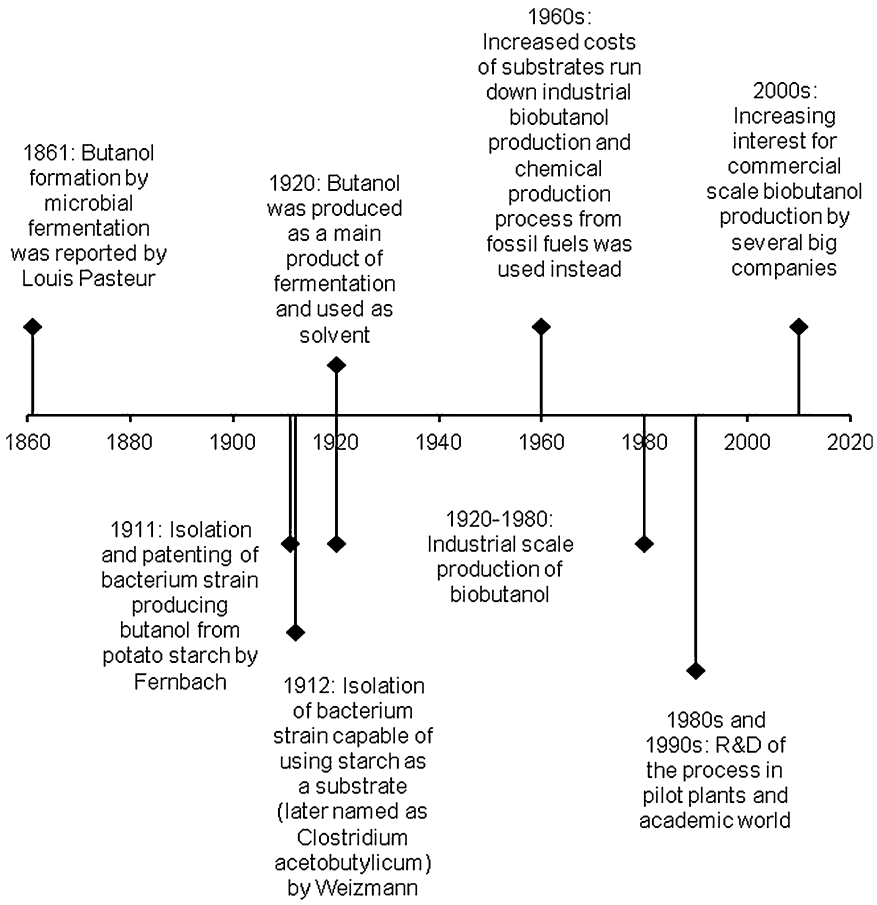


Fig. 17.1 Milestones of (bio) butanol production

17.4 Chemical Production Processes

17.4.1 Traditional Chemical Production Processes

Currently, butanol is almost exclusively produced from fossil fuels originated hydrocarbons via chemical routes. Traditional industrial chemical processes for butanol production are Oxo synthesis also known as hydro formylation (Eqs. 1, 2 and 3 in Fig. 17.2), Reppe synthesis (Eq. 4) and crotonaldehyde hydrogenation (Eqs. 5, 6 and 7) shown in Fig. 17.2.

In Oxo synthesis, carbon monoxide and hydrogen are added to propylene in the liquid phase. Catalysts such as cobalt (Co), rhodium (Rh), or ruthenium (Ru) are used. The first reaction step produces a butyraldehyde mixture, which is further hydrogenated to *n*-butanol and isobutanol. The catalysts and reaction conditions

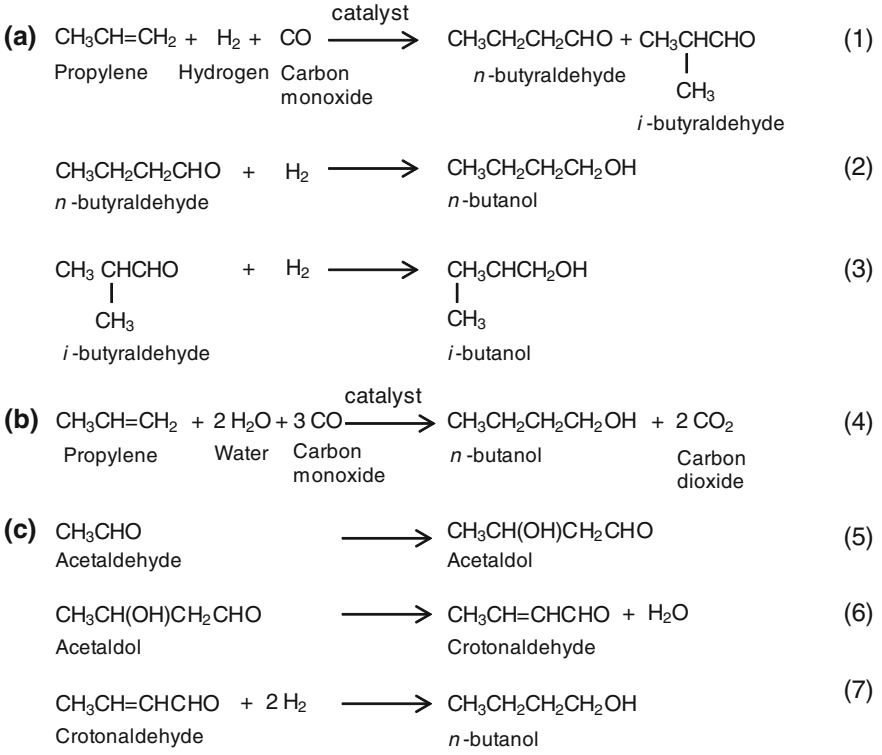


Fig. 17.2 Chemical synthesis of butanol via **a** Oxo synthesis, **b** Reppe synthesis and **c** crotonaldehyde hydrogenation

such as pressure and temperature used lead to different ratios of butanol isomers (Hahn et al. 2012; Falbe 1970). According to Chauvel and Lefebvre (1989), the required temperatures and pressures vary between 110–180 °C and 5–35 MPa for cobalt catalysts, while with rhodium processes are done at 70–120 °C and 1.5–30 MPa. Lower temperature and pressure conditions are used normally with modified catalysts such as cobalt hydrocarbonyl substituted by a phosphine or carbonyl rhodium modified by triphenylphosphine.

The Reppe process starts also from propylene and carbon monoxide, but water is used instead of H_2 and tertiary ammonium salt of polynuclear iron carbonyl hydrides as a catalyst. Carbon dioxide is produced in addition to *n*-butanol and isobutanol. Reaction takes place at lower temperatures and pressures (100 °C and 0.5–2 MPa) compared with the Oxo reaction, but the needed process technologies are more expensive. Thus, the Reppe process has not been found commercially successful (Hahn et al. 2012; Chauvel and Lefebvre 1989; Falbe 1970).

Hydrogenation of crotonaldehyde was the preferred process for butanol production prior to the development of the Oxo synthesis. Process includes steps of aldolization of acetaldehyde, dehydration of produced acetaldol to crotonaldehyde followed by hydrogenation to *n*-butanol. Both gaseous and liquid phase processes are developed, commonly in the presence of copper (Cu) or chromium (Cr) catalysts at 170–180 °C or at lower temperatures when nickel (Ni) catalysts are used (Hahn et al. 2012; Weissermel and Arpe 2003).

17.4.2 Novel Catalytic Routes with Renewable Feed stocks

Almost all of the industrially produced butanol has been manufactured by the Oxo reaction from fossil fuel-based materials. Renewable starting materials for the catalytic production routes are desirable, and thereby the interest to find proper catalysts for these processes has increased strongly during the past few years. Crotonaldehyde hydrogenation is assumed to have a more important role in the future due to the possibility to convert bioethanol produced by fermentation to acetaldehyde and further to *n*-butanol (Hahn et al. 2012). Calcium or strontium substituted hydroxyapatite catalysts, such as $Ca_{10}(PO_4)_6(OH)_2$ or $Sr_{10}(PO_4)_6(OH)_2$, have been found to be promising for the conversion of ethanol to *n*-butanol (Ogo et al. 2011; Tsuchida et al. 2006, 2008a, b). Moreover, zeolites (Ndou et al. 2003) and supported metal catalysts e.g. Ni/γ-Al_2O_3 (Yang et al. 2004) have been studied. Almost all ethanol-to-*n*-butanol processes have been reported to be operated in gas phase. Therefore, liquid-phase catalytic conversion of ethanol to 1-butanol is interesting (Riittonen et al. 2012). Table 17.2 summarizes some catalytic studies on the chemical conversion of ethanol to butanol.

In most heterogeneous *n*-butanol formation reactions presented, ethanol with carrier gas (N_2 or He) is introduced on the catalyst surface at temperatures from 200 to 450 °C. In these cases, the ethanol conversion has been varied between 2 and almost 100 % depending on catalytic material used. An autoclave reactor has been used in the direct catalytic valorization of liquid ethanol conversion to *n*-butanol. In these cases, the conversion of ethanol is between 2 and 18 % and the selectivity to butanol up to 62 % (Riittonen et al. 2012; Marcu et al. 2009).

The main challenges of the recent studies have been to find out novel and proper catalysts for the synthesis of butanol as well as to understand or clarify the reaction mechanism of *n*-butanol formation. To improve ethanol conversion, the effect of the catalyst composition has often been studied more closely. For example, the optimum amount of Cu addition on MgAl-oxide has been found to be between 5 and 10 wt %, the obtained maximum conversion of ethanol being around 4.1–4.5 % and selectivity to butanol from 40 to 42 % (Marcu et al. 2009). In addition, the partial substitution of Al_2O_3 by iron (Fe) has been shown to have an effect on the ethanol condensation (León et al. 2011b). It has been found that the product distribution in the catalytic conversion of ethanol is dependent on the catalysts. Therefore, several reaction mechanisms for catalytic ethanol reaction to

Table 17.2 Catalytic conversion of ethanol to butanol

Catalyst	Reaction conditions (phase, T, p)	Product composition	Conversion, *n*-butanol selectivity or yield	References
MgO/C (10 or 20 wt % Mg)	CH_3OH:C_2H_5OH in N_2, 1 Pa, 310–400 °C, 1.4 MPa, ambient T	H_2, CO_2, C_3H_6, acetylene, *i*-butane, *trans*- and *cis*-butene, C_2H_4, O_2, CH_4, CO, N_2	$X_{C_2H_5OH}$[a] = 15 % at 300 °C, $X_{C_2H_5OH}$ = 70–98 % at 360 °C, $X_{C_2H_5OH}$ = 91 % at 400 °C	Olson et al. (2004)
Ni/MgO (3 wt % Ni)	CH_3OH:C_2H_5OH in N_2, 360 °C, 1 Pa	H_2, CO_2, C_3H_6, acetylene, *i*-butane, *trans*- and *cis*-butene, C_2H_4, O_2, CH_4, CO, N_2	$X_{C_2H_5OH}$ = 47 %	Olson et al. (2004)
Mg–Al mixed oxides	200–450 °C, 5.5 vol % C_2H_5OH in He	Ethylene, acetaldehyde, 2-butenal	$X_{C_2H_5OH}$ ~ 90 % at 450 °C	León et al. (2011a)
Mg–Al 3:1 mixed oxides	12 vol % C_2H_5OH in N_2	Ethylene, acetaldehyde, butanol, butyraldehyde	$X_{C_2H_5OH}$ ~ 33 % at 350 °C $S^{b}_{butanol}$ = 38 %	Carvalho et al. (2012)
Fe/Mg–Al mixed oxides	200–450 °C, 0.1 MPa, 5.5 vol % C_2H_5OH in He	Butanol, 1,3- butadiene	$X_{C_2H_5OH}$ ~ 90–100 % at 450 °C	León et al. (2011b)
Cu/Mg–Al mixed oxides	50 ml C_2H_5OH, 200–260 °C, autogenic pressure, stirring	*n*-butanol, 1,1-diethoxyethane, acetaldehyde, ethyl acetate, butyraldehyde, methyl ethyl ketone, diethyl ester	$X_{C_2H_5OH}$ ~ 4.5 % $S_{butanol}$ ~ 40 %	Marcu et al. (2009)
Ca-P, Ca-V, Sr-P, Sr-V hydroxyapatite catalysts	300 °C, 1 Pa, 16.1 vol % C_2H_5OH in Ar	1-butanol, 2-butenol, acetaldehyde, crotonaldehyde	$X_{C_2H_5OH}$ = 5.8–7.6 % $S_{butanol}$ = 8.1–74.5 %	Ogo et al. (2011)
MgO, CaO, Ca/P	300–450 °C, 0.1 MPa, 16.4 vol % C_2H_5OH in He	Acetaldehyde, butyraldehyde, crotonaldehyde, 1-butanol, 1-hexanol, 2-ehyl-1-butanol, 1-octanol, 2-ethyl-1-hexanol, 1-decanol, 1-hexene etc.	$X_{C_2H_5OH}$ = max 26 % $S_{butanol}$ = max 76 %	Tsuchida et al. (2006, 2008a, b)

(continued)

Table 17.2 (continued)

Catalyst	Reaction conditions (phase, T, p)	Product composition	Conversion, *n*-butanol selectivity or yield	References
γ-Al_2O_3 MgO	450 °C, 0.1 MPa, 10 ml C_2H_5OH in N_2	Acetal, butanal, crotonal, 2-butanol, butanol, crotanol	Al: $X_{C_2H_5OH}$ = 82 % $Y^c_{butanol}$ = 0 % Mg: $X_{C_2H_5OH}$ = 56 % $Y_{butanol}$ = 18 %	Ndou et al. (2003)
Ni, Fe or Co/γ-Al_2O_3	200 °C, 0.1 MPa	Acetaldehyde, butaldehyde, ethanyl acetate, *n*-butanol	Ni: $X_{C_2H_5OH}$ = 19 % $S_{butanol}$ = 64 % Fe: $X_{C_2H_5OH}$ = 3 % $S_{butanol}$ = 0 % Co: $X_{C_2H_5OH}$ = 17 % $S_{butanol}$ = 23 %	Yang et al. (2004)
Ru, Rh, Pd, Pt, Au or Ni on Al_2O_3	Mini-reactor, up to 250 °C, up to 10 MPa, 1.5 ml C_2H_5OH in He	Acetaldehyde, diethyl ether, ethyl acetate, 1-butanol, 1.1-diethoxy ethane, 1-hexanol, 1-octanol	$X_{C_2H_5OH}$ = 2–18 % $S_{butanol}$ = 0–62 %	Riittonen et al. (2012)

[a] X: conversion, [b] S: selectivity, [c] Y: yield

butanol have been proposed based on Guerbet reaction involving a bifunctional catalyst with a base component and metal species. The catalyst promotes condensation reaction between alcohols. Three steps occurring in this reaction are: dehydrogenation of alcohols to the corresponding aldehydes, aldol condensation of the resulting aldehydes, and finally hydrogenation of the unsaturated condensation products to higher alcohols (Tompsett et al. 2011). For example, Ogo et al. (2011) succeeded with a four step mechanism, where 1-butanol formation over hydroxyapatite goes through the dehydrogenation of ethanol to acetaldehyde, the aldol condensation of acetaldehyde to crotonaldehyde, and the hydrogenations of crotonaldehyde, 2-buten-1-ol, and/or butyraldehyde to 1-butanol. According to Marcu et al. (2009), *n*-butanol formation over CuMgAl mixed oxides goes after the first step of ethanol dehydrogenation into acetaldehyde to self-condensation of acetaldehyde to butyraldehyde, and finally to the hydrogenation of butyraldehyde.

Another catalytic route to produce butanol is from fermentation products, esters (R_1COOR_2) to alcohols (R_1CH_2OH) and R_2OH. Hydrogenolysis of butyl butyrate ($C_3H_7COOC_4H_9$) to butanol over a $Cu/ZnO/Al_2O_3$ catalyst has been attracting industrial interest in recent years (Ju et al. 2010; Kim et al. 2011). The idea is to use 1 mol of butyric acid (C_3H_7COOH) and 1 mol of butanol from a fermentation process for esterification to butyl butyrate, which is further converted to 2 mol of butanol by hydrogenation. Hydrogenolysis of butyl butyrate has been studied to yield esters via dissociative adsorption on the $Cu/ZnO/Al_2O_3$ catalyst, producing C_3H_7CO and C_4H_9O fragments. The surface C_3H_7CO fragment is then hydrogenated via butyraldehyde to butanol and the counterpart C_4H_9O fragment is directly hydrogenated (Ju et al. 2010). The presence of zinc oxide (ZnO) in the $CuO/ZnO/Al_2O_3$ catalyst has been found to enhance the catalytic activity of Cu due to the dual function of ZnO (Kim et al. 2011).

17.5 Biochemical Production Processes

The biochemical production route of biobutanol is illustrated in Fig. 17.3. Based on the main products of the fermentation step, the process is also known as the acetone-butanol-ethanol (ABE) fermentation. A wide range of feed stocks including side products, wastes, and residues from agriculture and industry can be used. Upstream processing steps required before the fermentation step are pretreatment of feedstock biomass and hydrolysis by using enzymes or acids. If some inhibitors have been formed during the pretreatment, additional detoxification steps may also be needed. The anaerobic fermentation process is used to convert sugars to acetone, butanol and ethanol and in addition to acids and gases as by-products. The final products obtained from the fermentation are recovered and purified in downstream processing steps (Qureshi and Blaschek 2005).

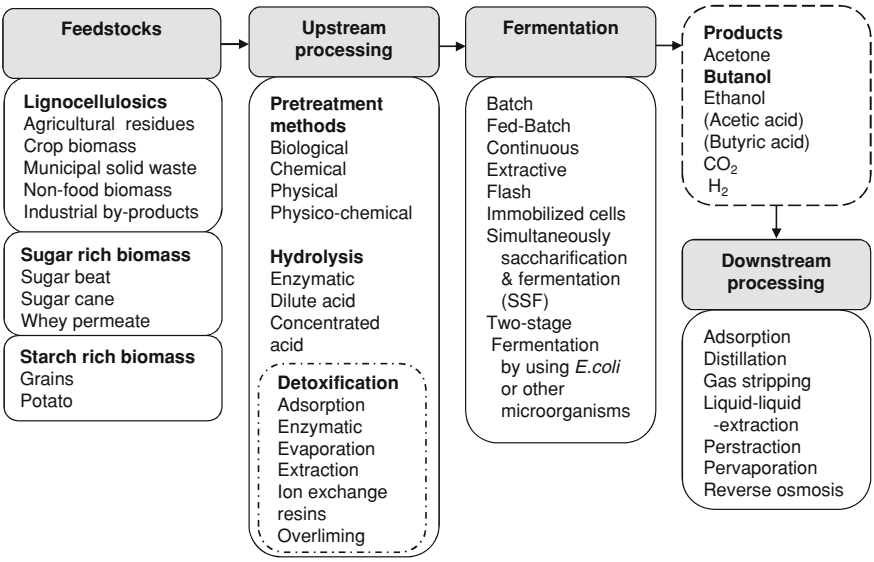


Fig. 17.3 Biobutanol production process with alternative feedstocks and processing techniques (—means process step required if inhibitors are produced during upstream processing—are products)

17.5.1 Feedstocks

A low price, good availability and supply together with the reasonable costs of transportation and upstream processing to gain fermentable sugars are the most important factors when selecting biomasses as raw materials for the processes (Lynd et al. 1999). Crop biomasses have drawbacks in seasonal availability and variations of quality and yields between growing periods. On the other hand, upstream processing of sugar and starch-based biomasses is usually easier than with lignocellulosics. Better availability with lower supply costs is, however, promoting the use of lignocellulosic biomasses. Additionally, the use of nonedible biomasses instead of foodstuff is desirable. By using by-products and waste materials, also sustainability, material efficiency, and waste minimization can be enhanced. Molasses, starch from potato and corn have been the traditional feedstocks used for industrial scale production of biobutanol (García et al. 2011). However, these starting materials have faced several obstacles as they can be used as food.

