Chapter 10
The Realm of Lipases in Biodiesel Production

Daniela V. Cortez, Cristiano Reis, Victor H. Perez and Heizir F. De Castro

Abstract Lipases are the enzymes known for the hydrolytic activity on carboxylic fatty ester bonds. The industrial interest in lipases is due to their application in a wide array of products: in detergents and cleaning products, in pharmaceutical applications, in the food industry, and on the production of biodiesel. Biodiesel, i.e. short-chain-acyl fatty ester, is mainly produced via the transesterification of fatty-acyl glycerides or esterification of fatty acids, both reactions with a short chain alcohol. Lipases can catalyze both said reactions with high specificity, producing biodiesel at high yields at low temperature. With the significant advances in biodiesel production over the last decades, coupled with a strong industrial partnership, the costs of utilizing lipases as catalysts have dropped significantly. The production of lipases became popularized in the industry due to advances not only in the reaction mechanisms, and in better understanding of lipase-producing microorganisms, but to cost-effective utilization practices. Immobilization is the practice responsible for the initial breakthrough innovation that allowed efficient reutilization of lipases, thus reducing the cost per batch. There was, and still there is, numerous advances in the development of immobilizing matrices and novel utilization pathways of immobilized enzymes available in the literature. More recently, other methods of using lipase in biodiesel production have been developed, e.g. via the utilization of whole-cell and fermented solid with lypolytic activity, and by the use of lipase in liquid formulations. Over the last years, there has been an increased interest in developing next-generation biodiesel, i.e., the one produced from alternative lipid feedstock, such as microbial and residual lipids, and by utilizing ethanol as acyl agent, instead of methanol. There has also been prominent advances in the reactor engineering aspect of lipase-derived biodiesel, by promoting more efficient batch processes, and the development of lower-cost continuous processing. The present chapter reviews the recent literature

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in the important field of using lipases in biodiesel production, and critically describes the opportunities and challenges present in such applications.

Keywords Lipase · Biodiesel · Immobilization · Transesterification
Hydro esterification · Batch and continuous processes

10.1 Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) are part of the family of hydrolases that act on the carboxylic ester chain and do not require any cofactors. Under conditions in which the availability of water in the medium is reduced, most lipases are capable of catalyzing reverse reactions such as esterification and interesterification (transesterification, alcoholysis and acidolysis), as well as the hydrolysis of triacylglycerols, among others (Hasan et al. 2009). The behavior of the induced-fit type of these enzymes makes it possible to convert a significant variety of artificial substrates, which often do not have naturally common structures-triacylglycerols (Faber 2011). Thus, lipases are among the most important biocatalysts used in chemical reactions in both aqueous and non-aqueous media. The reason of their importance is mainly due to their ability to utilize a broad spectrum of substrates, in addition to their robustness to operate within a wide range of temperature, pH and organic solvents, and their chemo, regio and enantioselectivities (De Castro et al. 2004; Faber 2011).

Daiha et al. (2015) analyzed the number of publications and patents related to the use of lipases based on some industrial sectors at different stages of development and with different technological levels. According to this publication, the use of lipases as biocatalysts has remained relevant to the industrial segment since the discovery of its potential with a projected increase in world demand of 6.2% per year, reaching USD 345 million in 2017. Among the possible applications of these robust biocatalysts, the enzymatic production of biodiesel is the one with the highest number of publications and records, including patents and scientific articles. Such data are also in agreement with other surveys that evaluated the different types of catalysts used in biodiesel synthesis. According to Pinto et al. (2005) and Quintella et al. (2009), the use of lipase as biocatalyst numerically exceeds the sum of the articles and patents referring to obtaining this fuel by all other types of catalysts.

