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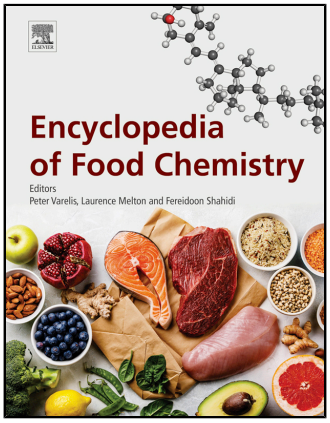

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Lipases for Biofuel Production

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Glossary

Aerobic gram-negative bacterial These are a group of gram-negative bacteria that able to grow under an air atmosphere and have an inner cell membrane surrounded by a thin layer of peptidoglycan which is a combination of proteins and sugars with the inner cell membrane unable to retain gram stain dyes typically used to identify bacteria.

Glycoproteins These are simply proteins with have sugar molecules attached and are usually found floating in or around the membrane of cells. The sugars may be attached to proteins in the endoplasmic reticulum, and in the Golgi apparatus.

Isoelectric point The isoelectric point of a molecules or surface refers to the pH at which a molecule or surface carries no net electrical charge.

Isoenzymes These are simply alternative forms of enzymes with different structures that are capable of catalysing the same reaction.

Moiety This is used to refer to a part of a molecule for instance the alkyl group of an organic compound responsible for the physical properties of a chemical compound.

Nonsporogenic Any fungi that does not produce spores.

Saprophytic This refers to the act of getting energy from dead or decaying matter. A saprophyte or saprotroph is therefore an organism which gets its energy from dead and decaying organic matter implying that saprophytes are heterotrophs.

Nomenclature

List of nomenclature used including all definitions and units.

kDa kilodalton
BCL *Burkholderia cepacia* lipase
CALB (CALA) *Candida Antarctica* lipase B (A)
CRL *Candida rugosa* lipase
GRAS Generally Recognised as Safe status
RML *Rhizomucor miehei* lipase
ROL *Rhizopus oryzae* lipase
TLL *Thermomyces lanuginosus* lipase

Introduction

Lipases (EC 3.1.1.3) are triacylglycerol ester hydrolases, considered as one of the two major classes of hydrolases, with the second class identified as the so called 'true' esterases (EC 3.1.1.1. carboxyl ester hydrolases). According to Vakhlu and Kour (2005), lipases were previously classified as serine hydrolases due to the presence of serine in the highly conserved domain of their active sites. This serine presents the only common feature shared by all identified lipases sequenced thus far (Vakhlu and Kour, 2005). Lipases are distinguishable from carboxyl esterases via spectral assessment of their substrates, since *p*-nitrophenyl palmitates are cleaved by lipases while *p*-nitrophenyl butyrates are cleaved by esterases. These two major classes of hydrolases can also be distinguished by assessing differences in stabilities in organic solvents, since lipases exhibit higher stabilities compared to esterases (Bornscheuer, 2002). Lipases are recognised as very important industrial inputs as illustrated by applications in the food processing industry (cheese ripening and flavour development) (Saxena et al., 1999), cosmetic industry (aroma production) (Metzger and Bornscheuer, 2006), bioenergy industry (biodiesel production), pollution remediation (oil spill management) (Nakamura and Nasu, 1990) and commercial cleaning industry (alkaline lipase from *Pseudomonas alcaligenes* M−1 used in removing oil stains) (Gerritse et al., 1998).

Lipases are characterised by molar weights ranging from 20 to 75 kDa with their optimal pH and optimal temperature conditions ranging from 6 to 8 and from 30 °C to 40 °C respectively (Giovana da Silva et al., 2012). Typically, the molecular weight of a lipases may be greater than 75 kDa, if the lipase has the capacity to form aggregates in solution (Gill and Parish, 1997). According to Gill and Parish (1997), the majority of lipases secreted by organism function extracellularly and are characterised by acidic glycoproteins containing between 2% and 15% carbohydrates and with the major glycoside residue being mannose. Most importantly, lipases are biocatalysts capable of acting on a range of substrates to enhance a multitude of reactions (Montero et al., 1993; Moura et al., 2017). Lipases are widely available in nature with extracts of lipases readily isolated from plant

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Table 1 Characteristics of some lipases obtained from different sources