17.5.2 Upstream Processing

The main components of lignocellulosic biomasses are cellulose (35–50 % of dry weight), hemicelluloses (25–35 %) and lignin (10–25 %) (Cherubini 2010). Cellulose consists of glucose, while hemicellulose includes sugars such as D-galactose, D-glucose, D-mannose, D-xylose, and L-arabinose. Clostridial bacteria can utilize all these sugars (Ezeji and Blaschek 2008), but the complex structure of feedstock biomasses is needed to be altered prior to fermentation to release the sugars for fermentation. Lignocellulosic biomasses require more severe upstream processing techniques than noncellulosic materials. This is because of the much more complicated structure of lignocellulosics: cellulose, hemicellulose, and lignin are bound together with chemical bonds hindering the upstream processing.

Several pretreatment techniques are available, including physical (e.g. chipping, grinding or milling), physico-chemical (steam expansion, ammonia fiber expansion, and liquid hot water treatment), chemical (dilute or concentrated acid hydrolysis, alkaline hydrolysis, and oxidative delignification), and biological (fungal pretreatments by for instance bacteria or brown, soft or white rots) methods. Numerous review articles are available with detailed information about these techniques (e.g. Kumar et al. 2009; Taherzadeh and Karimi 2008; Jørgensen et al. 2007; Mosier et al. 2005). The most desirable pretreatment technique is cost and energy efficient in addition to a good yield of fermentable sugars without the formation of inhibitors for subsequent hydrolysis and fermentation steps. Results of pretreatments should always be balanced against the costs of construction, materials, chemicals, and operation as well as the influence on the fermentation and downstream processing steps.

After the pretreatment, the obtained cellulose and hemicelluloses are broken down into glucose and other fermentable sugars by enzymatic or acid hydrolyses. Enzymatic hydrolysis is done with cellulase and hemicellulase enzymes produced by brown, white or soft rot fungi such as *Aspergillus*, *Phanerochaete* and *Trichoderma*, in addition to several species of bacteria, e.g. *Clostridium*, *Bacillus* and *Streptomyces* (Sukumaran et al. 2005). Enzymatic hydrolysis is done in mild conditions such as in the temperature range of 40–50 °C and pH of 4.5–5.0 (Taherzadeh and Karimi 2007), but slowness and cost of enzymes are hindering the industrial use of this method (Sánchez and Cardona 2008). Acid hydrolysis is performed typically by using dilute (0.75–5 wt %) or concentrated (10–30 wt %) solutions of sulphuric, hydrochloric, or nitric acid (Sánchez and Cardona 2008). Dilute acid pretreatment is done at elevated temperatures at around 140–190 °C compared to the lower temperatures e.g. 40 °C when concentrated acids are used (Taherzadeh and Karimi 2008). Disadvantages of acid hydrolysis include the cost and corrosiveness of acids, in addition to the need for acid recovery and neutralization of hydrolysates prior to the fermentation.

Undesirable degradation products inhibiting enzymatic hydrolysis or fermentation may be formed during the upstream processing steps. Inhibitors released during the processing of lignocellulosic materials include aliphatic acids (acetic,

formic, and levulinic acids), furan derivatives (furfural and 5-hydroxymethylfurfural), and phenolic compounds (syringaldehyde, ferulic and *p*-coumaric acid) (Larsson et al. 1999). Removal of inhibitors can be done by for instance adsorption with activated charcoal, ion exchange resins, evaporation, or solvent extraction. Another approach is the neutralization of inhibitors by enzymatic detoxification or overliming with calcium hydroxide (Huang et al. 2008).

17.5.3 Fermentation

Anaerobic fermentation is typically done by using some of the several Clostridial bacterium strains such as *Clostridium acetobutylicum* or *Clostridium beijerinckii*. Fermentation includes two stages. At first in the acidogenic phase, bacteria are growing and producing acetic and butyric acids, carbon dioxide (CO_2), and hydrogen (H_2) from pyruvate obtained from sugars by glycolysis (Fig. 17.4). At the end of the exponential growth phase, the metabolism of the bacteria changes and the produced acids are converted into acetone, butanol and ethanol, typically with the ratio of 3:6:1. This second phase is usually called the solventogenic phase.

Product concentration remains low, mainly because butanol is inhibiting the growth and metabolism of Clostridial bacteria. The maximum butanol concentration obtained from batch fermentation is typically about 18 g/L while the total solvent yield is around 26 g/L (Qureshi et al. 2010a).

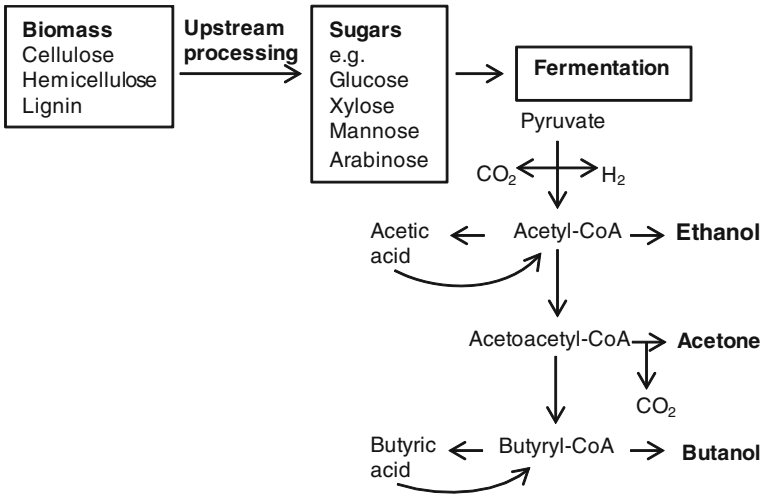


Fig. 17.4 Simplified metabolic pathway of Clostridial fermentation

Table 17.3 Challenges and suggested improvements of the biobutanol production process

Process step	Challenges	Suggested improvements	References
Feedstocks	Price, availability, composition, costs of cultivation, harvesting, transportation, and handling	Use of cheaper and more sustainable non-food raw materials such as easily available lignocellulosics, wastes or residues from industry or agriculture	Qureshi and Ezeji (2008)
Pretreatment	Costs of chemicals, equipments, and energy, efficiency (structural changes, sugar yield, formation of inhibitors)	Development of more efficient and/or novel methods, such as using of ionic liquids, use of detoxification methods	Kumar et al. (2009), Li et al. (2010a), Liu and Blaschek (2010)
Hydrolysis	Acid hydrolysis: cost of chemicals, corrosion of equipmentsEnzymatic hydrolysis: slowness, costs of enzymes	Combined pretreatment and hydrolysis steps (e.g. fractionation with chemicals, ionic liquids, hot water, supercritical conditions). Development of enzymatic hydrolysis to gain better selectivity and faster hydrolysis	Li et al. (2008), Jørgensen et al. (2007)
Fermentation	Low solvent yield, product inhibition, slow metabolism, and other limitations of *Clostridia*	Development of genetically modified bacterium strains (more efficient solvent production, better tolerance against solvents) and advanced fermentation techniques such as continuous fermentation and in situ product recovery (ISPR)	Qureshi and Ezeji (2008), Lee et al. (2008)
Downstream processing	Costs (equipments and energy), low product concentration versus high boiling point of butanol, energy intensiveness of distillation	Development of more energy-efficient methods for the recovery and purification of the solvents (e.g., membrane separation methods such as pervaporation)	Izák et al. (2008), Vane (2005, 2008)
Sustainability evaluation	Setting boundaries for evaluation and calculations, choosing of relevant indicators, lack of the process data, comparison to the other cases/evaluations is difficult	Development of guidelines and harmonization of evaluation processes. Generation of general databases and evaluation programs. Concentration not only to one index (e.g., greenhouse gas emissions) in the evaluation, but trying to take all sustainability aspects (environmental, economic, and social) into account	This work

17.5.4 Downstream Processing

Low product concentration and the boiling point of butanol (118 °C) are the main reasons for energy intensive and costly downstream processing. Costs can be reduced by using more economic and energy efficient separation techniques compared to conventional distillation. Adsorption (Qureshi et al. 2005), gas stripping (Maddox et al. 1995; Qureshi and Blaschek 2001a; Ezeji et al. 2003, 2004), liquid–liquid extraction (Groot et al. 1990; Qureshi and Maddox 1995), pervaporation (Qureshi and Blaschek 2000; Qureshi et al. 2001), perstraction (Grobben et al. 1993; Qureshi and Maddox 2005), and reverse osmosis (Garcia et al. 1986) are the most studied separation methods integrated with the ABE fermentation. The downstream processing is discussed in more detailed in the Sect. 17.6.4.

17.6 Recent Improvements of Biobutanol Production Processes

The biochemical production process needs to overcome several challenges before economically competitive and efficient industrial scale production is feasible. Table 17.3 summarizes the challenges and ideas suggested to solve the problems in question. The cost of substrates, low product yield in fermentation and the high price of up and downstream processing are the main limitations of the ABE process.

Finding a novel, easily available low-cost feedstock and improving pretreatment techniques are essential development areas for upstream processing to enhance the efficient and sustainable utilization of renewable feedstocks. Studies to increase the low yield of the fermentation process have been done by advanced fermentation practices and metabolic engineering of the bacteria strains, and by exploring novel microorganisms for the fermentation processes.

17.6.1 Novel Feedstocks

Since the cost of feedstock has a great impact on a price of the whole processing, novel low-cost feedstocks have been searched for the process. Recently studied raw materials for ABE fermentation include cassava (Thang et al. 2010; Tran et al. 2010), agricultural residues such as corn fiber (Qureshi et al. 2006, 2008a), non-edible barley straw (Qureshi et al. 2010a), corn stover (Qureshi et al. 2010b), wheat straw (Qureshi et al. 2008b, c), switchgrass (Qureshi et al. 2010b), and sugar maple hydrolysate (Sun and Liu 2012). In addition, industrial by-products including whey permeate (Qureshi and Maddox 2005), rice bran (Al-Shorgani et al. 2012; Lee et al. 2009), wheat bran (Liu et al. 2010), and wastewater algae (Ellis et al. 2012) have been tested. Renewable and economically feasible

feedstocks with good availability are desired and the use of by-products or residue materials can also improve the material use efficiency. A challenge with using these lignocellulosic materials is in the complex structure and requirements for efficient upstream and detoxification techniques to obtain sufficient amounts of sugars without compounds inhibiting the fermentation.

17.6.2 Upstream Processing

Costs of upstream processing can be reduced by using enhanced technologies having lower energy and cost requirements. A steam explosion and treatments with ammonia, dilute acid, lime, and liquid hot water are considered as the most potential and cost-effective methods for processing of lignocellulosics (Taherzadeh and Karimi 2007).

Kim and Hong (2001) have demonstrated that supercritical carbon dioxide ($scCO_2$) pretreatment improves enzymatic hydrolysis of aspen and southern yellow pine, while Narayanaswamy et al. (2011) observed an increased glucose yield from corn stover by $scCO_2$ treatment, but for switchgrass the treatment was ineffective. Other raw materials studied in $scCO_2$ pretreatment include e.g. avicel, recycled paper mix, sugarcane bagasse, repulping waste of recycled paper (Zheng et al. 1998), wheat straw (Alinia et al. 2010), and rice straw (Gao et al. 2010). An improved glucose yield is obtained due to the enhanced decomposition and increased pore sizes of biomass structure resulting in easier and more efficient enzymatic hydrolysis (Gao et al. 2010). Supercritical conditions enhance also the mass transfer and reaction rates, and can be combined with enzymes and ionic liquids (Wimmer and Zarevúcka 2010). In addition, CO_2 is low cost and nontoxic solvent, easy to separate after the pretreatment process, and enables pretreatment at lower temperatures compared to other chemicals used in the treatments (Kim and Hong 2001).

Recently, another advanced pretreatment method of lignocellulosics gained lot of attention, i.e. the use of ionic liquids (ILs) (Kilpeläinen et al. 2007; Tadesse and Luque 2011; Mäki-Arvela et al. 2010). The selective dissolution of biomass can be obtained by selecting IL having proper anions and cations. Tested lignocellulosics include corn cob (Li et al. 2010b), corn stover (Binder and Raines 2010), switchgrass (Li et al. 2010c; Zhao et al. 2009), wheat straw (Li et al. 2009), sugar cane bagasse (Kuo and Lee 2009), and rice straw (Ngyuena et al. 2010). Ionic liquids are considered in order to increase the sugar yield from hydrolysis while being a more selective and less energy demanding pretreatment method. However, challenges including the price and recyclability of ILs, corrosivity, and lack of toxicological data should be overcome prior to industrial scale use of ILs as a pretreatment method.

17.6.3 Fermentation

The fermentation step in biobutanol production can be operated in several different modes including batch, fed-batch, or continuous techniques. In addition, more enhanced ways such as the immobilization of bacterium cells have been used (Tripathi et al. 2010; Lee et al. 2008a; Huang et al. 2004; Qureshi et al. 2000, 2004). Some researchers have used separated reactors for acidogenic and solventogenic stages (Mutschlechner et al. 2000).

Fermentative biobutanol production is traditionally based on the use of spore-forming *Clostridium*. During the past decade, the tools of metabolic engineering have been commonly used for the strain modifications. Aims of the metabolic engineering are to increase butanol yield by

- extending substrate utilization range,
- increasing butanol yield on carbon source,
- enhancing selective production of butanol instead of mixed acids/solvents, and
- increasing inhibitor (e.g. butanol) tolerance of bacteria.

Metabolic engineering is done e.g. by inserting some enzyme genes from other bacteria for example to improve the *Clostridium* ability to utilize cheaper substrates. Other ways are to insert or knockdown/out the genes coding critical enzymes and thereby changing the metabolism of the bacterium, or to regulate some genes and improve the bacterium tolerance against inhibitors. Genetic modification can be done also to delay or restrict the sporulation of the bacterium, leading to enhanced production of solvents (Jin et al. 2011).

According to Lee et al. (2008b), the first successful metabolic engineering example of *C. acetobutylicum* ATCC 824 was done by the research group of Papoutsakis in the 1990s when they amplified the acetone formation pathway in bacteria. The fermentation with modified recombinant *C. acetobutylicum* led to increased final concentrations of solvents compared to parental strain (Mermelstein et al. 1993). Borden and Papoutsakis (2007) have reported that strains with a plasmid carrying a CAC1869 or a CAC0003 gene exhibited 13 and 81 %, respectively, increases in butanol tolerance compared to the plasmid control strain.

Although metabolic engineering tools are available for modifying *Clostridia*, there are many challenges like a relatively slow growth rate, spore-forming life cycle, formed by-products, and intolerance to oxygen and the formed solvents in using the bacteria for industrial production. To obtain industrially feasible production process there exists an interest to modify other, more user-friendly microorganisms for 1-butanol production. Recently, *n*-butanol biosynthetic pathway for 1-butanol production has successfully been done with engineered *Escherichia coli* and *Saccharomyces cerevisiae* (Atsumi et al. 2008; Inui et al. 2008; Steen et al. 2008). In these studies, the substitution of Clostridial enzymes with isoenzymes of a number of different organisms were tested. The results showed a successful butanol production but still there is necessity of increasing the yield. Although there are challenges in maintaining the necessary native metabolism

when expressing heterologous biosynthetic pathways, better knowledge of the metabolism, physiology, and genetics of these microorganisms benefits the design and construction of optimal pathways for biobutanol in the future (Atsumi et al. 2008; Inui et al. 2008; Steen et al. 2008).

Another way in order to get more feasible production processes is to use co-cultures of two different bacteria. Tran et al. (2010) have studied co-culture of *Clostridium butylicum* with *Bacillus subtilis*. The use of high amylase producing aerobic *Bacillus* increased the starch substrate utilization and ABE production. It was also shown that adding of costly reducing agents or flushing the medium with N_2 to ensure anaerobic conditions for *Clostridium* were not needed in the co-culture of oxygen consuming *Bacillus*.

Instead of using lignocelluloses and sugars as a carbon source, the direct conversion of CO_2 into 1-butanol has been studied. Lan and Liao (2011) demonstrated 1-butanol production directly from CO_2 and light by engineered cyanobacterium. In the study, the modified CoA-dependent 1-butanol production pathway was transferred into the *Synechococcus elongatus* PCC 7942. The pathway contained a heterologous expression of five enzymes needed for the conversion of acetyl-CoA to 1-butanol. Since the 1-butanol production pathway is derived from strict anaerobes, expressing the pathway in oxygen producing cyanobacteria is challenging. Oxygen removal by inhibiting the cyanobacteria's oxygen evolving capability after growth and the careful modulation of the heterologous enzyme expression is required. To produce one mole of 1-butanol via photosynthesis and Calvin-Benson cycle 48 photons are required. The photon yield is the same as for glucose and isobutanol, which makes it desirable to produce 1-butanol directly from CO_2 and light (Lan and Liao 2011).

17.6.4 Downstream Processing

Choosing of the best recovery method for biobutanol is challenging due to the complexity of the process. The selection should be balanced with many variables such as efficiency, energy requirements and other costs, economics, safety, and process simplicity. Membrane-based methods e.g. membrane evaporation, pervaporation, and reverse osmosis are considered as the most promising techniques (Izák et al. 2008; Ezeji et al. 2003). Especially pervaporation technique has recently received more attention when designing separation and purification steps for biofuel production and thereby pervaporation is introduced more detailed in this section.

Pervaporation is a separation technique used especially in the recovery of liquid components having low concentrations in feed solutions. Target compounds are diffused selectively through the membrane and desorbed to the permeate side as a vapor. Phase change occurs only with the permeating compound reducing the operational costs compared to the conventional distillation process. Driving force for the separation is a chemical potential gradient achieved by different partial

vapor pressures of compounds on the opposite sides of the membrane i.e. the feed side of the membrane is in atmospheric pressure while the permeate side is under vacuum or in low pressure (Schäfer and Crespo 2005).