Biodiesel is defined as the mono-alkyl ester derivative of long-chain fatty acids from lipid feedstocks (oils and fats). The renewability of biodiesel is associated with the replacement of fossil fuels in compression ignition engines or diesel engines (Knothe et al. 2010). The most widely used biodiesel production route in the industry is based on the alcoholysis reaction, also known as transesterification, of a lipid material with a short chain alcohol (e.g. methanol and ethanol). This reaction is considered the most industrially accepted route because it yields a product (biodiesel) with characteristics such as viscosity and cetane number close to those of diesel. The oils and fats when subjected to the transesterification process have their viscosity

values decreased significantly, so that the fuel obtained can be burned directly in diesel engines without the need for adaptation or modification of traditional diesel engines (Knothe et al. 2010). In order to obtain a high-quality biofuel, some technical characteristics are essential, such as: the transesterification reaction must be complete reflecting the total absence of remaining fatty acids, and the biodiesel produced must be of high purity, not containing traces of residual glycerin or excess alcohol from the transesterification reaction (Knothe et al. 2010).

Like any other enzyme-mediated reaction, lipase-catalyzed biodiesel production has a number of advantages over the chemical reaction, mainly due to the specificity and selectivity of the biomolecule, which makes the process less energy-intensive with respect to the raw materials and the reaction conditions (Gog et al. 2012; Meunier et al. 2017). In addition to high selectivity, lipases do not form soap as a by-product, and can be esterified free fatty acids and reused in more than one reaction cycle. The reaction requires little to no heat, since it occurs under mild pressure and temperature conditions, and it does not require costly purification costs (Gog et al. 2012; Christopher et al. 2014). At the end of the process, glycerol (lower phase) is separated from the biofuel (upper phase) by simple decantation. Deodorization and neutralization of the final product is usually not required as well (Ranganathan et al. 2008). The enzyme transesterification is applicable to crude and refined vegetable oils, fats, tallow and other fat residues, and various alcohols, such as methanol, ethanol, propanol, isopropanol, butanol, and isobutanol (Ranganathan et al. 2008; Gog et al. 2012; Christopher et al. 2014). The free fatty acids present in the oil are esterified and do not require purification steps of the raw material, therefore, oils containing triacylglycerols and free fatty acids are enzymatically converted into biodiesel because the lipases catalyze the transesterification and esterification simultaneously (Ranganathan et al. 2008; Gog et al. 2012; Meunier et al. 2017). The addition of organic solvent *(tert*-butanol, hexane, *n*-heptane, chloroform, 1,4-dioxane, isooctane) in the reaction medium can assist in solubility between the alcohol and the oil, facilitating mass transfer and enzymatic catalysis. Addition of solvent can also minimize the possible inhibitory effect of alcohol on lipase (Iso et al. 2001; Fu and Vasudevan 2009).

The search for enzymatic catalysts that promote reactions that can act competitively with the well-established chemical pathway has led increasingly to the establishment of new forms of lipase presentation, which are mostly characterized as being free, i.e. soluble, immobilized, bound to the mycelium, i.e. whole cells, or in the form of fermented solids with lipolytic activity. In this context, there is a consensus that obtaining more stable biocatalysts with properties that allow their reuse for several cycles, with the effective maintenance of the catalytic activity is the key to development of the activities in the field. In the case of the enzyme used in the soluble form, the search for solutions for this type of technology arises with the development of genetic engineering and cultivation techniques. Such advances have contributed to the reduction of the cost of liquid lipase, which allows the enzyme to be used only once, with results comparable to the conventional process, but more economically feasible (Zeng et al. 2017). In the vast majority of studies, besides the enzyme, there is a concern in the determination of the conditions optimized for the

reaction, with evaluation of the factors that directly interfere in the process. Thus, the literature related to the production of biodiesel by lipase is quite extensive and dynamic. The research in lipase-catalyzed biodiesel, motivated by the importance and opportunities that biofuel represents within sustainable development, is a major factor to the development of energy security alternatives and an energy grid based on renewable fuels. For these reasons, this chapter reviews the latest technologies within the area, presenting some examples, in order to highlight the great advance in related research (Fig. 10.1).