Source organism	*Organism source*	*Short description*
Burkholderia cepacia	Bacteria (Thakur, 2012)	BCL lipase is highly tolerant to methanol and displays particularly high conformational stability under conditions employed during transesterification reactions for biodiesel production (Sasso et al., 2016). The lipase source is an aerobic gram-negative bacillus and is one of the most thoroughly studied lipase sources.
Candida Antarctica	Fungi (Thakur, 2012)	The organism produces two lipases namely CALB and CALA, characterised by a similar optimum pH of 7. CALB (CALA) is also characterised by a molecular weight, an isoelectric point and a specific activity of 33 kD (45 kD), 6 (7.5) and 435 (420) respectively (Kirk and Christensen, 2002). Most importantly CALB and CALA differ in terms of their positional specificity toward triglycerides, with a positional specificity of *Sn*-3 and *Sn*-2 reported respectively (Kirk and Christensen, 2002). Both lipases are characterised by an almost uniform activity towards straight-chain primary alcohols and carboxylic acids (Kirk and Christensen, 2002).
Candida rugosa	Fungi (Thakur, 2012)	The organism generates lipases that contain at least five isoenzymes of varying proportions and varying stabilities. It is speculated that the existence of these five related but non-identical isoenzymes enhances the adaptability to different substrate sources (Benjamin and Pandey, 1998). The CRL is popularly used in fat-splitting operations in the food industry due to its proven Generally Recognised as Safe (GRAS) status (Gurung et al., 2013) where the organism is nonsporogenic, pseudo-filamentous, unicellular and non-pathogenic in nature (Benjamin and Pandey, 1998).
Rhizomucor miehei	Fungi (Thakur, 2012)	RML is used for many applications in the conversion of fats and oils and in chemical processes. In its free and immobilised forms it has a very high activity and good stability under diverse conditions (Rodrigues and Fernandez-Lafuente, 2010). The lipase also has a high enantiospecificity.
Rhizopus oryzae	Fungi (Thakur, 2012)	The lipase produced by this fungi, ROL, also has GRAS status, which is responsible for its wide acceptance in the food and biotechnology industry (Cantabrana et al., 2015). It is a potent saprophytic and pathogenic fungus which can produce a spectrum of metabolites, such as esters, polymers and bioalcohols (Ghosh and Ray, 2011).
Thermomyces lanuginosus (TLL)	Fungi (Maheshwari et al., 2000)	TLL is used in large amounts in detergents and various synthetic applications. It is characterised by a molar mass of 31.7 kDa and an isoelectric point of 4.4 (Jha et al., 1999). It is a widely distributed thermophilic fungus, thriving at temperatures up to 60 °C but fails at temperatures lower than 20 °C (Singh et al., 2003). It has the capacity to produce high levels of cellulase-free thermostable xylanase, which has wide biotechnological applications (Singh et al., 2003).
Pentaclethra macrophylla Benth	African bean seeds (Enujiugha et al., 2004)	This lipase is characterised by a high thermal stability with lipolytic activity reported at temperatures up to 80 °C (Enujiugha et al., 2004). Optimum temperature and pH conditions have been reported to be 30 °C and 7 respectively. High sensitivity of the enzyme to salts has however been reported with the presence of mercury chloride and sodium chloride significantly inhibiting the catalytic ability (Enujiugha et al., 2004).

and animal sources (Sevil et al., 2012). **Table 1** presents some examples of characteristics and sources of common lipases used in the biotechnology industry.

Lipases are also characterised by high bio–catalytic activities when acting on water-insoluble substrates, such as triglycerides composed of long-chain fatty acids, and are typically characterised by a high chemo-, regio- and stereoselectivity, in organic solvents (Barros et al., 2010). Crucially they are generally recognised as environmentally benign with their catalytic effect optimised when mild reaction conditions are imposed (Ferreira-Dias et al., 2013). The effectiveness of lipases in catalysing processes under mild condition also promotes its application in several reactions. Major reactions that can be catalysed using a lipase are presented in **Fig. 1**.

Fig. 1 shows that lipase can effectively catalyse acidolysis, alcoholysis, aminolysis, interesterification, glycerolysis and hydrolysis reactions, when a triglyceride molecule is the substrate (Pandey et al., 1999).