Selected membrane material determines the nature of the separation: organic compounds can be separated with hydrophobic membranes while hydrophilic membrane material is selected for water removal from the feed solution. Separation performance is evaluated usually by two key parameters: the membrane selectivity indicated as selectivity or enrichment factor, and the flux indicating the amount of compounds passing through the membrane. The experimental variables affecting of these parameters include feed composition, temperature, type, thickness and material of the used membrane, and the pressures in the feed and permeate sides.

Pervaporation has no negative effects on the microbes in the fermentation broth and it is a competitive method compared to other techniques (Qureshi and Blaschek 2001b). Further, pervaporation can be combined with fermentation as an in situ product recovery (ISPR) method and thereby an increased yield, productivity, and economy of the production process can be achieved (Izák et al. 2008; Qureshi et al. 2001). Recently, mainly model ABE-water solutions (Liu et al. 2011; Zhou et al. 2011; Thongsumak and Sirkar 2007; Liu et al. 2005; Huang and Meagher 2001) or real fermentation broths (Liu et al. 2011; Thongsumak and Sirkar 2007; Qureshi et al. 2001) have been used for pervaporation studies. Some researchers have also managed to integrate fermentation and pervaporation into a hybrid process (Liu et al. 2011; Qureshi and Blaschek 1999; Qureshi et al. 1992; Matsumura et al. 1992; Groot and Luyben 1987).

Polydimethylsiloxane (PDMS) membranes have been used in most of the published studies because of highly hydrophobic properties and good chemical, mechanical, and thermal stability of the material. In addition, the fabrication of these membranes is easy and economic (Li et al. 2010c). Other membrane materials used in the recent studies include for instance polyether block amide (PEBA) (Fouad and Feng 2008; Liu et al. 2005), polyvinylidene fluoride (PVDF) (Srivasan et al. 2007), and liquid membranes (Izák et al. 2008; Thongsumak and Sirkar 2007).

Several impermeable components such as acids, bases, salts, and sugars are present in fermentation broths and may have influence on pervaporation performance by causing fouling or blocking of the membrane. Electrolytes and sugars in the feed solution can also affect the driving force and mass transfer rate through the membrane. García et al. (2009a, b) have studied the influence of electrolytes on the butanol pervaporation. They did not observe a significant influence of the electrolyte (NaCl) on the pervaporation performance of PDMS-based membranes toward butanol.

17.6.5 Sustainability of the Process

The widely used definition of sustainability is of the United Nations' Bruntland Commission (WCED 1987): "Sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs". Corporate sustainability implies that companies have to maintain and grow their economic, social and environmental capital base, while actively contributing to sustainability in the political domain (Dyllick and Hockerts 2002). Sustainability can be covered by the triple bottom line approach (Bowell 2010) consisting of three impact areas: environmental, economic and social, or "people, planet and profits". Three elements of sustainability are in a triangle, indicating that there are not necessarily solid borders between these three aspects or other way is that all aspects are satisfied at the same time. This would indicate that all of the aspects of sustainability have the same value, and nothing else is sustainable than the ideal state of triple overlap. The problem arising when separating the three sides of sustainability into their own ellipses or pillars is that these aspects are interrelated and are not necessarily exclusive.

In addition to concerns about the environment, also legislation promotes the sustainability assessment in relation to the production of biofuels. In Europe, legislation under the Directive 2003/30/EC requires the use of biofuels or other renewable fuels for transport, with the goal of reaching a 5.75 % share by 2010. Under the Renewable Energy Directive (RED Directive) (2009/28/EC) on the promotion of the use of energy from renewable sources rises to a minimum 10 % in every Member State in 2020. The Directive also aims to ensure that as the use of biofuels in the EU is expanding, only sustainable biofuels are used, which can generate a clear and net green house gas (GHG) savings with no negative impact on biodiversity and land use. Renewable Energy Directive (RED) includes three relevant articles with respect to the sustainability: sustainability criteria for biofuels and bioliquids (Article 17), verification and compliance with the sustainability criteria (Article 18), and calculations of the GHG impact of biofuels and bioliquids (Article 19) (EU Directive 2009/28/EC, BioGrace 2011). It is expected that these sustainability criteria also call for reporting requirements on feasibility and applicability in relation to air, soil, or water protection.

Evaluation of sustainability is very complex and difficult issue and it should take into account not only environmental related questions as it seems to be done in legal requirements at the moment. To simplify the method, the following questions can be asked and answered to assess the three aspects of sustainability:

- Does the process produce waste?
- Are there any health and safety issues related to the harmful nature or amount of waste?
- What is the atom economy of possible reaction routes?
- Are the by-products saleable?
- Does the process use or generate materials that are potentially harmful?

- Are there any health and safety issues related to the harmful nature of chemicals used or generated in the process?
- Do the products produced contain harmful or hazardous materials (take into notice also the side products)?
- Do the processes require large deviation from ambient temperature and pressure?
- Are there any solvents and/or other auxiliary chemicals used in the processes or products?
- Are the used materials renewable or nonrenewable?
- Does the process use catalysts?
- Are the catalysts heterogeneous or homogeneous?
- Is there any health, safety, or environmental issues related to the used catalytic materials?
- Are there hazards related to the designed process (synthetic and formulation activities, involved operations, or reaction conditions)?

These type of questions can be used in an early process design phase and when defining the sustainability of a new process and comparing it to the existing ones. In biobutanol production, the question of raw material used is very important as the raw material will have a huge impact on all the three aspects of social, economic, and environmental issues; even if the renewable material is used. Energy and material efficiency are certainly better in novel catalytic reactions compared to traditional ABE fermentation process. However, the impact of the catalytic materials needs to be evaluated because in many cases rare and precious metals used have high social, economic, and environmental impacts depending on mining and processing procedures. The amount and quality of produced by-products may also have significant impacts. Additional recovery steps are usually needed, but if by-products are valuable positive economic impacts can be gained. More research and methodology development are required to ensure successful sustainability assessment to gain the best knowledge and results based on the triple bottom line.

17.7 Conclusions

There is a demand for the increased production and usage of biofuels from both environmental and a political point of view. Bioethanol and biodiesel are currently the most used biofuels in the transportation sector. New alternatives such as biobutanol are also needed to fulfill the usage demands in the future. In this chapter, biobutanol has been discussed as a potential option for future transportation biofuel. Biobutanol has better environmental and fuel properties when compared to more commonly used biofuels such as ethanol.

The economic and feasible biobutanol production in industrial scale depends on several aspects including substrate cost, product yield, efficiency and expenses of the separation and purification steps of biobutanol, and utilization of by-products

formed. By using novel catalytic routes, the chemical production process of butanol can be based on renewable raw materials instead of petrochemicals. Research related to the synthesis of bioethanol or butyric acid to biobutanol for finding optimal catalysts and process conditions is going on actively. The biochemical, fermentative production of biobutanol can be enhanced by finding economical and sustainable raw materials (e.g., biomasses classified as by-products or wastes), by using metabolic engineering and modified bacterium strains to gain better product yields from fermentation, and by developing more energy efficient upstream and downstream processing techniques. The efficient use of raw materials and utilization of all products of the process are also vital. Moreover, sustainable process design also considers the economic, environmental, and social impacts caused by the whole production process chain and uses materials and techniques with least negative impacts.

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Chapter 18
Life-Cycle Environmental Impacts of Biofuels and Co-products

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Abstract Issues of energy independence and security, global climate change, and the depletion of fossil resources drive research into biofuels and bioproducts. While emerging biofuels and biorefineries pursue lower carbon transportation fuels, careful consideration of a wide range of potential environmental impacts is necessary to avoid unintended consequences. These concerns can be addressed by holistic life-cycle evaluation of bioenergy/biofuel supply chains from raw materials acquisition, to fuel conversion and end use. Life cycle assessment (LCA) is a promising tool for assessing the environmental sustainability of these biofuels. This chapter discusses current biofeedstocks and fuels, introduces the methodological framework of LCA, and explores challenges, critiques, benefits, and applications of LCA in evaluating the environmental performance and sustainability of emergent biofuels and co-product systems. An analysis of algal biodiesel production is presented as a case study, and the broader implications and potential of LCA to inform decision making are explored.

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18.1 Introduction

Life cycle assessment (LCA) is a technique used to quantify the environmental impacts of a product or service that incorporates and aggregates the related resource consumption, emissions, and impacts across the various stages of the product's life. LCA has been touted as a relevant tool for assessing the environmental sustainability of emerging technologies, and has had significant application in the developing field of biofuels and bioproducts. LCA can be used to identify process inefficiencies and environmental "hotspots" along a supply chain; therefore, facilitating efficient eco-design, and helping support environmentally sound decision making. For these reasons, LCA is a germane and powerful tool for assessing emerging biofuel technologies. The goal of this chapter will be to present the challenges, benefits, and applications of LCA in evaluating biofuels and related coproducts. The chapter will include a discussion of current biofeedstocks/biofuels and provides a broad understanding of the methodological framework and role of LCA in evaluating the environmental performance and sustainability of emergent biofuels. The chapter is organized as follows: Sect. 18.1 provides a basic description of the environmental, economic, and political motivation for biofuels. Additionally, Sect. 18.1 compares different biomass types including first generation, second generation, and third generation feedstocks, and examines different conversion pathways to produce biofuels. Section 18.2 presents a detailed description of the LCA framework and methodology, examines the application of LCA to biofuels, and reviews different environmental sustainability metrics. Section 18.3 offers an overview of several previous biofuel LCA studies representative of 1st-, 2nd-, and 3rd-generation feedstocks and fuels, and also examines the challenges associated with the application of LCA methodologies to biofuels. A case study of algal biodiesel is provided in Sect. 18.4. Section 18.5 provides a summary of the chapter with consideration of the broader role of LCA in the development of sustainable fuels.

18.1.1 Political, Economic, and Environmental Motivations for Biofuels

The dawn of the twentieth century brought the rapid expansion and development of the petroleum, natural gas, and coal industries. Since then, the world's economies have become highly dependent on fossil resources to meet the demands of industry and to maintain consumer quality of life. Resultant depletion of fossil reserves has the potential to limit global economic growth, complicated by a current reliance on nonrenewable sources of energy that may threaten long-term global economic prosperity. In addition to these economic considerations, increased global demands for energy and transportation fuels as well as concerns

over global climate change have driven researchers to identify alternative liquid fuels which are more sustainable and carbon–neutral.

Accordingly, biofuels have gained widespread attention at academic, industrial, regulatory and political levels, and to some degree among the general public. Scientists have focused on identifying the most promising biofuel feedstocks and are investigating various biochemical and thermochemical conversion pathways to transform these feedstocks into useful fuel products. Research, development, and the subsequent commercial deployment of biofuels all have the potential to create new jobs and provide extensive economic benefits.

Rising atmospheric concentrations of anthropogenic carbon have become a mounting international concern as indicated by national and international responses to the work of the Intergovernmental Panel on Climate Change (IPCC 2007). A growing body of evidence suggests that increasing concentrations of greenhouse gases, brought on by human activities, are causing harmful and long-term global climate change, stimulating worldwide efforts for greenhouse gas (GHG) reductions. Since the transportation sector constitutes approximately 15 % of global GHG emissions (EPA 2010), displacing traditional transportation fuels with low-carbon or carbon–neutral biofuel alternatives presents one avenue for mitigating and stabilizing global atmospheric levels of anthropogenic carbon.

Policy makers are playing a larger role in the development and implementation of biofuels by establishing regulatory policies and mandates for renewable fuels. In 2007, the United States passed the Energy Independence and Security Act (EISA), which mandates annual domestic production targets for renewable transportation fuels. By the year 2022, production must reach 36 billion gallons including at least 21 billion gallons from cellulosic ethanol and advanced biofuels (Sissine 2007). Similarly, in 2008 the European Union (EU) passed the Renewable Energy Directive (RED), which mandates that 20 % of gross national energy consumption be derived from renewable sources by the year 2020 (Ismail and Rossi 2010). Additionally, in 2009 the EU passed a revised fuel quality directive (FQD) which requires transportation fuel suppliers to reduce life-cycle GHG emissions by 1 % each year culminating in a 10 % overall reduction by 2020. As has been discussed, technological, economic, and political factors will all play critical roles in the evolution and commercial viability of emergent biofuel industries.

18.1.2 Comparison of Potential Feedstocks, Fuels, and Conversion Pathways

Commercial biofuel production is a multifaceted issue: Identifying and assessing potential biofuel feedstocks remain an arduous process, as the use of some bio-feedstocks raises complex ethical questions and may have unintended economic and environmental ramifications. A myriad of different biofuel conversion

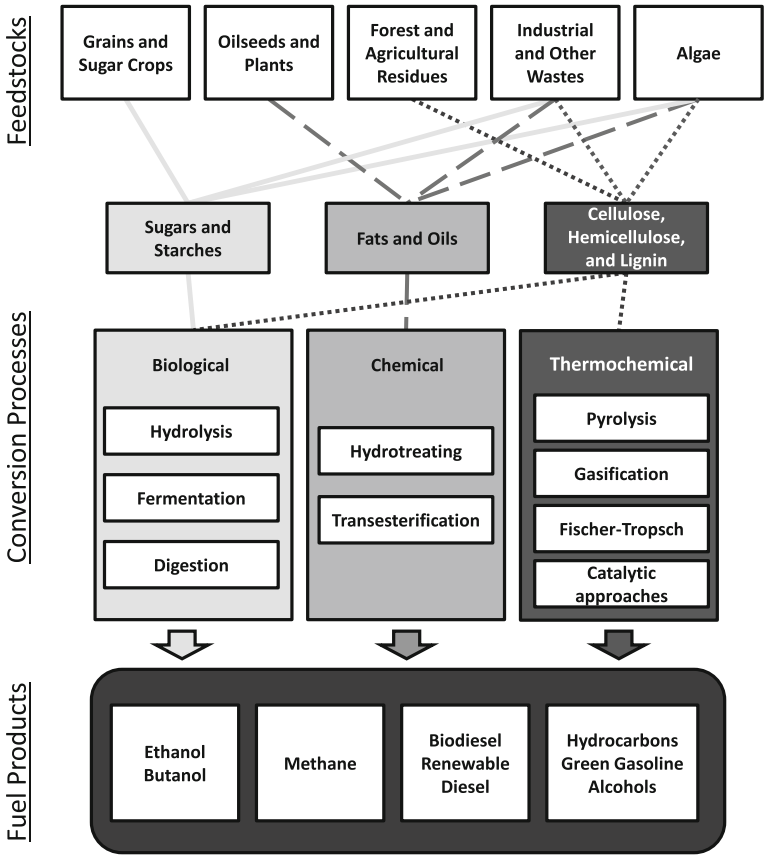

Fig. 18.1 Alternate biofuels production routes: feedstocks, conversion pathways, fuel products and coproducts

pathways and associated coproducts are possible, see Fig. 18.1. In addition, the mode of biofuel production can influence the quality of the resultant fuel as well as its related economic, environmental, and energetic impacts. This section will compare different biofeedstocks and examine different thermochemical and biochemical conversion pathways for producing biofuels.

18.1.2.1 First Generation Biofuels

First generation biofuels, also known as conventional biofuels, are derived from sugar, starch, animal fats, and plant or vegetable oils. Common first generation biofuels include: bioalcohols, bioethers, green diesel, vegetable oils, biogas, syngas, and solid biofuels (Naik et al. 2010). First generation biofuels are typically

produced from the fermentation of grains and crops with a high sugar or starch content, such as corn, sugarcane, sugar beats, wheat, or barley to produce bioethanol, or by transesterification of oils extracted from crops such as soybean, rapeseed, canola, mustard seed, palm, coconut, and sunflower to create biodiesel. Alternative biochemical pathways are being investigated for converting biomass into heat, electricity, or fuel utilizing a wide range of different technologies.

Despite the many advantages of first generation biofuels various economic, political, environmental, and social issues have hindered their widespread adoption. In recent years, political and scientific actors have raised concerns that the use of first generation biofuels may result in further environmental degradation (Pimentel et al. 2007), including potential loss of biodiversity, adverse impacts on water resources, soil erosion and depletion, accelerated deforestation, and land-use impacts (Sims et al. 2008).

Existing research has also reported that the direct and indirect land use change effects may possibly negate the carbon dioxide reduction potential of first generation biofuels resulting in overall higher life-cycle GHG emissions relative to baseline petroleum fuels (Fargione et al. 2008; Melillo et al. 2009; Searchinger et al. 2008). Additionally, thermodynamic and energy analyses have shown mixed results concerning the energetic balance of some first generation biofuels (Patzek 2004; Pimentel et al. 2007; Patzek and Pimentel 2005; Hammerschlag 2006).

Furthermore, without government subsidies and grants, biofuels are currently not cost competitive with more established transportation fuels (Pimentel et al. 2007), making them an expensive option for mitigating GHGs. Additionally, there is growing concern that displacing farmland and food crops for biofuel production may lead to inflation of global food prices (Timilsina et al. 2012; Nonhebel 2012; Pimentel et al. 2007), as many biofuel cultivars—including corn, soybean, and sugarcane—are primarily used for animal feed and/or human consumption. For these reasons researchers have investigated producing biofuels from alternative, nonfood crops.

18.1.2.2 Second Generation Biofuels

Many of the issues and shortcomings of first generation biofuels (*conventional biofuels*) are being addressed with second generation biofuels (*advanced biofuels*) (Eisentraut 2010; Sims et al. 2008). While first generation biofuels are generated from sugars, starch, and oils produced from arable crops, second generation biofuels are derived from forest and agricultural residues, lignocellulosic biomass, industrial wastes, and nonfood crop feedstocks. Second generation biofuels include: biomethanol, bioDME (DiMethyl Ether), biohydrogen, biomethane, butanol and isobutanol, DMF (2,5-DiMethylFuran), HTU (Hydro Thermal Upgrading) diesel, wood diesel, and mixed alcohols (Sims et al. 2010; Naik et al. 2010). Common biochemical pathways for second generation biofuels include the use of pretreatments, such as enzymes and microorganisms, to break down and extract the sugars contained in lignocellulosic biomass, which can then be

fermented to produce ethanol and other alcohols. Thermochemical pathways such as gasification, pyrolysis, and torrefaction of second generation biofeedstocks (Sheehan 2009) can be used to produce syngas of when bio-oil can be fermented or chemically reformed into various fuel products, including ethanol, synthetic diesel, or aviation fuel (Sims et al. 2008).

Despite their apparent advantage over first generation biofuels, second generation biofuels must overcome many technological and economic challenges before their commercial deployment (Williams et al. 2009). Development of these biofuels at a commercial scale has remained challenging, because many of these feedstocks cannot be produced all year long and can only be harvested periodically (Eisentraut 2010). Furthermore, second generation biofuels have also yet to prove cost competitive at a commercial scale. Improvements in biomass cultivation, processing, and conversion efficiencies will be crucial both to increasing biofuel performance and for cost reduction. While thermochemical conversion pathways commonly utilize mature, proven technologies to produce a wide range of synthetic fuels, current biochemical pathways are less technologically mature and may present greater potential for cost reduction through process improvements over time. Further, R&D will be required to optimize these conversion systems and to identify which thermochemical and biochemical conversion pathways are best suited for commercial-scale biofuel production.