10.2 Lipase Properties for Biodiesel Synthesis

Lipases are a broad group of enzymes with several industrial applications. These biomolecules are characterized by their versatility of catalysis of hydrolysis and synthesis reactions, often in a chemo, regional and enantioselective way (Kapoor and

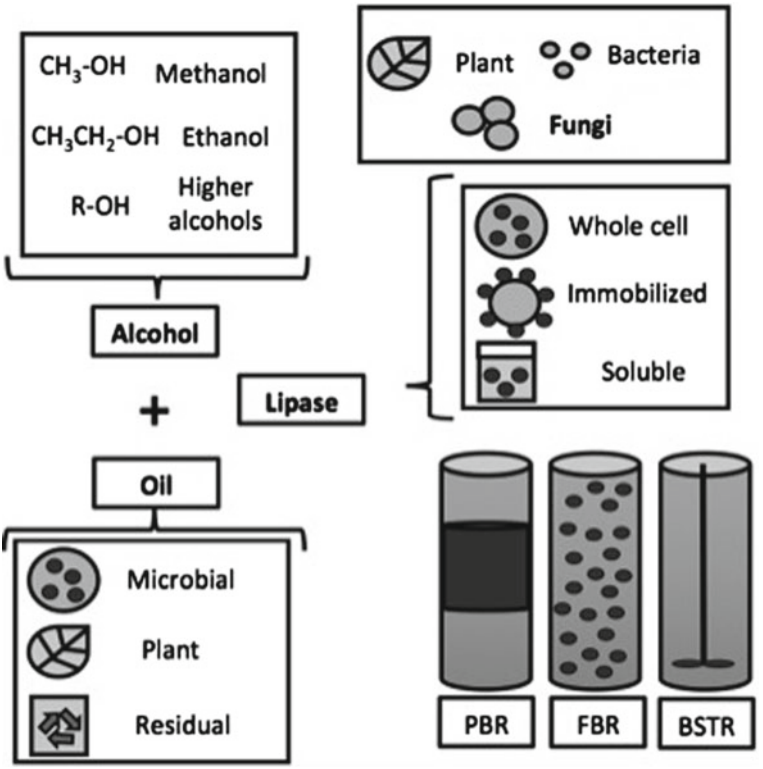

Fig. 10.1 The worldwide energy consumption for different time period (Adapted and modified from IEO 2017, an open source article)

Gupta 2012). Lipases can be found in animal and plant tissues, as well as in microorganisms (Ribeiro et al. 2011; Freire and Castilho 2008). Among the lipase-producing sources, the microbial one is the most industrially used, due to simpler isolation procedures from the fermentation broth, and by the fact that they are generally more stable and have more diversified properties than lipases from other sources (Jaeger and Reetz 1998). Lipases can be produced by bacteria, yeasts, actinomycetes and fungi, the latter being the most used in industrial processes. Microorganisms with the potential to produce lipases originate from a variety of habitats, including the marine environment, the Antarctic environment, vegetable oils and residual oils and dairy industries, contaminated soils, plants, and rotten foods. In this way, nature offers an extraordinary and potential collection of sources of microbial lipases (Cortez et al. 2017). Among the species using in the industrial scale, a few fungi belonging to the *Aspergilllus*, *Mucor*, *Rhizopus*, *Geotrichum*, *Penicillium*, and *Thermomyces* genus, as well as the *Candida* yeasts, and, *Bacillus*, *Pseudomonas* and *Burkholderia* bacteria stand out when compared to other strains (Treichel et al. 2010). With respect to the animal source of lipase, porcine pancreatic tissue is the most commonly found, mainly due to the stability of the isolated enzyme (Mendes et al. 2012). In the case of plant sources, a variety of seeds and beans from oil crops and cereals (Barros et al. 2010), as well as in latex plant tissues, e.g. from *Carica papaya* (Villeneuve 2003; Mazou et al. 2016), can be utilized as a feedstock to lipase extraction. Comparatively speaking, the use of plant lipases is much less developed than those from microbial origin, but plant-based enzymes can also be envisaged as biocatalysts for lipid bioconversions. Lipases are available in large amounts in the latex of some plant species, which can yield a relatively inexpensive source, though still underdeveloped (Mazou et al. 2016). However, the industrial biodiesel production by plant lipase is still a challenge due to the slow transesterification kinetics and lower yield when compared with microbial lipases (Cambon et al. 2009; Mounguengui et al. 2013).