From **Fig. 1**, the interesterification reaction involves the exchange of an acyl radical between the parent triglyceride and a 'donor' triglyceride. If however the donor is a free fatty acid, then the reaction is considered as an acidolysis reaction. The exchange of acyl radicals is typically undertaken to improve the properties of the parent triglyceride molecule. On the other hand the displacement of the acyl moiety between the triglyceride and an alcohol (ROH) serves to generate biodiesel. Similar acyl moiety exchanges between the triglyceride and glycerol ($C_3H_5(OH)_3$) is called a glycerolysis reaction. Such glycerolysis reactions serve to generate useful mono-($C_3H_5(OH)_2.(OOCR'')$) and di-(($C_3H_5(OH).(OOCR'')_2$) glycerides, which are useful products used in the food industry since both products can be used as food stabilisers in air-in-liquid emulsions like cake batter or ice cream.[1] The hydrolysis reaction serves to split the triglyceride molecule into its glycerol backbone and constituent fatty acid (R''COOH) fragments. The aminolysis reaction serves to spilt the parent triglyceride by reacting the triglyceride with ammonia (NH_3) or amines ($R{-}NH_2$) to form a fatty acid amide (R''CONHR).

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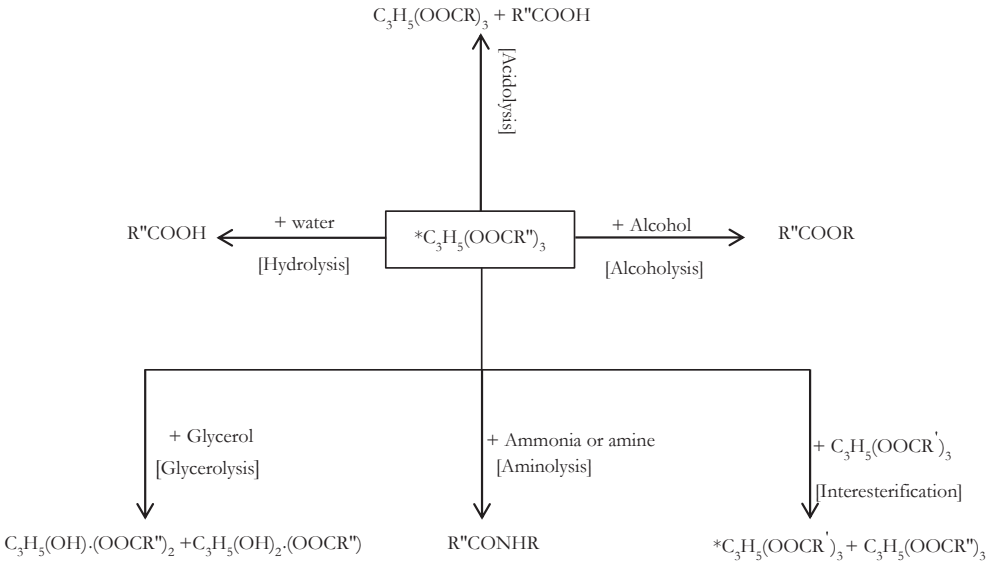

Figure 1 Lipase catalysed reactions involving triglycerides as the substrate (where *$C_3H_5(OOCR'')_3$ represents the parent triglyceride and $C_3H_5(OOCR')_3$ represents the 'donor' triglyceride in the interesterification reaction).

According to Ribeiro et al. (2011) most lipase catalysed reactions are initiated by a nucleophilic attack on the carbon present in the ester bond of the susceptible substrate by an hydroxyl group in the serine residue of the active site of the lipase. Nucleophilic attack results in the formation of an acyl–enzyme complex and the release of alcohol from the triglyceride substrate. The regeneration of the lipase is achieved via the hydrolysis of the acyl–enzyme complex. This process may occur via complex or simple mechanisms, depending on several factors, such as temperature, presence of inhibitors and substrate concentration (Artioli, 2008).

The ability of the lipases to catalyse acidolysis, alcoholysis, interesterification, glycerolysis and hydrolysis reactions has been explored extensively in the biofuel industry as the basis for the development of an environmental friendly production process. According to Alonso et al. (2005), the versatility of lipases in enhancing the hydrolysis of ester-carboxylate bonds at the organic-aqueous interphases and esterification–transesterification reactions in water-restricted environments is responsible for its extensive use in the biodiesel (biofuel) industry.

Lipase Application in Biodiesel Production

The utilisation of lipases in catalysing transesterification (alcoholysis) reactions for biodiesel production processes was first reported by Mittelbach (1990). Mittelbach demonstrated the utilisation of *Pseudomonas fluorescens* lipase to enhance the alcoholysis of sunflower oil using methanol (Mittelbach, 1990). Over the years, more research has been undertaken to explore the activity of lipases in biodiesel production processes when different oil substrates are utilised (Rodrigues et al., 2016; Amini et al., 2017).