18.1.2.3 Third Generation and Drop-In Replacement Biofuels

Third generation biofuels—fuels produced from microalgae (Dragone et al. 2010)—do not suffer from many of the major drawbacks associated with first or second generation biofuels, and in recent years have gained increased consideration as sustainable liquid–fuel alternatives. Microalgae are considered as an ideal feedstock for next generation biofuels due to their ability to be cultivated on nonarable land, high productivity (Chisti 2007), high lipid content, and semi-continuous to continuous cultivation and harvesting cycles. Microalgae have the potential to utilize wastewater, as well as CO_2 from industrial flue gas, for growth (Benemann 1997; Golueke CG 1965; Ho et al. 2011; Kadam 2001). Additionally, the production of microalgae needs not displace food, animal feed, and other arable-crop derived products. Microalgal derived fuels have the potential to act as "drop in" replacements for petroleum fuels since their chemical composition may prove compatible with the existing transportation fleets as well as current fuel storage and delivery infrastructure. Furthermore, researchers are exploring the application of modern technologies such as genetic modification to optimize the growth, resilience, and oil yield of new algal strains.

Open raceways ponds (ORP) and photo-bioreactors (PBR) are two standard growth configurations currently considered for the mass cultivation of microalgae (Jorquera et al. 2010). While PBR systems have better control over algae growth parameters, lower risk of contamination of the algal culture, and higher volumetric growth rates as compared to ORP, high capital and operating costs limit their

commercial potential (Xu et al. 2009). Due primarily to low capital costs, ORPs have gained increased consideration from industrial, governmental, and academic sectors. Between 1978 and 1996, the US Department of Energy's (DOE) aquatic species program studied algal biochemistry, strain selection, and the pilot–scale production of algal biodiesel using ORP systems (Benemann and Oswald 1996). More recent research has primarily focused on the production of algal biodiesel via transesterification and has identified CO_2 provisioning, fertilizer supply, and biomass drying as the primary rate-limiting factors in algae-to-fuel systems (Clarens et al. 2010; Kadam 2001; Lardon et al. 2009). A variety of cultivation, harvest, and conversion options have been examined (Brentner et al. 2011; Clarens et al. 2011; Soratana and Landis 2011) to produce multiple algal fuel and bioenergy products such as biomethane, renewable diesel, green aviation fuel (Agusdinata et al. 2011), and bioelectricity. Further research will be required to identify which cultivation, harvesting, conversion, and fuel-upgrading pathways are most energetically favorable and which are most economically and environmentally advantageous. A case analysis of algal biodiesel production is presented in Sect. 18.4.

Understanding and assessing the far-reaching impacts and implications of emerging biofuels—before their widespread implementation—will be critical for assuring the long-term sustainability of these fuels. Failure to address the potential risks and impacts associated with biofuels may have long-standing environmental consequences, and may jeopardize the successful adoption and commercial viability of these bioresources. Comprehensive analysis from a systems perspective that consider the full range of environmental impacts can address these concerns. One widely popular systems analysis technique is LCA, which is discussed next.

18.2 LCA Methodology and Framework

18.2.1 LCA Approach

LCA is a systematic technique for quantifying and assessing the total environmental impacts of a product or process across all phases of the product's life cycle, as standardized in ISO 14040 (ISO 2006). The considered life cycle phases generally include: raw material extraction, input conversion, product manufacturing, packaging, transport, product use, and disposal or recycling at end of life. LCA's four distinct but interdependent steps are (1) Goal and scope definition, (2) Life Cycle Inventory (LCI) development, (3) Life Cycle Impact Assessment (LCIA), and (4) Interpretation and Improvement Analysis which are illustrated in Fig. 18.2.

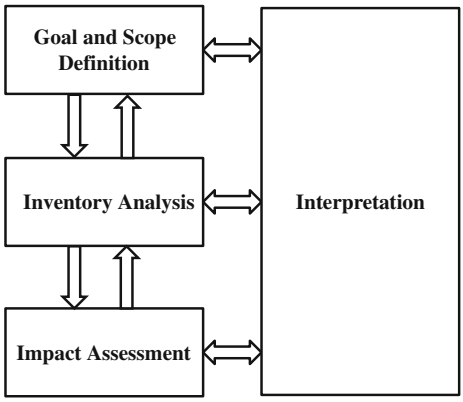

Fig. 18.2 Life cycle assessment framework as standardized in ISO 14040

18.2.1.1 Goal and Scope Definition

The first step of performing an LCA includes defining the purpose of the study, its scope, and its context including the audience for whom it is intended. The temporal, spatial, and production chain boundaries of the system under study must be established, methods to be used identified, functional unit chosen, and impact categories of interest enumerated (Baumann and Tillman 2004). Purposes of LCA studies are often to compare products or to identify component processes responsible for the greatest contributions to life cycle environmental impacts. For example, the LCA might be designed to compare corn ethanol, soy biodiesel and their respective coproducts, or to analyze a single complete production pathway for the generation of bioelectricity from corn stover.

Boundary definition identifies what processes are included in the system to be modeled by the inventory analysis. This begins by setting the overall scope of the analysis in terms of the cycle life stages to include: from raw material extraction to capital equipment, from simple manufacturing to delivery, use and disposal. For example "cradle-to-grave" scope implies production, use and disposal phases, whereas "cradle-to-cradle" implies production, use and recycling. In fuel LCA, scope is often stated in alternate terminology relating to traditional petroleum fuel extraction processes: well-to-gate, well-to-pump, and well-to-wheels.

Defining the boundary is a balance between including too many of the connections to the greater economy (increasing work, time, and cost of the analysis) versus including too few component processes which could lead to incomplete or erroneous results. Restricting the scope of the study limits the extent of data collection, but also may limit the validity of results. Specific cutoff criteria for negligible contributions should be selected based on the percent contribution of an element to the total mass, energy, or environmental burden to the overall system.

The choice of a functional unit is more than setting a production quantity, it defines a fair basis for comparison and evaluation. It arises from the function of the product system, rather than the quantity of the product itself. Thus beverage packages (of varying sizes) may be evaluated not by bottle count, but by eight-ounce

servings delivered. Similarly, for fuel systems the functional unit may be defined in terms of gallon of ethanol equivalent (based on energy content), per megajoule (MJ) energy, person-kilometers traveled, or vehicle-kilometers traveled (VKT).

Among the important methodological considerations to be decided as part of the scoping process is the selection of allocation method. Allocation describes the method for splitting inventory flows and environmental impacts of a process across multiple coproducts produced in the examined system. Allocation and its ramifications will be discussed in greater depth below.

18.2.1.2 Life Cycle Inventory Analysis

The LCI analysis step consists of defining and quantifying all relevant flows of energy and materials into, through, and from the system (ISO 2006). Essentially, a flow model is built reflecting all the processes included within the system boundary including resource extraction, production processes, transport, use, and waste management. Input and output data are collected and documented for each process being modeled including flows of raw materials, energy, products and coproducts, wastes, and emissions to air and water. For cradle-to-grave studies, data collection is particularly extensive as it must include all upstream processes (resources extraction, production, and transport) as well as downstream processes (product use and disposal). While some process data may be available in public or commercial databases—such as Ecoinvent (Ecoinvent Centre 2007; Frischknecht 2005), GREET (ANL 2010), US LCI (Deru and NREL 2009), and ELCD (ELCD/ILCD 2012)—or through the use of software tools—including SimaPro (SimaPro 7.3.3/Pre Consultants 2012), GABI (GaBi 5/PE International 2012)—data collection can be resource and time intensive, especially when modeling site-specific processes and new technologies.

Once complete unit process data have been assembled, aggregate resource use and pollutant emissions can be calculated to determine environmental loads per functional unit for the overall system. The time-intensive process of assembling a complete LCI is complicated by processes that produce more than one product. In this case, shares of the inventory must be assigned to each coproduct through the allocation procedure(s) selected during the goal and scope phase.

18.2.1.3 Life-Cycle Impact Assessment

The purpose of LCIA is to translate the resource and emissions flows identified in the LCI into their potential consequences for the environment and human health. It consists of a two-step process of impact classification and quantitative characterization. The classification step links each LCI flow with its respective impacts on resource use, human health, and the environment (ISO 2006).

The characterization step calculates the magnitude of the associated impacts in terms of a reference unit for each category. For example, though carbon dioxide,

methane, and nitrous oxide each have different strengths as GHGs, their global warming potential (GWP) can be measured and aggregated in the common unit of CO_2 equivalents. Specifically, 1 kg methane has a global warming impact equivalent to 25 kg of CO_2 and 1 kg nitrous oxide has a global warming impact equivalent to 298 kg of CO_2(IPCC: M.L. Parry 2007; IPCC: B. Metz 2007). Therefore, the GWP characterization factors for methane and nitrous oxide, relative to CO_2, are 25 and 298, respectively. These factors enable the LCA practitioner to aggregate the GWP impacts from these disparate substances into one combined measure. Similar characterization factors for a vast number of chemical substances and impact categories are available in the literature and through public and commercial databases.

This process of classifying, characterizing, and aggregating related results into a series of midpoint indicators, which summarizes each type of life-cycle environmental impacts, facilitating the comparison of complex systems. Normalization of impact categories allows for the comparison of tradeoffs between different environmental impacts. A midpoint impact assessment method developed by the US Environmental Protection Agency (EPA), the Tool for the Reduction and Assessment of Chemical and other environmental Impacts (TRACI) translates the environmental loads identified by the life-cycle inventory into 11 specific impact categories: ozone depletion, global warming, acidification, eutrophication, tropospheric ozone (smog) formation, ecotoxicity, human health criteria-related effects, human health cancer effects, human health noncancer effects, fossil fuel depletion, and land-use effects (Bare et al. 2003). Alternative midpoint assessment methods include: ReCiPe (Goedkoop et al. 2009), USEtox (Querini et al. 2011; Rosenbaum et al. 2008), CML 2001, EDIP 2003 (Dreyer et al. 2003), Ecological scarcity 2006 (Frischknecht et al. 2009), Greenhouse Gas Protocol (Ranganathan et al. 2004; Sundin and Ranganathan 2002), Ecological footprint (Huijbregts et al. 2008).

Additional weighting and normalization steps may be used to further aggregate midpoint indicators into endpoint or damage indicators such as ReCiPe (Goedkoop et al. 2009), Eco-indicator 99 (Dreyer et al. 2003), Impact 2002+ (Jolliet et al. 2003), and EPS 2000. The various LCA tools have been evaluated extensively in the literature (Landis and Theis 2008; Kulkarni et al. 2005; Dreyer et al. 2003; Olsen et al. 2001; Whittaker et al. 2011). While single metrics may conveniently summarize damages to human health and the environment, facilitating interpretation by decision makers, the LCA practitioner's choice of end-point valuation scheme is subjective and controversial, and is often skipped in favor of a midpoint analysis. The chosen LCIA approach should correspond with the goal and scope of the study.

18.2.1.4 Interpretation

During the interpretation phase of the LCA process, the process is evaluated and validated, conclusions are drawn from the preceding LCI and LCIA, and recommendations made based on inventory and impact assessment data. In addition, the

interpretation phase should include the validation of results, and can often lead to iterative revisions to and improvements in the other LCA phases. Validation of the results is essential and may include analysis of variability and uncertainty in the model using a variety of qualitative sensitivity analysis techniques as well as quantitative statistical procedures. As the LCA process requires, the aggregation of data from multiple processes with varying degrees of uncertainty, statistical techniques offer the most robust treatment for uncertainty in the assessment.

Conclusions may include the identification of the product or process with lowest impact(s) or identification of specific subprocesses of greatest impact, so-called "hot spots" which can then be targeted for improvement, enabling businesses to both increase product quality and reduce harmful environmental impacts.

18.2.1.5 Attributional versus Consequential LCA

LCA models may also differ in the approach employed to address the material and energy flows in the system under investigation. The attributional LCA (ALCA) methodology, which has been utilized for a vast majority of biofuel LCA studies, attempts to quantify the flow of resources and emissions from a product system and its subsystems. Emissions and their related impacts are attributed to the final product by one of several available methods, including allocation strategies, and system expansion. ALCA determines the magnitude of environmental burdens which can be attributed to a product or service, and which processes within the production chain account for the greatest share of impacts. Researchers have argued that it is not fully possible to draw conclusions on future changes by using only ALCA (Ekvall and Weidema 2004; Schmidt 2008; Weidema et al. 1999).

In contrast, consequential LCA (CLCA) methodology, aims to explain how the physical flows to and from the technosphere may change in response to a change in the life cycle of the product or service. CLCAs attempt to consider a much broader system boundary. The most commonly employed form of a CLCA considers the use of economic models. These models track monetary, material, and energy flows across economic systems. In a CLCA, the consequential effects of production are added within the system as resources enter or leave the economic market, thereby changing the supply–demand dynamics, and potentially causing changes in resource use and associated environmental impacts. In other words, the system under investigation is expanded to include the consequences due to changing some production patterns outside the narrowly defined life cycle of interest. This is generally accomplished using marginal data and is accounted for on the basis of price elasticities of supply and demand (Lesage et al. 2007; Lund et al. 2010; Reinhard and Zah 2009; Sanden and Karlstrom 2007). Such change-oriented LCAs focus on the broader environmental impacts of alternate courses of action, such as the replacement of the existing technologies with a new technical option. For example, in an algae-to-biodiesel consequential LCA, the main desired product is the biofuel. However, the production of glycerol as a byproduct may result in the replacement of glycerol production via other methods involving greater

consumption of nonrenewable resources. Similarly, the coproduction of animal feed may displace other sources of animal feed existing in the market. As with any attempt to predict the future, CLCA faces inherent challenges due to data and modeling limitations as well as inherent uncertainty.

18.2.1.6 The LCA Approach—Challenges and Benefits

LCA studies have been criticized for various reasons including (1) incomplete or obsolete process LCI data, (2) inappropriate system boundaries, (3) poor allocation procedures, or incomplete accounting for byproducts. The assumption that emissions can be scaled linearly may not correspond to processes that exhibit a range of emissions, depending on scale or temperature or other factors. Also LCA results may either not be generalizable beyond the specific geographic location and time period of the analysis, or they may include process data that are outdated or inappropriate to the specific location. The specific application of LCA to fuel systems is discussed below and further in the case study in Sect. 18.4.

18.2.2 LCA and Fuels

LCA methodologies are increasingly essential to evaluating the sustainability of both traditional and alternative fuel products. Many life-cycle studies of biofuels concentrate primarily on energy analysis and greenhouse gas emissions, though other impacts such as water consumption, eutrophication potential, and land-use change are of increasing interest. This section will present the major components and metrics of life-cycle energy analysis as well as a discussion of life-cycle GHG analysis.

18.2.2.1 Major Energy Metrics

A variety of metrics are used to quantify the consumption of primary energy in the production of biofuels (Murphy et al. 2011b; Cleveland 2010; Chwalowski 1996). In contrast to the process energy used directly in a given process, primary energy represents the total of all energy resources consumed to produce the materials and energy used in the process. For example, operating a 40 W light bulb for 1 h would use 40 W-hours of process energy (electricity), while the total primary fuels consumed to generate and transmit that required electricity would be on the order of 108 W-hours (assuming a primary energy factor of 2.7) with wide variability due to specific geographic location, technologies, and above all the specific mix of primary fuels used to generate the electricity.

Several principle energy metrics are defined and summarized in Table 18.1. One key measure considered for biofuels is the net energy balance (NEB) also

Table 18.1 Key energy metrics

Name	Abbreviation	Formal definition
Net energy balance	NEB	$\sum \text{Energy}_{\text{output}} - \sum \text{Energy}_{\text{input}}$
Energy return on investment	EROI	$\frac{\sum \text{Energy Output}}{\sum \text{Energy Input}}$
Net energy ratio	NER	$\frac{\sum \text{Energy Output}}{\sum \text{Energy Input}}$
Fossil energy ratio	FER	$\frac{\sum \text{Energy Output}}{\sum \text{Fossil Energy Input}}$
Energy breeding factor	BF_{en}	$\frac{\sum \text{Energy Output}}{\sum \text{Nonrenewable Energy Input}}$

known as net energy value (NEV). NEB is defined as the difference between the energy in the biofuel product (and coproducts) and the total primary energy required to produce the fuel. A positive net energy balance is one criterion for sustainable fuel product. The energy return on investment (EROI) and net energy ratio (NER) represent ratios of the total energy in the biofuel product and its coproducts to the total primary energy required to produce them. NER values greater than one correspond to a positive energy balance.

A variation of energy return on investment, fossil energy ratio (FER) considers only the consumption of nonrenewable fossil fuel resources and is defined as the ratio of the sum of energy content of the products over the primary fossil fuel inputs consumed during production. This metric considers only the nonrenewable fossil fuel components of primary energy and serves to evaluate how much of fuel product is generated per unit investment of fossil fuels. FER values greater than 1 are net fossil energy positive, reflecting more energy in the products than the fossil energy consumed during production. Thus, FER is a measure of the renewability of the fuel. The energy breeding factor, an analogous metric, represents the energetic return per unit of nonrenewable energy consumed.

18.2.2.2 Criticisms of Energy Metrics

Net energy balance and related metrics have been criticized for aggregating different types of energy with no adjustment for their differing quality or value (Murphy et al. 2011a; Liska et al. 2009; Liska and Cassman 2008; Murphy et al. 2011b; Dale 2007). The implicit assumption that all energy carriers are equal and can be added together is contradicted by the observed wide variation between energy carrier prices. Compare, for example, the per million BTU prices for the following fuels: $2/mmBTU for coal, $10/mmBTU for petroleum, and $24/mmBTU for electricity. These fuels are clearly valued not just for their energy content, but for the services that they can provide, that is the differing utility or usefulness of coal, petroleum, and electricity (Dale 2007). Despite their intuitive appeal, ratios that assume all energy forms are fungible can be at best of only limited value. The choice of energy metrics must be informed by a clear

understanding of the goal(s) of the analysis. If the policy goal is to increase domestic energy security policy-driven metrics such as petroleum displacement ratio or FER should be considered. If the goal is to reduce GHG emissions to increase climate security, metrics such as GHG emissions per vehicle-kilometer traveled are more appropriate (Dale 2007).

A recent study proposed a set of nine complementary energy metrics for comparing biofuel systems. These metrics span three categories of evaluation (Lavigne and Powers 2007)

- energy consumption (from fossil, petroleum, and renewable sources)
- energy security (NEV, %foreign consumed, and % domestic consumed), and
- energy resource consumption (% renewable consumed, energy efficiency ratio, and net energy resources for transportation)

In general, a move toward the use of more transparent and holistic methodologies for the evaluation of biofuels can better reflect the multiple energy, environmental, economic, and security goals.