Specificity is an important feature of lipases, being controlled by their molecular properties, substrate structure and by factors that affect enzyme-substrate binding (Antczak et al. 2009). Lipases can then be classified according to their positional specificity, i.e. non-specific or 1,3-specific, or according to their fatty acid specificity (Lotti and Alberghina 2007; Antczak et al. 2009). Substrate specificity consists in the ability to distinguish structural features of fatty acid chains such as the length, number, position or configuration of unsaturated bonds, the presence of branching, as well as the nature of the fatty acid chain, i.e. fatty acid, alkyl ester or glycerol ester. In the reaction of triacylglycerols and alcohols, lipases also distinguish the size and type of alcohol used in the reaction (Antczak et al. 2009; Faber 2011). The *sn*-1,3-specific lipases such as those produced by *Rhizopus oryzae* (Ban et al. 2002), *T. lanuginosus* (Nordblad et al. 2014) and *M. circinelloides* (Carvalho et al. 2015a) have been reported to efficiently catalyze transesterification of vegetable oils with conversion yields greater than 90%. Such high conversion of transesterification reactions is due to the efficient transfer from the residue acyl to specific positions on the glycerol molecule. Though being *sn*-1,3-specific enzymes, such lipases are effective for cleaving TAG fatty acids in the *sn*-1,3 positions promoting the migration of the acyl residues in the *sn*-2 position terminal (*sn*-1 and *sn*-3) in

glycerol (Antczak et al. 2009). On the other hand, the migration of the acyl group may also be influenced by the polarity of the solvent used in the transesterification reaction. According to Li et al. (2010) the reduction of solvent polarity increases the acyl group migration rate constants due to the favorable influence of the dispersion of the charge on the transition state, leading to varied yields of biodiesel.

The mechanism of lipases, unlike other enzymes, is significantly complex and dependent on certain structures typical of the biomolecule. In addition, the water content has a primary effect on its behavior, directly affecting the hydration of the enzyme or indirectly altering the nature of the reaction medium (Salis et al. 2007). Thus, the selection of suitable conditions for the performance of a lipase catalyzed reaction must follow a careful manipulation of the environment of the biocatalyst in such a way that the productivity of the system is maximized by the total potentiality of the enzyme activity (Reis et al. 2009). Using appropriate solvents and controlling the water content in the reaction medium can efficiently increase the activity levels close to their maximum potential. When water is replaced by an organic solvent, changes in the native conformation of the enzyme can occur both in the tertiary structure and in the more prominent secondary structures (α-helix and β-sheet), thus causing its destabilization. In order to ensure a catalytically active enzymatic conformation in organic media, the enzyme molecule must have a defined hydration layer, separating the solvent from contact with the surface of the protein and contributing to the increase of its internal flexibility (Klibanov 2001). Another way to protect the native configuration of the enzyme is through its immobilization on solid supports (Villeneuve et al. 2000; Hanefeld et al. 2009). Immobilization refers to the location or confinement of the enzyme. The selection of the immobilization method should be based on certain parameters, such as global activity of the immobilized derivative, regeneration and inactivation characteristics, cost of the immobilization procedure, toxicity of the immobilization reagents and desired final properties of the immobilized enzyme (Guisán 2006; De Castro et al. 2008; Hanefeld et al. 2009; Meunier et al. 2017).