This is largely due to the ability of lipases to act on a wide range of oils including low grade oils characterised by a high free fatty acid content, such as dissolved air flotation sludge oil extract (Okoro et al., 2017a), without initiating unwanted side saponification reactions occurring (Rodrigues et al., 2016; Okoro et al., 2017b). **Table 2** presents some examples of previous work that have explored lipase utilisation in biodiesel production processes.

Lipase Immobilisation

Table 2 suggests that high biodiesel yields are feasible when lipases in different forms are used as biocatalysts. However current applications have suggested the preference of the immobilised lipase forms as a means of circumventing several biofuel production limitations identified when lipases in their free forms are utilised (Zhao et al., 2015). Some of these limitations include an escalation of the production costs due to the difficulty of lipase recovery and reuse and the increased possibility of production promoting product contamination with residual enzyme (Ribeiro et al., 2011). Other benefits arising from the application of immobilisation techniques includes an improvement in the thermal stability, pH stability of the lipase and an enhanced tolerance of the lipase to chemical species in the reaction mixture (Zhao et al., 2015; Silva et al., 2013). Such improvements in the properties of the lipase via the application of immobilisation methods will increase the applicability of lipase in continuous biofuel production systems as a result of the enhanced lipase adaptability (Silva et al., 2013).

Lipase immobilisation, in the simplest terms may be considered as the confinement of the lipase in a region or a defined space while simultaneously ensuring that the catalytic activity of the lipase is maintained (Tan et al., 2010). Lipase immobilisation is usually achieved by tethering the lipase to a suitable supporting matrix which must be hydrophilic, inert (to the lipase), biocompatible, resistant to microbial attack, resistant to compression and readily accessible at a low cost (Mohamada et al., 2015). According to Cao

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Table 2 Biodiesel production using lipase as catalyst

Substrate	*Alcohol used*	*Lipase*	*Form*	*Temperature (° C)*	*Reaction time (h)*	*Performance (%)*	*References*
Ocimum basilicum (sweet basil) seed oil	Methanol	*Candida Antarctica*	Immobilised on acrylic resin	47	68	94.58 (yield)	Amini et al. (2017)
Jatropha oil	Methanol	*Thermomyces lanuginosus* (TL) 1,3 specific lipase	Free	35	24	80.7(yield)	Bueso et al. (2015)
Jatropha oil	Ethanol	*Chromobacterium viscosum*	Immobilised on Celite-545	40	8	92(yield)	Shah et al. (2004)
Waste cooking oil	Methanol	*Rhizomucor miehei*	Immobilised on textile cloth	45	–	91.08 (yield)	Chena et al. (2009)
Soybean	Methanol	*Rhizomucor miehei* displaying Pichia pastoris	Used as a whole cell biocatalyst (Pichia pastoris).	55	72	83.14(yield)	Huang et al. (2012)
Palm oil	Isobutanol	*Candida antarctica B*	Immobilised on granular activated carbon	40	45	100(conversion)	Naranjoa et al. (2010)
Waste cooking	Methanol	*Thermomyces lanuginosus* (Lipozyme TL IM)	Immobilised on hydrotalcite and zeolites	45	105	95 (yield)	Yagiz et al. (2007)
Rape seed oil	Methanol	*Saccharomyces cerevisiae*	Immobilised on immobilised on Mg—Al hydrotalcite	45	4.5	96 (conversion)	Zeng et al. (2009)
Tallow and Grease	Ethanol	*Burkholderia cepacia*	Immobilised on a phyllosilicate sol–gel	50	18	94 (yield)	Hsu et al. (2001)
Canola	Methanol	*Thermomyces lanuginosus*	Immobilised onto hydrophobic microporous styrene–divinylbenzene copolymer	50	24	97 (yield)	Dizge et al. (2009)
Palm oil	Methanol	*Aspergillus niger*	Used as a whole-cell biocatalyst	40	72	87 (yield)	Xiao et al. (2010)
Soybean oil	Methanol	*Rhizopus oryzae ATCC 24563*	Used as a whole cell biocatalyst	35	6	97(conversion)	Lin et al. (2011)
Olive oil	Methanol	*Candida antartica lipase B*	Immobilisation on an amphiphilic matrix	50	24	90 (yield)	Lee et al. (2010)
Soybean oil	Methanol	*Pseudomonas flurescens*	Free	35	90	90(yield)	Kaieda et al. (2001)
Babassu oil	Ethanol	*Burkholderia cepacia*	Immobilised on SiO_2–PVA	40–50	48	100(yield)	Da Rós et al. (2010)