18.2.2.3 Greenhouse Gas Emissions and LCA

Concerns over the specter of global climate change have resulted in international and national legislation designed to limit the release of GHGs throughout national economies, particularly from the transportation sector. As a result, reduction of GHG emissions has arisen as one of the primary drivers for the biofuels industry. In the United States context, the current renewable fuel standard (RFS2) requires the production and blending of renewable fuels into the US fuel mix. RFS2 requires reductions in life-cycle GHG emissions of these renewable fuels as compared to the petroleum fuels they replace. For example, advanced biofuels must achieve at least a 50 % reduction in GHG emissions across their life cycle (EPA 2010).

A first step in modeling GHG emissions is to execute a carbon balance, that is, to determine the flows of carbon into, through, and out of the production system. The LCA practitioner must be cognizant of the biogenic and anthropogenic sources of elemental carbon moving through the cultivation, fuel production, and combustion chain. While petroleum fuel combustion releases "new" atoms of carbon into the atmosphere that had previously been stored in underground formations over geologic timescales, biofuel combustion does not necessarily release "new" CO_2. Atmospheric CO_2 which is fixed into plant biomass via photosynthesis, converted to fuel, and returned to the atmosphere during fuel combustion, does not result in the addition of any new—that is anthropogenic—atmospheric carbon dioxide, and therefore has no impact on global warming.

Carbon accounting can become problematic when comparing studies with different system boundaries. For example, a study of biomass cultivation might indicate that atmospheric carbon is fixed by photosynthesis, and therefore "sequestered" in the biomass product, potentially resulting in net negative GHG

emissions. A second study of biomass cultivation and conversion may show that the same biogenic carbon is again embedded between two coproducts, fuel and residual biomass. While biofuel combustion *per se* may be carbon neutral, the fate of the carbon embedded in the coproduct must also be considered. For example, while residual biomass used as a soil amendment may sequester carbon in the earth for several years, residual biomass combusted for the generation of bioelectricity results in immediate release of the fixed carbon resulting in no true sequestration. A well-to-wheel LCA of the complete biofuel cultivation, production, and combustion system also requires the consideration of both direct and indirect life-cycle emissions of all GHGs including methane, nitrous oxide, and CFCs and anthropogenic CO_2. Additionally, the choice of allocation method can have a strong impact on the LCA results.

18.2.2.4 Allocation and Coproducts

For an integrated system that produces more than one product, system expansion and allocation are alternative techniques for allowing the resource consumption and emissions to be equitably split between the coproducts. System expansion refers to expanding the conceptual boundary of the studied system, such that it is credited for emissions avoided from another system. That is, the primary system (S1) generating primary product (P1) and coproduct (P2) is credited in an amount equivalent to the avoided emissions; that is, the emissions which would have been generated had P2 been produced in its own virgin system (S2).

According to ISO 14040, when allocation cannot be avoided by using system expansion or increasing the detail of the model, it should split environmental loads among the system's "different products or functions in a way which reflects the underlying physical relationships between them" (ISO 2006). Relevant physical relationships may include mass, volume, and energy content. When a physical basis for allocation cannot be determined, it should be based on another relation between the coproducts, such as their proportional economic value. Additionally, ISO 14040 requires a sensitivity analysis to be carried out when multiple allocation procedures are possible. As different allocation methods can yield widely varying LCA results (Kaufman et al. 2010; Azapagica and Cliftb 1999; Luo et al. 2009), this variability can be captured and effectively communicated through a sensitivity analysis.

Beyond allocation, issues of coproducts scale may also arise. An almost trivial case occurs when the production of the coproduct exceeds that of the primary product either in terms of mass or value. An alternative case particularly suited to the study of biofuels and other nascent industries occurs when the scale of production increases to industrial scale to meet the growing demand for the primary product. Concurrent coproduct production may surpass its own market demand. This could result in dramatic drops in market price for the coproduct to the point where any additional marginal production must be treated as waste.

18.3 Biofuels LCA: Selected Applications

This section provides an overview of several previous LCA biofuel studies including an updated report on the life-cycle performance of corn ethanol, soybean diesel, and a comparison of algal bioenergy to other 1st- and 2nd- generation biofuels. Issues and challenges with previous biofuel studies are addressed.

18.3.1 Corn Ethanol

The rapid expansion of the US ethanol industry over recent years has brought about significant advancements in biomass harvesting, processing, and conversion technologies, resulting in a plentitude of corn-ethanol studies (Hill et al. 2006; Farrell et al. 2006; Hsu et al. 2010; Kendall and Chang 2009; Kim et al. 2009; Kim and Dale 2005, 2008; Patzek 2004; Patzek and Pimentel 2005; Pimentel et al. 2007). These technological improvements and process efficiencies have had a significant impact on the environmental and energetic performance of corn ethanol. Liska et al. (2009) examined the impacts of technological maturation and system evolution on the life-cycle energy efficiency and GHG emissions of corn ethanol. The life-cycle energy efficiency and GHG emissions for various corn-ethanol systems were evaluated utilizing updated values for crop yields and agricultural management, bio-refinery operations, and coproduct utilization, which were compared against prior studies based on older process technologies. The results indicate that corn ethanol has the potential for 48–59 % GHG reduction as compared to traditional gasoline, approximately 2–3 times greater than previously reported values. Additionally, ethanol-to-petroleum output-to-input ratios were found to range from 10:1 to 13:1. It was hypothesized that a ratio of 19:1 could be achieved if farmers adopted alternative agricultural and soil management practices. The net energy ratio (NER) of corn ethanol was determined to range from 1.5 to 1.8, significantly higher than prior reported values of approximately 1.2. The authors also considered corn ethanol production utilizing a closed-loop bio-refinery coupled with an anaerobic digestion system. Under this scenario the NER was found to be 2.2 coupled with a 67 % reduction in GHG emissions. The conclusions of this study highlight the importance of technological maturation and ongoing innovation in emerging bioenergy systems, and reveal that corn ethanol's updated environmental and energetic performance is competitive with projected values for cellulosic-ethanol and other advanced biofuels.

18.3.2 Soy Biodiesel

In 1998, the US National Renewable Energy Laboratory (NREL) conducted the first complete energetic LCA of US soy biodiesel (Sheehan et al. 1998). This landmark study constructed an energy inventory for soy biodiesel based on 1990

agricultural data. This analysis was updated in both 2009 and 2011 to reflect technological improvements in soybean farming, processing, and biodiesel conversion, utilizing agricultural data from 2002 to 2006, respectively. In the most recent analysis, Pradhan et al. (2011) compared the updated energetic performance of soy biodiesel against the two prior studies, and discussed the influence of technological development and agricultural advancements on life-cycle inventories and related energy balances. The findings indicate that the Fossil Energy Ratio (FER) for soy biodiesel has substantially increased over time: from 3.2 in the 1998 NREL report, to 4.56 in the 2009 study, and finally to 5.54 in the 2011 report. The authors attribute this gradual increase in FER to increased soybean yields, improvements in soybean crushing, and advancements in biodiesel conversion operations. The results indicate reductions in energetic inputs (normalized per unit volume of biodiesel) for agricultural operations, soybean crushing, and transesterification of 52, 58, and 33 %, respectively, as compared to the 2002 report. The authors partially attribute the reductions in the energetic inputs for agricultural operations to a decrease in pesticide use resulting from the recent adoption of genetically engineered (GE) soybeans. Additionally, reductions in the energy consumption in biomass conversion and processing are largely attributed to increased efficiency and optimization of the soybean crushing and transesterification facilities and operations. These conclusions suggest that continued advancements in the agricultural sector and in the biofuels industry will allow for further optimization of the biofuel supply chain, leading to potentially lower biofuel production costs and increased biofuel FER values over time.

18.3.3 Algae-Derived Bioenergy

In recent years, microalgae have received substantial interest as a potentially sustainable source of bioenergy. While microalgae derived fuels do not exhibit many of the drawbacks of first generation or advanced biofuels, recent studies have indicated that third generation biofuels may exhibit high upstream energetic and environmental impacts. Researchers have suggested that industrial symbiosis, such as the use of industrial flue gas and wastewater effluent in algae cultivation, as well as alternative production scenarios, such as the production of biomethane via anaerobic digestion of algal biomass, may alleviate concerns over algae's environmental performance (Soratana et al. 2012; Borkowski et al. 2012; Vasudevan et al. 2012; Edward et al. 2012; Campbell et al. 2011; Collet et al. 2011; Murphy and Allen 2011; Yang et al. 2011; Singh and Olsen 2011; Brentner et al. 2011; Sander and Murthy 2010; Ferrell and Sarisky-Reed 2010; Batan et al. 2010; Sialve et al. 2009; Huesemann and Benemann 2009; Chisti 2008). In a 2011 study, Clarens et al. performed a well-to-wheel LCA of algae-derived biofuel and bioelectricity and compared the results against other biofuels (Clarens et al. 2011). In this study, multiple algal nutrient provisioning, harvesting, and conversion scenarios were modeled. Algae's performance was then evaluated against other biofuel feedstocks

in terms of equivalent VKT normalized per hectare of cultivated land. The results reveal high dependence of the energetic balance and environmental performance of algal energy systems on the mode of bioenergy production. The direct combustion of algal biomass for bioelectricity outperformed scenarios involving anaerobic digestion or algal biodiesel production, yielding a maximum EROI of approximately 4.92. Additionally, scenarios involving the direct combustion of algal biomass generally produced higher VKT than either switchgrass or canola. Given the large variability in algae's energetic and environmental performance, careful consideration of the algae-to-energy supply chain is required to ensure the long-term sustainability of emerging algal bioenergy systems.

18.3.4 Issues and Challenges to LCA of Biofuels

Biofuel LCAs often examine different system boundaries, utilize diverse functional units, and present results using a host of various sustainability and energy metrics. This creates difficulties in comparison between studies. Because LCA is becoming an increasingly prevalent tool for informing environmental and political decision-making progress toward a unified and standardized LCA framework for assessing biofuels and their associated coproducts is crucial. Factors such as coproduct allocation and substitutability, model assumptions, and data quality can highly influence LCA results for biofuels.

Another key issue with biofuel LCA is addressing uncertainty and variability (McKone et al. 2011). Common methods for assessing uncertainty and variability include both the use of Monte Carlo simulations to determine model uncertainty based on known or assumed underlying probability distributions, and the One-Factor-At-a-Time (OFAT) method in which input parameters are varied separately to determine their relative influence on the LCA results. However, these methods can be time- and resource-intensive, and may be constrained by issues of data quality and availability. Additionally, due to data averaging and the assumption of linear relationships, traditional LCA models of resource use and emissions generation cannot fully capture the effects of spatial (Yazan et al. 2011) and temporal variation, nor do they account for industrial dynamics and technological change. Similarly, the spatial (Yazan et al. 2011), temporal, and dynamic complexities of environmental response are not fully captured by traditional LCA.

Although sustainability indicators such as life-cycle GHG emissions and energy consumption are commonly studied in biofuel LCA, examination of the entire range of environmental impact categories is necessary for comprehensive understanding and to avoid unintended shifting of environmental burdens from one impact category to another. Furthermore, no generally accepted impact categories or standard methodology exist for reporting direct and indirect water consumption or land use, which are important issues meriting further study. The complexities of applying LCA methodologies to biofuels are illustrated in the following brief case study of algal biodiesel.

18.4 Case Study: LCA of Algal Biodiesel

A comparative well-to-pump LCA was conducted to investigate the life-cycle GHG emissions and FER of algal biodiesel derived from algae cultivated in open raceway ponds. Comprehensive evaluation of production pathways allows for a greater understanding of the potential tradeoffs, environmental impacts, and technical practicality of different algae production options. Additionally, prior algal biofuel LCAs have shown mixed results regarding algae's environmental and energetic performance, often as a consequence of differences in system boundaries and model assumptions. Holistic evaluation of multiple algal biofuel production pathways within the framework and assumptions of one study can address these issues.

18.4.1 Methodology

This study examines the production of algal biodiesel produced via transesterification and the effects of varying the use of the residual biomass coproduct. The system boundary begins with the cultivation of algal biomass and extends to the delivered fuel product, capital costs for cultivation, extraction, and fuel conversion were excluded. As the ultimate fuel product was to be combusted in the existing vehicle fleets, combustion processes were assumed to be equivalent to those used for the existing petroleum fuels and were excluded from this analysis (Huo et al. 2008). The functional unit of this study was chosen as one megajoule (MJ) of biodiesel at pump, allowing easy comparison with petroleum diesel. It was assumed that biomass cultivation and fuel production would occur in an integrated open raceway pond biorefinery located in Phoenix, Arizona, and would be co-located with an industrial source of waste flue gas. The biofuel production chain is shown in Fig. 18.3, which illustrates the major subsystems: cultivation, primary and secondary harvest, drying, oil extraction, fuel upgrading, and three alternate use-pathways for the residual biomass.

The extraction subsystem model was developed using data from the Ecoinvent database (Ecoinvent Centre 2007), initially on a per-pound-of-oil-extract basis, which was subsequently scaled to the per-MJ-fuel functional unit. Biodiesel requires approximately 1 lb. bio-oil per lb. of output. For mature processes such as oil extraction life-cycle data are readily available from the peer review literature as well as databases including GREET (ANL 2010), Ecoinvent (Ecoinvent Centre 2007), and USLCI database (Deru and NREL 2009).

Data for molecular composition of the de-oiled biomass were based on Lardon et al. (2009) and data on energy density and molecular composition of algal and soy meals came from the 2011 GREET model (ANL 2010). The use of waste industrial flue gas was modeled as a source of CO_2 (Benemann 1997). Algae were assumed to be grown under nitrogen deprivation conditions to maximize lipid

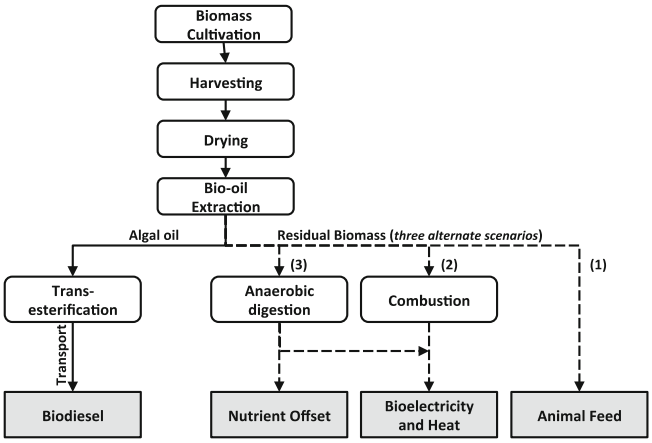

Fig. 18.3 System diagram for production of algal biodiesel and coproducts

content while reducing the requirement for synthetic fertilizers (Cho et al. 2011; Converti et al. 2009; Illman et al. 2000). The resultant algal biomass was modeled using a mean fractional composition of 38.5 % lipids, 52.9 % carbohydrates, and 6.7 % proteins. Biodiesel can be produced only from the lipid fraction of the biomass, leaving the remaining 60 % as a coproduct or as waste. This study considers several alternative uses for the residual de-oiled algal biomass (DOAB), including as an animal feed product (modeled through the displacement of soy-meal feed); as a combustion fuel for the creation for bioelectricity and heat; and as a feedstock for anaerobic digestion, which produces bioelectricity, heat, and recycled nutrients.

18.4.2 Results

For allocation purposes in the animal feed scenario, DOAB molecular composition was compared with that of soybean meal and soy beans to develop displacement ratios based on the protein content of each. Additional information and extended results can be found in a related study published in the proceedings of the 2012 IEEE-ISSST (Borkowski et al. 2012).

Primary fossil energy use across the component processes of the algal biofuel production system is indicated as inputs for three separate scenarios in Fig. 18.4. The corresponding outputs column for each scenario shows one MJ of produced biodiesel fuel product, along with any proportional coproducts. The largest contributions to primary fossil energy consumption arise from heating processes involved in biomass drying and biooil extraction, followed by electricity used in harvesting, cultivation, and oil extraction.

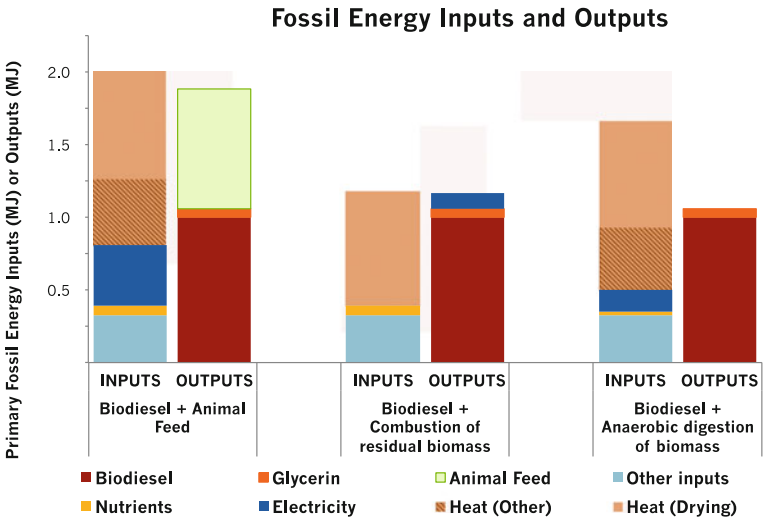

Fig. 18.4 Fossil energy inputs and outputs normalized per MJ biodiesel

Comparing the baseline animal feed scenario to that where the residual biomass is combusted for heat and power, we see a 42 % decrease in net fossil fuel consumption as well as the generation of approximately 0.1 MJ surplus bioelectricity per MJ of biofuel produced. The anaerobic digestion case shows smaller reductions in heat and electricity consumption, no surplus of bioelectricity, as well as a reduction in synthetic fertilizer consumption due to nutrient recycling. These three alternate scenarios may be more easily compared by examining the ratio of outputs to fossil energy inputs, that is, via the FER values as summarized in Fig. 18.5. The residual biomass combustion shows the highest FER value of 0.99, followed by the animal feed scenario (FER: 0.94), and by the anaerobic digestion scenario, which exhibits the lowest FER value of 0.63.

The modeled GHG emissions do not directly correspond to FER results as can be seen in Fig. 18.5. The anaerobic digestion scenario shows GHG emissions of 93 g of CO_2 equivalent per megajoule, which is on par with those of petroleum-derived diesel. The animal feed scenario shows emissions of 115 g CO_2 eq/MJ, which is higher than the petroleum diesel benchmark. The lowest GHG emissions correspond to the biomass combustion scenario with 39 g CO_2 eq/MJ, in which the greatest avoidance of electricity and natural gas consumption is achieved.

Given that under 40 % of the cultivated biomass is lipids (oils) by weight, the variation in GHG emission levels and FER values between scenarios is not surprising. The fate of the residual biomass coproduct, representing the majority 62 % of the mass, clearly has significant effect on LCA results. Comparing the anaerobic digestion and animal feed scenarios shows a clear example of the trade-offs revealed by LCA, while the anaerobic digestion scenario exhibits lower (*better*) GHG emissions than the animal feed case, it also exhibits lower FER, that is,

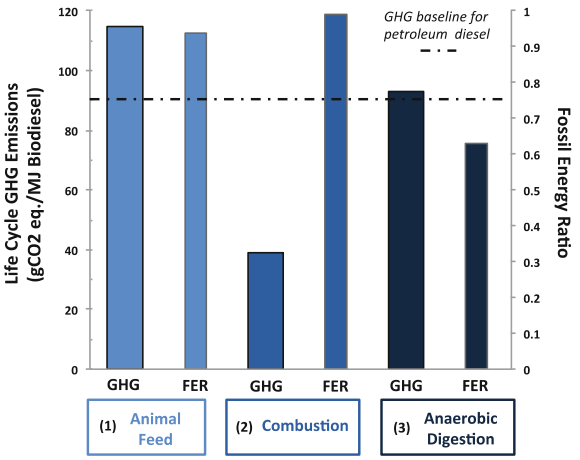

Fig. 18.5 Life-cycle GHG emissions and fossil energy ratio of biodiesel production under various residual biomass scenarios

worse energetic performance. The combustion scenario is most attractive with both lower GHG emissions and highest FER results.

Though the purpose of a given study may be to examine the environmental impacts of the biofuel products, an accurate LCA must consider the complete system including all coproducts as well as the inevitable trade-offs between different impact goals. Considering the high variability in the energetic and environmental performance of algal biodiesel in just the three coproduct scenarios studied here, LCA clearly offers the potential to evaluate the multiplicity of alternative feedstocks and production pathways discussed in Sect. 18.1 and to inform the sustainable development of nascent algal biofuel industries.

18.5 Conclusions: The Broader Role of LCA in the Development of Sustainable Biofuels

As discussed earlier, the adoption of biofuels/bioenergy has the potential to decrease dependence on foreign oil, reduce consumption of nonrenewable resources, mitigate GHG emissions and global climate change, and boost the domestic economy. Holistic evaluation of various biofuel supply chains from a life cycle perspective can help to indicate the degree to which these objectives are met. Additionally, given the multitude of potential biofeedstocks and biofuel conversion pathways, LCA represents a useful tool both for comparing environmental sustainability among pathways and for guiding the sustainable development of biofuel industries. Policy makers are adopting life cycle thinking and LCA metrics into renewable fuel regulation and legislation. Scientists are using systems analysis, such as LCA, to identify process inefficiencies and hotspots for targeted improvement along myriad possible biofuel supply chains.

While LCA is increasingly valued by researchers, practitioners, and decision makers, it currently faces multiple methodological challenges. These include issues of data quality, uncertainty, system boundaries, spatial and temporal data resolution, scale, and system dynamics as well as trade-offs inherent to optimizing competing goals. To address these issues, researchers are developing increasingly sophisticated models and metrics. New tools for analyzing geo-spatial and regional/localized impacts are helping to quantify impacts on local watersheds and to disentangle the complexities of indirect land-use change. Issues of data quality and availability are being met with the development of commercial and private databases. Some of the shortcomings of traditional LCA are being met with alternative forms of LCA such as Economic Input Output Life Cycle Assessment (EIO-LCA), Ecologically based Life Cycle Assessment (Eco-LCA) (Baral et al. 2012), and hybrid approaches (You et al. 2012; Acquaye et al. 2011).

By insisting on an LCA "seal of approval", or seeking a single reductive metric to answer the complex questions of sustainability, decision makers fail to utilize the true potential of LCA (McKone et al. 2011). LCA can best serve the nascent biofuels industry when it remains an iterative process, much like the scientific method itself. Iterative LCA has the potential to coevolve with technology while providing the basis for adaptive planning through an ongoing symbiotic collaboration among the LCA practitioner, the basic scientist, the R&D community, and decision makers in industry, finance, and government. Vital roles exist for

- Scientists and engineers in research and technology development—targeting hotspots for process improvement, emission reduction, and efficiency improvement
- LCA practitioners—identifying areas of uncertainty and variability, posing new questions, and driving the coevolution of LCA models with new technology
- Decision makers—organizing information, resolving conflicting goals, and working to understand aggregated impacts while gaining a deeper understanding of the underlying issues, risks, and potential benefits of the studied system and broader ramifications across its life cycle.

Building on the common basis of life cycle thinking, LCA models and methodologies can guide the development of emergent biofuels industries onto a path that is simultaneously economically viable, energetically efficient, and environmentally responsible.

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Chapter 19
The Principle and Applications of Bioelectrochemical Systems

Zhiyong (Jason) Ren

Abstract Bioelectrochemical system (BES) is a unique technology that uses microorganisms to covert the chemical energy stored in biodegradable materials to direct electric current. Compared to traditional chemical and environmental technologies, BES offers a flexible platform for both oxidation and reduction reaction oriented processes, because any biodegradable substrate, especially waste materials, can be oxidized in the anode chamber, and the generated current can be directly harvested as electricity or used to produce value-added chemicals, desalinate salt water, and remediate contaminants. This chapter reviews the microbiological and technological principles of the BES technology and discusses the different functions and recent developments of systems.

19.1 Introduction

19.1.1 Biomass Energy Plays a Vital Role to the Future of Alternative Fuels

Worldwide concerns on energy depletion, environmental pollution, and climate change are driving the search and development of alternative energy sources for fossil fuels. The replacement of fossil fuels requires the use of a myriad of energy sources and carriers that are renewable and clean and suited to meet different end uses. Biomass-based bioenergy is particularly attractive in this context because of its relatively low cost, plentiful supply, and environmentally benign production.

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Biomass energy is a captured form of solar energy that is stored in biomass, such as those from agricultural and forestry resources, municipal solid waste, and the dissolved or suspended organic matter in wastewater. The great abundance and availability make biomass the primary resource for renewable energy production. The US Departments of Agriculture and Energy estimated the annual availability of 1.3 billion dry tons of biomass feedstock in the US, which could displace 30 % or more of the country's present petroleum consumption (Perlack et al. 2005). The US Energy Information Administration estimated that the biomass share of the US renewable energy consumption was 53 % in 2010, which was largely contributed by the increased amount of biofuel production (Fig. 19.1). The Energy Independence and Security Act requires at least 10 % annual increase in alternative fuel consumption till 2015 (US Congress 2007). Biomass also represents 66 % of the renewable energy capability in the European Union in 2007, which is also expected to expand in the next decade (Europe Energy Portal 2012).

Depending on the end-use application, different biomass resources have to be converted to a variety of energy carriers such as ethanol (Mielenz 2001), butanol (Green 2011), biodiesel (Powlson et al. 2005), hydrogen (Ni et al. 2006; Ren et al. 2007a), and biogas (O'Sullivan et al. 2005), etc. Only recently, this versatility has been extended to direct energy or chemical production through microbial electrosynthesis in bioelectrochemical systems (BESs) (Ren et al. 2007b; Rismani-Yazdi et al. 2007).

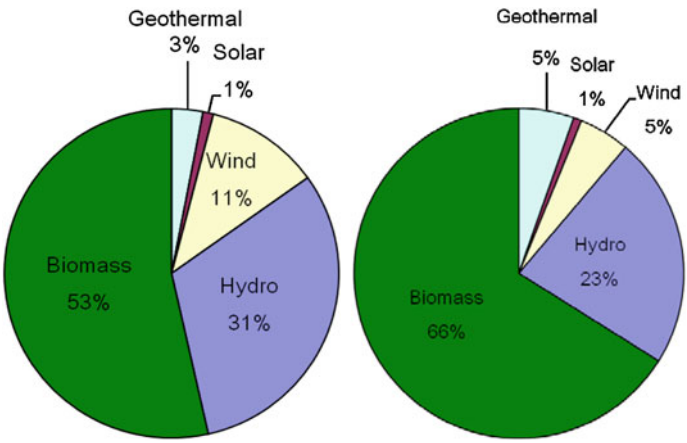

Fig. 19.1 Renewable energy source in (**a**) the United States (2010), and (**b**) the European Union (2007)

19.1.2 BES is a Unique Technology for Biofuel and Biochemical Industry

BES is a unique technology capable of converting the chemical energy stored in biodegradable materials to direct electric current using living microorganisms. This phenomenon was first reported a century ago in 1911, when Potter found that *Saccharomyces cerevisiae* was able to generate a voltage of 0.3–0.5 V from glucose using a platinum electrode (Potter 1911). The research and development of this concept had been stagnant till the turn of the century, as only a handful of articles were published before 2001, but since 2002, the research productivity has experienced an exponential growth, resulting in more than 2,000 articles in the past decade (Fig. 19.2).

The recent blossom of BES research and development was largely due to its great potential and versatility. While many existing technologies have only one or two functions, the BES platform has more than a dozen functions that have been discovered. BESs use electrochemically active bacteria (EAB) to catalyze the oxidization of organic and inorganic electron donors in the anode chamber and deliver electrons to the anode. The electrons can be captured directly for electricity generation (Bond et al. 2002; Liu et al. 2005a) or supplemented by external power input for producing value-added chemicals, such as H_2, peroxide, and organics (Logan et al. 2008; Luo et al. 2011; Nevin et al. 2010; Rozendal et al. 2009). The electrons can also be used in the cathode chamber to remediate contaminants such as uranium, chlorinated solvents, and perchlorate (Aulenta et al. 2008; Butler et al. 2010; Gregory and Lovley 2005). The potential across the electrodes can also drive water desalination (Cao et al. 2009; Jacobson et al. 2011; Kim and Logan 2011b; Luo et al. 2011). Figure 19.3 shows the schematic of a BES reactor, with anode conducting substrate oxidation reaction and cathode performing reduction reaction. Based on the different functions, the BES platform has been specified into many different names that researchers name them MXCs, where X stands for the

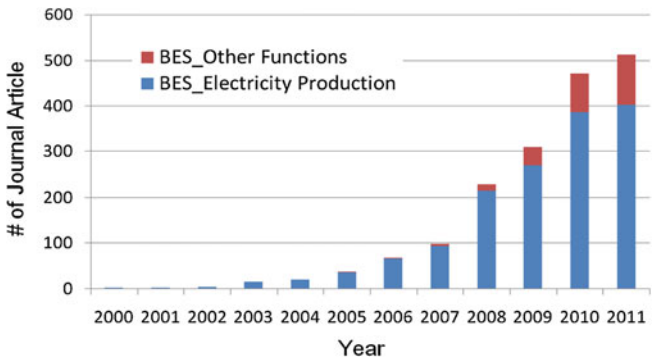

Fig. 19.2 The number of published journal articles on BES or microbial fuel cells (*Source* Scopus/Google Scholar, March 2012)

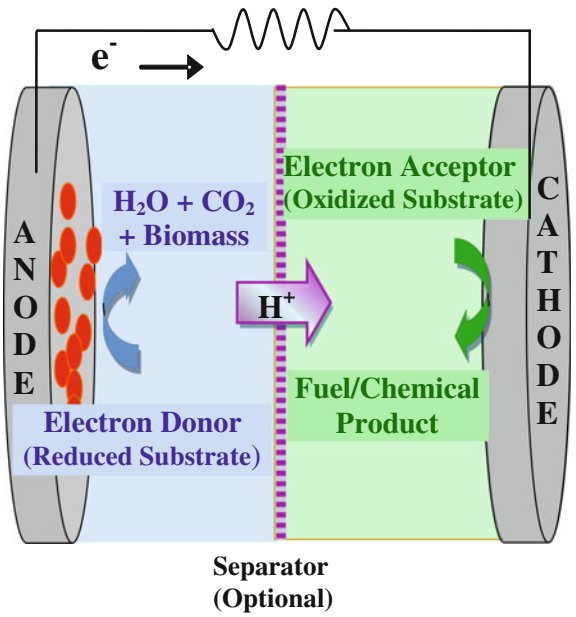

Fig. 19.3 Schematic of a BES reactor demonstrating general oxidation reaction in the anode chamber and reduction reaction in the cathode chamber

different applications (Harnisch and Schroder 2010; Torres et al. 2010). Table 19.1 summarizes the main Xs to demonstrate the versatility of this platform technology.

19.2 Extracellular Electron Transfer by EAB

A BES consists of at least an anode and/or a cathode, where microorganisms function as biocatalysts. The uniqueness of BES is not only related to the special extracellular electron transfer (EET) to solid electron acceptors (anodes) or from solid electron donors (cathodes), but also comes from how different functional groups interact with each other and form a robust microbial ecological structure. On the anode side, EABs oxidize biodegradable substrates and reduce the anode as insoluble electron acceptor. On the cathode side, EABs may accept electrons from the solid cathode and reduce external electron acceptors, such as O_2, NO_3^-, and CO_2, etc. There have been several terms for microorganisms capable of interacting with electrodes, especially for anode oxidizers, such as exoelectrogens (Logan 2009), electricigens (Lovley 2006), anodophiles (Park and Zeikus 2003), anode-respiring bacteria (Torres et al. 2009), and EAB (Chang et al. 2006). Here, we use EAB to represent both anode-reducing bacteria and cathode-oxidizing bacteria.

Table 19.1 Summary of main types of BES processes

Main type of BES	Electron donor for anode oxidization	Electron acceptor for cathode reduction	Products	References demonstrated term
Microbial fuel cell (MFC)	Any biodegradable material	O_2, $K_3Fe(CN)_6$, other oxidants	Electricity	Kim et al. (1999), Tanaka et al. (1983)
Microbial electrolysis cell (MEC)	Any biodegradable material	H^+,CO_2, organics	H_2, H_2O_2, CH_4, NaOH, Struvite	Cheng et al. (2009), Cusick and Logan (2012), Liu et al. (2005b), Rabaey et al. (2010), Rozendal et al. (2009)
Microbial chemical cell (MCC)	Any biodegradable material	CO_2, organics	Organics	Nevin et al. (2010), Steinbusch et al. (2010)
Microbial remediation cell (MRC)	Any biodegradable material	Any biodegradable oxidant	Reduced/non-toxic chemicals	Butler et al. (2010), Morris et al. (2009)
Microbial desalination cell (MDC)	Any biodegradable material	O_2, $K_3Fe(CN)_6$, other oxidants	Desalinated water	Cao et al. (2009), Jacobson et al. (2011), Kim and Logan (2011a), Luo et al. (2011)

19.2.1 Drawing Electrons from EAB to the BES Anode

19.2.1.1 EET by Pure Culture EABs

The microbial EET to external insoluble electron acceptors, such as metal oxides have been studied for many years, but how EABs interact with the anode electrode is still in the early stage of understanding. It is hypothesized that bacteria can either transfer electrons through immobilized structures or using mobile electron shuttles. Some exoelectrogens including *Geobacter* and *Shewanella* species establish a direct contact strategy for efficient electron transfer. Take *Geobacter sulfurreducens* as an example, studies showed that this model EAB strain requires a series of periplasmic and outer membrane *c-type* cytochromes to directly transfer electrons from inner membrane to the outer cell surface (Lovley 2006; Marsili et al. 2010). But these proteins are not always sufficient, because it also produced pilus-like appendages, called nanowires for direct attachment (Reguera et al. 2005). Microarray analysis of gene expression and gene deletion studies suggests that the electrically conductive pili and the *c-type* cytochrome OmcZ are essential for optimal current production, with the pili serving for long-range cell-to-cell electron conduction and OmcZ functioning as an 'electrochemical gate' to promote electron transfer onto the electrode (Fig. 19.4, left) (Logan 2009; Lovley 2011; Summers et al. 2010).

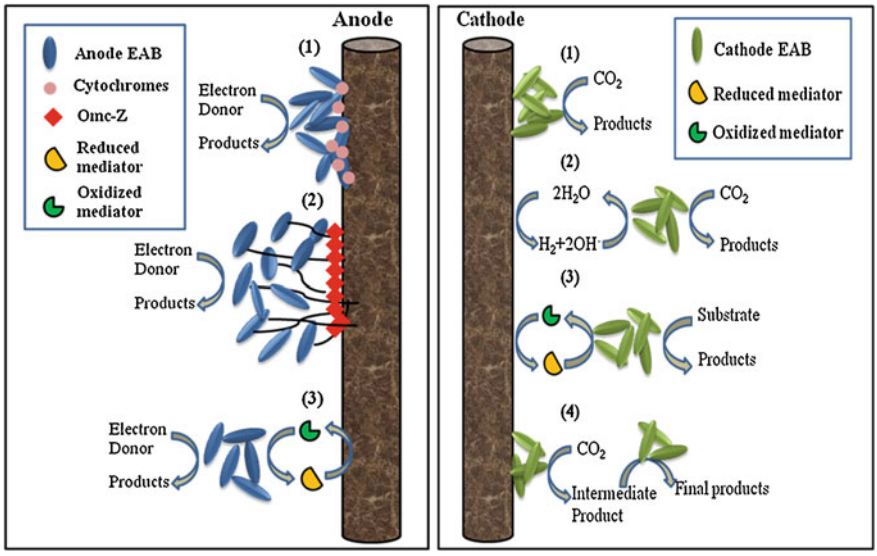

Fig. 19.4 *Left* Anode EET mechanisms (*1*) direct contact through cytochromes; (*2*) direct transfer through nanowires; (*3*) indirect transfer through mediators; *Right* cathode EET mechanisms (*1*) direct contact with the electrode; (*2*) through H_2 conversion; (*3*) through mediators; (*4*) through other intermediate products

Compared to *Geobacter* species, which are known to conduct direct EET to the anode, *Shewanella* species were reported to make both direct electrode contact through conductive filaments and indirect electron transfer via mediators, such as riboflavin or flavin ademine mononucleotide (FMN) (Gorby et al. 2006; Marsili et al. 2008; von Canstein et al. 2008). *Shewanella oneidensis,* another model EAB, has been reported to produce conductive nanowires, but those pili are longer and arrayed in bundles (Bretschger et al. 2007; Gorby et al. 2006). The electron transfer by *S. oneidensis* from the periplasm to the electrode involves a series of out-surface *c-type* cytochromes, in which *MtrC* plays a key role, because it is located on the outside of the membrane and capable of donating electrons in a broad potential range. Studies also suggest that the cytochromes may also be responsible for the reduction of flavins, which are released from the cell and serve as an electron shuttle for Fe(III) and electrode reduction (Biffinger et al. 2009; Marsili et al. 2008).

Compared to direct EET conducted by a few species of bacteria, many other bacteria can produce and use soluble redox mediators or electron shuttles, which transport the electrons from the cell to the electrode. For example, *Pseudomonas* species can produce phenazines as extracellular electron shuttles (Rabaey et al. 2005), and many other bacteria can use externally provided mediators, such as neutral red, anthraquinone-2,6-disulphonate (AQDS), and some humics (Milliken and May 2007; Park and Zeikus 2000). However, since the production and utilization of shuttles require a lot of energy, it has been considered less favorable than direct electron transfer.