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(2006), several factors influence the performance of an immobilised lipase such as the pore size of the carrier, the microenvironment of the carrier and the strength of the hydrophobic partition. These factors influence the extent of catalytic activity retention, the lipase stability and the rate of action on a hydrophobic substrate respectively. Thus although the introduction of immobilisation strategies will enhance the ease of the lipase separation for recovery and reuse (Ribeiro et al., 2011), leading to lower operating process cost, the technique and nature of immobilisation must be properly selected based on specific processing targets.

Immobilisation Methods

There are five major lipase immobilisation methods identified in the literature, namely, covalent, adsorption, cross linking (or aggregation), entrapment and encapsulation methods (Sirisha et al., 2016; Bickerstaff, 2009). These methods have been summarised and illustrated in **Table 3** and **Fig. 2** respectively.

Future Prospects

It is recognised that the utilisation of lipases in enhancing reactions provides a pathway that is comparatively more environmentally benign compared to reactions that utilise chemical catalysts (acid or alkaline). The high cost of lipase has however thus far limited its use in large scale processes (Poppe et al., 2015). Lipase high cost is exacerbated by lipase deactivation that typically occurs in the presence of alcohols for biodiesel production thus limiting lipase reuse options (Abdelmoez and Mustafa, 2014). Although there have been reports of an improved recoverability of the lipase for reuse via the incorporation of immobilisation techniques, current immobilisation systems are still limited by several factors. Some of these factors include possible losses in catalytic activity of the lipase, high cost of more suitable lipase supports, reduced stability of the immobilised lipase catalyst in oil–water subsystems and increased mass transfer restrictions in the immobilised lipase reaction system (Zhao et al., 2015).

Genetic engineering has therefore been proposed as a tool that can enable improved yields of better performing lipase strains at reduced production cost. This is because lipases can be genetically engineered to have high catalytic activities, to be stable in organic solvents, and enhance product recovery (Abdelmoez and Mustafa, 2014). Examples of successful recombinant DNA technology application include the improvements introduced to *Aspergillus oryzae* and *Pichia pastoris* strains which have been genetically modified to express the properties of several microbial lipases which can be used in biodiesel production (de Simone et al., 2016). The utilisation of whole cells as biocatalyst is also expected to reduce lipase catalysed biofuel production processes since the need for

Table 3 Major enzyme immobilisation methods

Immobilisation methods	*Short discussion*
Adsorption bonding [Fig. 2A]	This immobilisation bonding method is recognised as the most straightforward immobilisation method since lipase attachment is achieved in the absence of any chemical transformations. The tethering of the lipase to the support or carrier is achieved using weak intermolecular forces such as van der Waals or dispersion forces (Jegannathan et al., 2008). It is the most widely used immobilisation technique due to its simplicity and low cost (Lukovic et al., 2011). This immobilisation method is however limited by changes in ionic strength during the catalysing process, which may lead to lipase desorption (Rai, 2012). The lipase may also be susceptible to microbial or proteolytic attack.
Entrapment bonding [Fig. 2B]	Entrapment describes the immobilisation of the lipase via the 'sealing' of an enzyme within a polymeric network (Brena and Batista-Viera, 2006). The polymetric network enables the transfer of substrate and products across the polymeric network barrier while retaining the lipase within the polymeric network (Mohamada et al., 2015). This immobilisation method is considered as irreversible since the separation of the lipase from the polymetric matrix may require the destruction of the matrix (Mohamada et al., 2015). This immobilisation method is simple and is characterised by no intrinsic changes in lipase properties from chemical modification. This method is however limited by the possibility of enzyme leakage and diffusional constraints of the products and reactants. Furthermore the mass of lipase that can be immobilised within the polymetric network is clearly limited (Mohamada et al., 2015; Brena and Batista-Viera, 2006).
Encapsulation bonding [Fig. 2C]	Encapsulation is the confinement of the enzyme within a porous membrane forming a bilayer (Jegannathan et al., 2008). Encapsulation does not require any interaction between the catalyst and the support, with the size of the pore opening of the support considered crucial as it determines the mass transfer resistance of the reactants and products while also preventing any losses in the encapsulated lipase (Zhao et al., 2006). Several reports have however suggested that such encapsulation may occasionally lead to the inactivation of the lipase molecule (Dwevedi, 2016).
Covalent bonding [Fig. 2D]	This method is based on the formation of covalent bond between the lipase molecule and the support material using side chain amino acids such as histidine with the reactivity of the immobilised lipase influenced by the functional group that is present in the side chain (Sirisha et al., 2016). In cases where the bonds existing between the lipase molecule and the support are due to the presence of strong electrostatic (ionic) forces, such an immobilisation method is referred to as ionic bonding (Tor et al., 1989). The utilisation of such strong (covalent) bonds may however lead to significant changes in the conformational and catalytic properties of the lipase molecule (Dwevedi, 2016).
Cross linking [Fig. 2E]	This method involves the utilisation of bi- or multifunctional reagents to stimulate the aggregation of lipase molecules such that the lipase molecule does not undergo any denaturation (Talekar et al., 2012). This method ensures that the activity or the catalyst productivity is not negatively influenced since the cross linking agent is typically characterised by a molar mass that is large compared to the molar mass of the lipase molecule (Talekar et al., 2012). There is however increased possibility of catalyst loss due to the non-regulation of the aggregation reaction and reduced reactivity due to limitations caused by diffusion (Dwevedi, 2016).