19.2.1.2 Microbial Consortiums for Converting Complex Substrates

With tremendous electron donor versatility, BESs have been used to convert many different substrates into energy and chemicals. Besides simple sugars and derivatives, many complex waste materials have also been utilized, such as different wastewaters, starch, protein, even cellulose, and landfill leachates (Pant et al. 2010). Results from different studies also reveal a very broad diversity of the microbial community. Take MFC for electricity production as an example, while reactors using marine sediments showed the enrichment of *Deltaproteobacteria* of the family *Geobacteraceae* (Bond et al. 2002), another MFC inoculated with marine sediments showed the community was dominated by *Gammaproteobacteria* with the main species of *S. affinis* (Logan et al. 2005). Inoculation of an MFC with river water led to the enrichment of *Betaproteobacteria*, while inoculation with wastewater resulted in the dominance of *Alphaproteobacteria* (Phung et al. 2004). *Geobacter, Shewanella, Pseudomonas*, and *Rhodoferax* are the main isolates whose electrochemical activities have been widely studied. Although these bacteria have been found to develop various strategies to conduct EET, all of them can only use easily degraded organic substrates, such as glucose, acetate, or lactate as electron donors. Experiments indicated that electrons derived from the oxidation of organic matter are almost fully recovered as electricity in these pure culture

MFCs, showing 80–96 % coulombic efficiency (CE) (Bond and Lovley 2003; Chaudhuri and Lovley 2003). However, they hardly can adapt to the natural environment efficiently, because the substrates they need are not prevalent naturally and have to be produced by other metabolisms such as fermentation. On the other hand, even though some fermentative bacteria such as *Clostridium butyricum* have been isolated from MFCs, they cannot be a strategy for efficient power production either, because most of the electrons remain in fermentation products that do not readily react with electrodes without anaerobic respiration (Park et al. 2002). Although it is possible that there are bacteria that can completely oxidize complex polysaccharides such as cellulose or starch to carbon dioxide and release electrons to electrodes, they cannot compete with the consortium of fermenters and acid oxidizing, electricity producing bacteria owing to thermodynamic considerations (Rezaei et al. 2009). The thermodynamic rationale is not the amount of energy available per mole of electron donor metabolized, but rather is the amount of energy released per electron transferred (Mcinerney and Beaty 1988). This is consistent with the finding that the majority of glucose was fermented in Fe(III)-reducing sediments instead of being directly oxidized to CO_2 with Fe(III) oxide as the electron acceptor (Lovley and Phillips 1989).

Therefore, the best strategy to extract electrons from complex organic compounds, including those found in most wastes and biomass, requires the cooperation of a consortium of polymer degrading fermentative bacteria and electrochemically active microorganisms. The fermenters (such as *Clostridium* spp.) break down the complex organic matter such as cellulose, starch, and proteins into fermentable sugars and amino acids and further into fatty acids and solvents, then the EABs (such as *Geobacter* spp.) oxidize the fermentation products to carbon dioxide, with the anode serving as the electron acceptor (Freguia et al. 2008; Parameswaran et al. 2009; Ren et al. 2007b, 2008). Studies also found that positive syntrophic interactions between homo acetogens and EABs led to higher CE when ethanol was used as the electron donor, and when competitive electron consumption process, such as methanogenesis, was suppressed (Parameswaran et al. 2009, 2010).

The biofilm on a BES anode is different from conventional biofilm systems, because unlike many systems where the biofilm tends to grow thicker in order to reach to electron donor (e.g. organics) and acceptor (i.e. O_2) in the bulk fluid, the BES anode biofilm has to keep a balance in thickness, because the bacteria need exposure to both bulk solution for electron donors and the anode for efficient electron release. The viability and stability of anode biofilm catalysts, therefore, directly determine system performance. Recent findings show that there is a time-scale discrepancy between MFC electrochemical response and anode biocatalyst variation. A time-course characterization shows that power output from MFCs could stabilize within several days, but the anode biofilms continued evolving during the 7-month long operation. Both biofilm architecture and community composition shifted dramatically, from rod-shaped, dispersed *Geobacter* species to the emergence of filamentous like *Pseudomonas* species (Ren et al. 2011a). Other studies also found similar differences by operating MFCs under different anode

potentials or external resistors, because the potential regulates the availability of the MFC anode as the electron acceptor for microbial electron transfers, and therefore affects the biocatalyst activity and electrochemical performance (Ren et al. 2011b; Torres et al. 2009).

19.2.2 Feeding Electrons from BES Cathode into Microorganisms

Whereas findings on EET from bacteria to the anode electrode become abundant, the information about the reverse process—transferring electrons from the cathode into microbes—is very limited. The ability of microorganisms to accept electrons from electrodes as an electron donor offers great potentials in microbial electrosynthesis. While renewable and cost-effective electricity can be derived from solar, wind, or waste materials, the electrons can be microbially converted into value-added energy and chemical products. However, little is known about the mechanistic information on the electron transfer and the cathodic communities involved in the process (Lovley and Nevin 2011; Rabaey and Rozendal 2010; Rosenbaum et al. 2011).

There have been several hypothesized pathways that assess how bacterial cells accept electrons from the electrodes. Figure 19.4 (right) shows the four mechanisms discussed in a recent review article (Rabaey and Rozendal 2010). The most attractive way of achieving EET from cathodes is through direct contact, which means that single or multiple layers of biofilm attach on the cathode surface and conduct direct catalysis. This direct approach decreases overpotentials and avoided expensive electron mediators, and therefore more efficient in electron transfer. Several studies demonstrated that the cathodic current supply for respiration can be used in reducing many contaminants, such as nitrate (Clauwaert et al. 2007), perchlorate (Butler et al. 2010), chlorinated solvents (Aulenta et al. 2008), and uranium (Gregory and Lovley 2005). Recently, research was focusing on using the cathode for chemical production, and several studies have demonstrated the production of acetate, methane, ethanol, and other organic chemicals. The details can be found in Sect. 19.4.3. The main challenge may come from the slow mass transfer within biofilm, because it may limit the substrate and product movement in and out of the biofilm, and the potential accumulation of acid or base products as well as alcohols may decrease production rate and inhibit biofilm growth (Rabaey et al. 2011).

The indirect approach of cathodic EET is through soluble or miscible electron mediators, which often involves in both planktonic cells and attached biofilms. Similar as electron shuttles used for anode EET, mediators such as neutral red, AQDS, and methyl viologen (MV) can provide an conduit for electrons to enter into microbial cells (Rabaey and Rozendal 2010). Such mediated electron transfer can be facilitated by increasing the shuttle concentration and can be regulated

based on the shuttle middle point potential. The shuttles can also be reused many times in closed or cycled reactors systems. However, the problems of using electron shuttles come from their potential toxicity to microorganisms and instability under different environmental conditions, such as pH, temperature, and salinity. It is also difficult to use the mediators in continuous flow systems due to the loss of such molecules.

19.3 Parameters in Evaluating BES Performance

19.3.1 Electrode Potential and Power Output

All BES processes share a same principle—EAB in the anode chamber oxidize biodegradable substrates and generate electron flow (i.e. current) to reduce the electron acceptors in the cathode chamber. Therefore, the most direct way to evaluate the performance of a BES is the measurement of circuit voltage (V). The current (I) passing through an external resistance (R_{ext}) can be measured using a current meter, or simply calculated based on the Ohm's law:

$$I = \frac{V}{R_{ext}} \tag{19.1}$$

The V represents the direct output of a reactor, and it can also be describes as:

$$V = OCV - IR_{int} \tag{19.2}$$

where OCV is the open circuit voltage of the reactor, and R_{int} is the overall internal resistance. Generally, R_{int} represents the total current-related resistance loss, which is the sum of system ohmic resistance caused by the resistance of the electrodes and electrolyte, the activation loss caused by biochemical reactions on the electrode surface, and the charge transfer resistance caused by the limitation of mass transfer and concentration polarization (Logan et al. 2006). R_{int} is considered to connect with R_{ext} in series. The power density (P) from an MFC is inversely proportional to the total system resistance squared according to:

$$P = \frac{V^2 R_{ext}}{(R_{int} + R_{ext})^2} \tag{19.3}$$

The traditional approach of reporting power density from a BES or MFC reactor was to operate the reactor with a static R_{ext} or applied potential then transiently obtaining polarization data by changing the R_{ext} at a 5–30 min interval or conduct a voltammetry sweep (Logan et al. 2006; Lyon et al. 2010; Ren et al. 2011b). Figure 19.5 shows typical polarization and power density curves obtained from a lab scale MFC reactor, and it shows that the MFC voltage is practically inversely proportional to the output current, and there exists a pair of voltage and

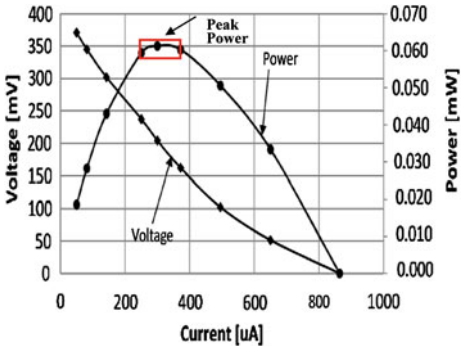

Fig. 19.5 Polarization curve and power density curve obtained from a lab scale microbial fuel cell reactor

current that delivers the maximum power when R_{ext} is equal to R_{int} (Pinto et al. 2011; Ren et al. 2011b).

Compared to other alternative energy systems, BES/MFC is a small power system due to its thermodynamic limitation. The MFC anode potential is generally around −0.3 V (versus Normal Hydrogen Electrode, NHE), which is set by the respiratory enzymes of bacteria that metabolize electron donors. Take acetate (5 mM, pH = 7) as an example:

$$\begin{aligned} &\text{Anode: } 2\ HCO_3^- + 9\ H^+ + 8\ e^- \rightarrow CH_3COO^- + 4\ H_2O \\ &OCP_{anode} = -0.296\,V \end{aligned} \tag{19.4}$$

The cathode potential is around +0.8 V when oxygen is used as the terminal electron acceptor (Logan et al. 2006; Meehan et al. 2011).

$$\text{Cathode: } O_2 + 4\ H^+ + 4\ e^- \rightarrow 2\ H_2O \quad OCP_{cathode} = +0.80\,V \tag{19.5}$$

Such characteristics determine that the voltage of an air–cathode MFC is generally less than 0.8 V and the current output is usually in the range of a few mA due to overpotential and other losses. Other chemical oxidants, such as ferricyanide or permanganate could provide a higher cathode potential, but air cathodes have been widely adopted in MFCs because it is free and sustainable (Logan 2008; Ren et al. 2007b). Higher power generation using single or multiple MFCs can be achieved by applying electronic harvesting systems and developing larger stack systems. (Aelterman et al. 2006; Dewan et al. 2008; Park and Ren 2012; Wang et al. 2012).

Power density has been a key parameter to demonstrate the performance of a reactor. Traditionally, the power output is normalized to projected electrode area, defined as surface power density, so it is possible to compare the performance of different systems. However, such parameter sometimes may not represent a fair comparison when different systems use different electrode configurations and materials. For example, when replacing carbon cloth ($\sim$100 m^2/m^3) anode with high surface area brush anode ($\sim$9,600 m^2/m^3), the normalized cathode power density increased from 0.6 to 2.4 W/m^2, but the normalized anode power density did not change that much (Logan et al. 2007). Therefore, more and more studies

begin to use volumetric power density, which is the power output normalized by the reactor volume, because it represents the reactor overall power output and aligns with reactor size and water treatment capability. In general, the higher the electrode density, the better the reported volumetric power density, but very high electrode surface areas may pose another challenge of clogging when actual wastewater is used as the substrate.

19.3.2 Current Density and Chemical Production Rate

For BESs that target for chemical production and desalination, current density is considered more important than power density. The current density reflects the substrate oxidation rate and directly correlates with the production of chemicals. The more and faster the electrons flow from the substrate to the electrode, the more and faster chemicals can be synthesized on the cathode. For example, hydrogen gas production in microbial electrolysis cells (MECs) can be expressed as a direct function of the volumetric current density. The maximum volumetric H_2 production rate Q_{max} (m^3H_2/m^3d), is directly proportional to the current density (Logan et al. 2008):

$$Q_{\max} = \frac{I_v(\mathrm{A/m^3})r_{\mathrm{cat}}[(1C/s)A](0.5\mathrm{molH_2/mol})(86400\mathrm{s/d})}{F(9.65\times10^4\mathrm{C/mol})c_g(\mathrm{molH_2/L})(10^3\mathrm{L/m^3})} = \frac{43.2\,I_v\,r_{\mathrm{cat}}}{Fc_g(T)} \quad (19.6)$$

where I_v (A/m^3) is current density and averaged over a specified time period, c_g (mol/L) is the molar density of gas at a standard temperature (298.15 K) and standard pressure (1 bar), and 43.2 results from the given units. Thus, when hydrogen is captured efficiently, increasing the H_2 production rate depends solely on increasing current. MEC H_2 production rates have reached 3.12 m^3 H_2/m^3 d with an applied voltage of 0.8 V, and a maximum of $I_v = 186$ A/m^3 at $V = 0.6$ V was reported (Logan et al. 2008).

Microbial desalination cell (MDC) is a newly developed BES process, which inserts a third desalination chamber in the middle of the anode and cathode chamber and uses the electric potential to drive desalination (Cao et al. 2009). Detailed description and applications of MDCs are provided in Sect. 19.4.4. Studies show MDCs removed less total dissolved solid (TDS) at high power output (near peak point in the power density curve, 42.3 % TDS removal, 70 mA, 15.6 W/m^3) than at high current generation mode (near far right point in the power density curve, 60.1 % TDS removal, 143 mA, 1.1 W/m^3), but the high power operation produced more electricity with high energy efficiency (EE) (Jacobson et al. 2011). This suggests that the MDC operation mode maybe adjusted based on the treatment goals, with high power mode for organic removal and power generation under low salinity condition, which high current mode for more efficient desalination.

19.3.3 CE and EE

19.3.3.1 CE and Coulombic Recovery

CE is a common measure that represents the fraction of electrons removed from the electron donor that are recovered as current through the external circuit. Coulombic recovery (CR) represents the electrons recovered based on the total electron donor input. CE can be calculated as a ratio of total recovered coulombs (C_p) obtained by integrating the current over time to the theoretical coulombs (C_t) that can be produced from the removed substrate. For MFCs operated in batch mode, CE can be calculated as (Logan et al. 2006):

$$\mathrm{CE} = \frac{\int_0^t \mathrm{I}\mathrm{d}t}{\mathrm{F}bv\Delta C} \tag{19.7}$$

where F is Faraday's constant (96,485 C/mol), b is the number of electrons produced per mole of substrate ($b = 24$ for glucose, $b = 8$ for acetate), v is the volume of anolyte (L), and ΔC is the concentration difference of the substrate (mol/L) from time 0 to t.

For continuous flow reactor at steady-state condition, the current output is assumed constant during certain of time period, so the CE can be simplified into:

$$\mathrm{CE} = \frac{I}{\mathrm{F}bQ\Delta C} \tag{19.8}$$

where Q is the anolyte flow rate (L/s) and ΔC is the concentration difference between the influent and effluent (mol/L).

19.3.3.2 EE

EE is a measure that represents the energy recovered from BESs as compared to the theoretical heat energy embedded in the removed substrate. The form of recovered energy can be electricity, H_2, and other chemicals, depending on the function of the BES reactor. For microbial fuel cells, the EE is the ratio between the power produced and the combustion energy of the consumed substrates as:

$$\mathrm{EE} = \frac{\int_0^t VI\mathrm{d}t}{\Delta H\Delta CQ} \tag{19.9}$$

where ΔH is the combustion energy of the substrate (J/mol).

For chemical production such as H_2 generation in MECs, an external voltage is generally applied to overcome the thermodynamic limitations. In this case, the EE can be calculated based on the energy content of the product produced (H_2), compared to the energy input in the consumed substrate as well as the energy input from the external power source.

19.4 Applications of BES Technology

Compared to traditional chemical and environmental processes, which generally has one function, BESs offer a great platform technology, because it provides both oxidation and reduction approaches for chemical and energy production, waste treatment, contaminant remediation, and water purification. On the anode side, BESs can theoretically oxidize any biodegradable substrate and extract electrons to the anode. In addition to simple sugars and derivatives, many complex waste materials have been utilized such as different wastewater, biomass, landfill leachate, petroleum hydrocarbon, and marine sediments (Pant et al. 2010). On the cathode side, any reduction reaction-based chemical production can be achieved, including both organic synthesis and inorganic production (Pant et al. 2012), and any electron acceptor type of contaminants can potentially be reduced using the electrons supplied from the cathode (Lovley 2011; Rosenbaum et al. 2011). Here, we discuss a few application areas that BES holds great potentials for commercialization.

19.4.1 Electricity Production and Waste Treatment

The main advantage of using BESs in wastewater treatment is its potential to convert traditional energy intensive treatment processes into energy gaining processes while still achieving treatment objectives. Current wastewater treatment processes consume high amount of energy for aeration and sludge treatment. Wastewater treatment accounts for about 3 % of the US electrical energy load, which is approximately 110 terawatt hours per year, or equivalent to 9.6 million households' annual electricity use. Meanwhile, the energy content embedded in wastewater is estimated about 2–4 times the energy used for its treatment, so it is possible to make wastewater treatment self sufficient if full-scale BES can be integrated in wastewater treatment plants (McCarty et al. 2011).