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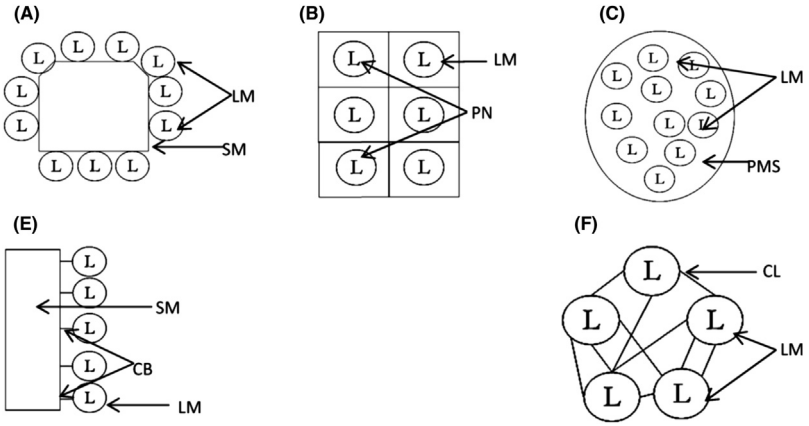

Figure 2 An illustration of the major immobilisation methods (LM represents lipase molecule; SM represents support matrix; PN represents polymetric network; PMS represents porous support matrix; CB represents covalent bond; CL represents the cross linkage).

lipase recovery and purification is avoided (Soares et al., 2017). Future modifications in existing immobilisation technologies such as the use of biopolymers as mobilisation supports will also enhance lipase recovery and improved product separation while also enhancing the environmental performance of biofuel production processes (Ravindra and Jegannathan, 2015).

Another possible future prospect is the incorporation of intensification approaches, such as the utilisation of ultrasonic and microwave radiation to optimise the activities of immobilised lipases (Subhedar and Gogate, 2017). This is because the application of ultrasonic and microwave radiation provide a low cost option for energising the lipases (Subhedar and Gogate, 2017). Finally, appreciating that the performance and economics of lipase catalysed biofuel production processes are dependent on a proper integration of the aforementioned improvements any future lipase aid biofuel production technology must incorporate an optimisation protocol with the production cost identified as an important objective function (Luna et al., 2016).

Conclusion

This chapter has highlighted the role of lipases as important biocatalysts in several industries, largely based on their abilities to reduce waste generation and thus lead to favourable environmental outcomes. The importance of lipases to the biofuel industry has been emphasised. Published studies that investigated the applicability of lipases (free and immobilised forms) in the biofuel industry were also highlighted. Enhanced performances of reactions catalysed using immobilised lipases compared to the lipases in free forms, were reinforced and discussions highlighting the benefits of lipase immobilisation, presented. Major immobilisation techniques were also briefly explained. As explained in this chapter, although the utilisation of lipases in catalysing biofuel production processes has many associated advantages, several limitations persist that have thus far hindered large scale applicability. It was suggested that these limitations are largely due to the high cost of lipases, with future perspectives focused on reducing the unit lipase cost. These future perspectives were therefore initially discussed.

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