Researchers have demonstrated that BESs can produce energy from almost all kinds of waste streams, including municipal, brewery, food processing, paper recycling, agricultural, and refinery wastewater (Pant et al. 2010). The power output is dependent on the biodegradability of the substrate, conversion efficiency, and loading rate. Higher power was usually achieved from simple substrates, while much lower electricity was found from recalcitrant wastes. For example, by using a 4 cm^3, air–cathode reactor and a sludge inoculum, the maximum power density achieved from acetate, brewery wastewater, and swine wastewater was 766, 205, and 225 W/m^2 (projected anode area), respectively (Cheng et al. 2006; Feng et al. 2008; Min et al. 2005). The advantages of using BESs to upgrade or replace current biological treatment units, such as aeration basin or trickling filter, may come from the following aspects:

1. *The elimination of aeration.* For traditional activated sludge system, aeration can amount to 45–75 % of plant energy costs, so the conservation of aeration tank to BES units can significantly save energy cost. Lab-scale comparisons on energy consumption between an aeration reactor and a BES using raw wastewater samples showed that both reactors were able to reduce COD from ~1,100 to ~30 mg/L in batch or continuous operations. However, the aeration reactor used an average 2.1 kWh/m^3 (wastewater) in electricity, while the MFC did not use any aeration energy and produced a maximum 168 W/m^3 (wastewater) in power density (Huggins et al. 2012). Other studies showed that a maximum 65 W/m^3 (reactor) of power density could be achieved with an organic loading of 0.6–1.5 kg COD/m^3/day (Pant et al. 2010).
2. *The production of useful products*. As demonstrated above, BESs can produce direct electricity during treatment process. With simple modifications, other value-added products, such as H_2, CH_4, caustic soda, peroxide, or organic chemicals can be produced. Figure 19.6 shows two studies on either directly adding MFC units to aeration tanks for electricity production or building new MFC systems to treat brewery wastewater and produce caustic soda for onsite disinfection.
3. *Reduced solids production.* The treatment and disposal of sludge amount up to 60 % of the total operation cost of a treatment plant. Because MFC is a biofilm based system, the cell yield of electrochemical active bacteria (0.07–0.16 gVSS/gCOD) was much less than the activated sludge (0.35–0.45 gVSS/gCOD), so the MFC process reduces sludge production by 50–70 % (Logan 2008). Many studies discussed that by converting aeration basin into MFC, second clarifiers may be reduced in size, converted to solid contact basin, or even eliminated.
4. *Nutrient removal.* Nutrient removal has become more critical to the treatment objectives of most plants. Compared to organic removal research, little work has been done in investigating nutrient removal from MFCs. Lab-scale studies have shown that nitrogen can be removed by up to 83 % due to both microbial nitrogen assimilation and coupled nitrification (anode) and denitrification

Fig. 19.6 **a–b** Converting aeration tank to MFC by submerging electrodes inside (advanced environmental technologies, USA). **c** Tubular MFC system for brewery wastewater treatment and electricity/caustic production (U of Queensland, AU)

(cathode) in single-chamber MFCs. A recently study by Cusick and Logan demonstrated that phosphorus can be recovered as struvite ($MgNH_4PO_4$–$6H_2O$) together with H_2 in MECs (Cusick and Logan 2012).

19.4.2 Value-Added Chemical Production and Carbon Sequestration

19.4.2.1 Chemical Production by MECs

For traditional MFC systems, bacteria on the anode oxidize electron donors and transfers electrons to the anode to produce current. If O_2 is present in the catholyte, the electrons will combine with O_2 and produce H_2O. However, current generation will not be spontaneous if no oxygen is available as the electron acceptor. For MECs, a small voltage can be applied externally to allow hydrogen gas generation at the cathode through the reduction of protons (Fig. 19.7) (Liu et al. 2005b; Logan et al. 2008). Many studies confirmed that the external voltage can be as low as 0.2 V, though in practice 0.6–0.8 V have been used. Despite the additional power input, MECs show great advantage for H_2 production, because the voltage used in MECs is much less than the 1.8–2.0 V used in industrial water electrolysis, and the substrates for MECs can be from renewable and waste materials. Compared to fermentative H_2 production, which has a yield limitation of 4 mol H_2/mol glucose, the hydrogen yield from MECs can reach up to 11 mol H_2/

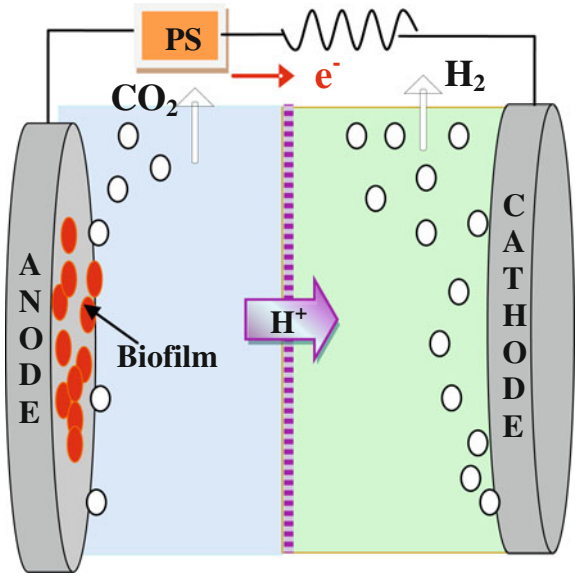

Fig. 19.7 Schematic of a MEC for H_2 production. An external power source (PS) is shown to provide additional voltage for H_2 evolution in the cathode chamber

mol glucose with a production rate of more than 1 $m^3/d/m^3$ reactor (Cheng and Logan 2007). Recently, a new process called microbial reverse-electrodialysis electrolysis cell (MREC) eliminated the requirement of external power for H_2 evolution by combining two driving forces in one system—a thermodynamically favorable oxidation of organic matter by EABs on the anode and the energy derived from the salinity gradient between seawater and river water (Kim and Logan 2011a).

By using similar strategies in MECs, many inorganic chemicals have been produced in the cathode chamber. Rozendal et al. investigated hydrogen peroxide generation, and they found at an applied voltage of 0.5 V, a lab-scale MEC produced 1.9 ± 0.2 kg H_2O_2/m^3/day at a concentration of 0.13 ± 0.01 wt % and an overall efficiency of 83.1 ± 4.8 % (Rozendal et al. 2009). The same group later used a similar approach to produce alkaline solutions as disinfectants for onsite use. They found that by using acetate as the electron donor in the anode, the MEC generated up to 1.05 A in current at 1.77 V applied voltage, which allowed for the production of caustic to 3.4 wt % (Rabaey et al. 2010).

19.4.2.2 Chemical Production and Carbon Sequestration Through Microbial Electrosynthesis

Microbial electrosynthesis is an emerging direction in BES research and development. The success of this process will have the potential to address the storage and distribution problems associated with many renewable energy forms, such as solar, wind, and natural gas, because microorganisms can use the electrons delivered by the electrode to reduce carbon dioxide into a variety of organic compounds, especially those with multiple carbons that are precursors for desirable value-added chemicals or liquid transportation fuels (Lovley and Nevin 2011; Rabaey et al. 2011).

As a new direction in BES research that was only introduced in 2010, there have been limited findings in microbial electrosynthesis. One of the first studies demonstrated such possibility is by Nevin et al., who showed that biofilms of *Sporomusa ovata* growing on graphite cathode surfaces consumed electrons with the reduction of carbon dioxide to acetate and small amounts of 2-oxobutyrate. Electrons appearing in these products accounted for over 85 % of the electrons consumed (Nevin et al. 2010). Traditionally, acetogenic bacteria can reduce carbon dioxide to acetate and other multicarbon extracellular products with hydrogen as the electron donor, and in an attempt of replacing hydrogen with electrode as the donor, the same research group found many acetogenic bacteria, such as *C. ljungdahlii, C. aceticum, S. sphaeroides,* and *Moorella thermoacetica* were all able to consume electrical current and produce organic acids. In general, acetate was the primary product, but 2-oxobutyrate and formate were also formed (Nevin et al. 2011). Studies also showed that methanogenic microorganisms were able to reduce carbon dioxide to methane in the BES cathode chamber (Cheng et al. 2009), and ethanol can be produced by reducing acetate at the cathode, but some processes

required the addition of mediators, such as MV (Steinbusch et al. 2010). Efforts on genetically engineering microorganisms for facilitated electron update and organic synthesis are underway, and the elucidation of the mechanisms for electron transfer from electrodes to microorganisms will greatly help the understanding and development of the technology (Lovley and Nevin 2011; Rabaey et al. 2011).

19.4.3 Environmental Remediation

BESs offer both oxidation and reduction approaches to remediate underground contaminations. Unlike wastewater treatment or chemical production, the BES used in remediation can be a single or an array of electrodes without using enclosed containers. The electrodes serve as inexhaustible electron acceptors/donors to stimulate and enhance microorganisms to simultaneously degrade biodegradable pollutants and, under circumstance of enhanced bio-oxidation, produce electricity. Another advantage of BES for remediation is that this technique does not require any chemical addition or energy input, and the produced current can potentially power wireless sensors and serve as a real-time bioremediation indicator.

Petroleum hydrocarbons, especially BTEX compounds (benzene, toluene, ethylbenzenes, and xylenes) have caused widespread groundwater and soil contamination associated with fuel spills and leaking from underground storage tanks (EPA 2004). Traditional remediation practice either heavily relies on energy-intensive or cost-intensive technologies such as thermal extraction, soil vapor extraction, bioventing, or expensive chemical oxidation (ChemOx) (Soares et al. 2010). Though bioremediation has been considered as low-cost and environmentally friendly, current in situ bioremediation is often limited by the availability of electron acceptors (e.g. O_2, NO_3^-) in the subsurface environment. By using electrode as a channel linking underground hydrocarbon as the electron donor and upground O_2 as the electron acceptor, studies have shown that the degradation of diesel carbon (C8–C25) was increased by 164 % (from 31 to 82 %) than the open circuit control within 21 days of operation. Another study showed that by converting an existing groundwater monitoring well into a BES and use hydrocarbon as the only available substrate, a BES removed 24 % of hydrocarbon within 66 days, 12 times more than in the control experiment without electrodes (Morris and Jin 2012; Morris et al. 2009). In addition, the BES produced a voltage from 25 to 190 mV, with the maximum power density of 2.2 W/m^3. Similar studies on biodiesel, ethanol, 1,2-dichloroethane, pyridine, and other contaminants were also reported, demonstrating that BES can be a viable approach for petroleum hydrocarbon degradation and energy production (Lovley 2011).

Conversely, oxidative contaminants can be removed using the electrode as the electron donor. However, different from the anode oxidation process which is highly microbial dependent, such remediation can be realized through either direct electrochemical or bioelectrochemical reductions. Chlorinated solvents, such as

trichloroethene (TCE) and perchloroethene (PCE), are almost entirely anthropogenic, and many are highly toxic or carcinogenic. Studies showed that a negatively polarized solid-state electrode could serve as an electron donor, with or without electron shuttles, for the reductive dechlorination of TCE to ethene by a mixed culture of microorganisms (Aulenta et al. 2008). Similar approaches have been used to reduce perchlorate, an emerging contaminant in drinking water due to its damage to thyroid gland (Butler et al. 2010). Other heavy or radioactive metals such as Cr(VI) (Wang et al. 2008), copper (Ter Heijne et al. 2010), and uranium have also been tested in lab-scale experiments. For example, Wang et al. found that Cr(VI) could be completely removed after 150 h operation, with a initial concentration of 100 mg/L (Wang et al. 2008). The maximum power density of this system was 150 mW/m^2. Gregory and Lovley reported that negatively poised electrode can serve as an alternative electron donor for U(VI) reduction by a pure culture of *G. sulfurreducens* and microorganisms in uranium-contaminated sediments (Gregory and Lovley 2005). The advantage of using electrode as opposed to organic electron donor delivery is that U(VI) can be reduced to U(IV), which remains as a stable precipitate on the electrode. When the electrode is pulled from the contaminated sediments, the precipitated uranium can be permanently removed from the subsurface and recovered and reused.

19.4.4 Water Desalination

Water desalination is a recently developed function in BESs. By inserting a pair of ion exchange membranes between the BES anode and cathode chamber to form a middle chamber containing saline water, the MDC utilizes the electric potential generated across the electrodes to drive desalination. Specifically, when bacteria in the anode chamber oxidize biodegradable substrates and produce current and protons, the anions (e.g., Cl^-) in the middle chamber migrate to the anode and the cations (e.g., Na^+) are drawn to the cathode for charge balance. The loss of ionic species from the middle chamber results in water desalination without any external electricity input or higher water pressure (Fig. 19.8) (Cao et al. 2009; Luo et al. 2012b). Desalination can also be achieved by replacing ion exchange membranes by a forward osmosis membrane and withdraw pure water from wastewater to the draw solution (Zhang et al. 2011).

The MDC process carries great potential in desalination systems, because it can either be used as a stand-alone process or serve as a pretreatment for conventional desalination processes such as reverse osmosis (RO) to reduce salt concentration of RO feed, and minimize energy consumption and the membrane fouling potential. Current desalination technologies, such as RO and electrodialysis (ED) are energy and capital intensive. Even the most advanced large-scale seawater RO units require 3–7 kWh/m^3 for water desalination, while conventional multi-stage flash evaporation requires 68 kWh/m^3. In contrast, the MDC system is considered to be an energy gaining process, because it converts the biochemical energy stored in

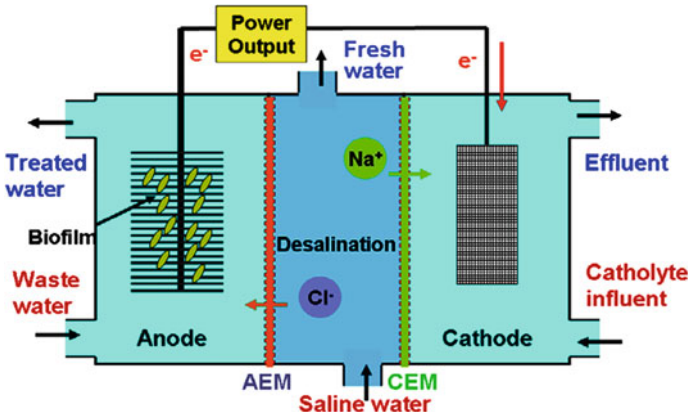

Fig. 19.8 Schematic of a three-chamber BES/MDC reactor for simultaneous wastewater treatment (anode), desalination (*middle chamber*), and energy reduction

wastewater to electricity or hydrogen gas. Lab-scale MDC studies showed that 180–231 % more energy can be recovered as H_2 than the reactor energy input when desalinating 5–20 g/L NaCl solutions (Luo et al. 2011; Mehanna et al. 2010), and a recent study calculated that a liter-scale MDC can produce up to 58 % of the electrical energy required by downstream RO systems (Jacobson et al. 2011).

Current MDC and associate processes focus on reactor stack development to increase desalination efficiency (Chen et al. 2011; Kim and Logan 2011b), and system characterization and operation optimization to improve performance (Luo et al. 2012a). In traditional MDC configurations, although salts get removed from the middle chamber, they become concentrated in the anode and cathode chambers, resulting in an increase of the volume of saline water. This concern becomes more imperative when wastewater is treated as the anolyte. Although the addition of ions (or TDS) increases wastewater conductivity and benefits electricity generation, the increased salinity may affect effluent water quality and prevent subsequent beneficial use of treated wastewater (Luo et al. 2012b). In addition, the AEM between the anode and middle chamber inhibits the free transfer of H^+ accumulated in the anolyte to other chambers, which causes a significant pH drop in the anode chamber and pH increase in the cathode chamber. A recently developed microbial capacitive desalination cell (MCDC) may eliminate the problem completely (Forrestal et al. 2012). The MCDC incorporated the concept of capacitive deionization and uses two cation exchange membrane assemblies to separate the three chambers (Fig. 19.9). Results showed that MCDC's salt removal efficiency was 7–25 times higher than traditional capacitive deionization processes. Moreover, all of the removed ions in the MCDC were adsorbed in the activated carbon double layer capacitors without migrating to the anolyte or catholyte, and the electrically adsorbed ions could be recovered during assembly regeneration. The two cation-exchange membrane based assemblies allow the free transfer of protons across the system, and thus prevented significant pH changes observed in traditional MDCs.

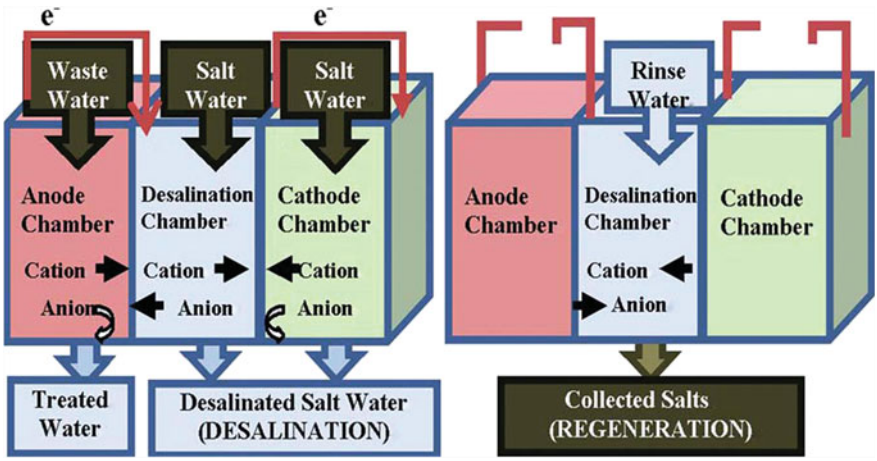

Fig. 19.9 Schematic of an MCDC reactor that addresses salt migration and pH fluctuation problems, allows salt recovery, and improves desalination efficiency by integrating with capacitive deionization

19.5 Conclusion

In less than a decade of research and development, the functionality of BESs has expanded dramatically and the performance has improved exponentially. The power density from MFCs has increased by orders of magnitude, from less than 1 mW/m^3 to ~500 W/m^3 (or 6.9 W/m^2), making it feasible for commercialization (Logan 2010). The projected volumetric wastewater treatment capacity of BES based on lab-scale studies can reach to 7.1 kg chemical oxygen demand (COD)/m^3 reactor volume/day, which is even higher than conventional activated sludge wastewater treatment systems (~0.5–2 kg COD/m^3 reactor volume/day) (Rozendal et al. 2008). Moreover, many new functions have been developed, making BES a great platform technology for many different applications.

However, BES experiments have typically been performed on a small scale, varying from mainly milliliters to the largest of 1,000 L (Cusick et al. 2011; Logan 2008). To achieve practical implementation, BESs still need to be scaled-up by several orders of magnitude to at least cubic meter scale, and reactor configurations have to be easily integrated with current wastewater infrastructure. Several researchers have aimed at developing pilot systems, but they have encountered multiple challenges, including water leaking, low power output, influent fluctuation, and unfavorable products (Cusick et al. 2011; Keller and Rabaey 2008). Some key challenges that need to be addressed including reducing material costs, developing scalable reactor configurations, reducing system internal loss and improve power output, effectively harvesting and delivering usable power, and increasing chemical production rates. Multiple reviews have summarized the progresses of BES system development and provided insights in further directions

(Logan 2010; Lovley 2011; Pant et al. 2012; Rozendal et al. 2008; Wang et al. 2012; Wei et al. 2012).

The versatility makes BES a unique platform technology for many different applications, including but not limited to chemical and energy production, wastewater treatment, environmental remediation, water desalination, and remote sensing, etc. However, it is still not clear where the BES can contribute the most to the current environmental infrastructure and chemical industry. There has been minimum evaluation of BESs regarding their life cycle in terms of function selections or comparisons with established technologies which they can complement (Foley et al. 2010; Pant et al. 2011). It has been assumed that the most environmental benefits from BESs come from the displacement of fossil fuel dependent resources (i.e. grid electricity, or chemical manufacture) through co-product production (i.e. electricity, chemicals) from renewable sources, but the energy and environmental footprints of BESs have to be clearly quantified before implementing large-scale applications.

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