The use of immobilized enzymes is known to offer several advantages compared to their use in free form (Sheldon 2007). In addition to a more convenient approach of handling the enzyme, it provides ease of separation from the reaction mixture and enables its use in repeated and continuous runs (Sheldon 2007; Adlercreutz 2013). Furthermore, immobilization is often linked to enhanced thermal stability and it is essential to perform on non-conventional medium reactions (Sheldon 2007; Adlercreutz 2013; Sheldon and Van Pelt 2013). Enzymes have been immobilized by different techniques, including adsorption, covalent attachment or entrapment (Ansari and Husain 2012; Meunier et al. 2017) on several matrixes. The factors involved on the features are directly related to the potential industrial applications, such as the required mechanical strength, chemical and physical stability, hydrophobic character, enzyme load capacity and cost for each application (Adlercreutz 2013; Es et al. 2015). In addition, the properties of supported enzyme preparations are governed by the properties of both the enzyme and the carrier material. The interaction between the two provides an immobilized enzyme with specific chemical, biochemical, mechanical and kinetic properties (Tacias-Pascacio et al. 2017). A wide

variety of natural, organic or inorganic synthetic materials with different characteristics such as size, shape, porosity, hydrophobicity and density have been tested for lipase immobilization (De Castro et al. 2008, 2010; Cazaban et al. 2017). However, comparative studies show significant differences in the performance of immobilized lipases in different substrates and show that the immobilization of lipases is still a complex challenge, since the extent of immobilization depends on the structure of the enzyme, on the immobilization method and on the type of support (De Castro et al. 2008, 2010; Tacias-Pascacio et al. 2017). In many cases, supports that provide high activity and stability of the enzyme have serious limitations of mechanical resistance and pressure drop, which make them unviable for use in some types of reactors (Yahya et al. 1998; Poppe et al. 2015a). Supports with high mechanical strength are desirable particularly in systems with agitation. The presence of solvents may require supports with high chemical resistance. The application of substrates with adequate internal geometries is quite attractive, since it reduces the diffusional limitation effects of the substrates to the active sites of the immobilized enzyme (Zanin and Moraes 2014; Poppe et al. 2015a). Considering that the loss of activity is a matter of time, the support must also be easily regenerated and reused (Yahya et al. 1998; De Castro et al. 2008).

The most recommended supports for immobilization of lipases for their subsequent use as a biocatalyst in biodiesel synthesis are hydrophobic, microporous styrene-divinylbenzene copolymer (STY-DVB) (Dizge et al. 2009), polypropylene (Bosley and Moore 1994), Sepharose (Villeneuve et al 2000), silica functionalized with organosilanes (Cazaban et al. 2017) matrices, among others. The amount of enzyme adsorbed on such supports is generally high, and an increase in adsorption is usually followed by increase in observed enzyme activities. Hydrophilic supports tend to compete with the enzyme for the water available in the reaction. When the lipase and the support are fully hydrated, the hydrophilic support leads to a higher water concentration in the environment of the enzyme favoring hydrolytic reactions (Villeneuve et al. 2000). Another benefit of the hydrophobic matrix is its ability to limit the adsorption of glycerol as byproduct formed during the transesterification reaction (Lima et al. 2015). It has become common knowledge that glycerol has a negative effect on lipase activity and stability by reducing the diffusion of the hydrophobic substrate to the active site of the lipase (Dossat et al. 1999). This undesirable effect of glycerol greatly shortens the operational stability of the catalyst and consequently influences the economic viability of the process. Since the glycerol issue could increase the production cost and affect the process design, it needs to be taken into account when immobilized lipases are used for large scale biodiesel production. Several articles and reports available in the literature deal with different lipase immobilization techniques, characterization of the activated complexes and applications in reactions in non-aqueous medium, according to the reviews published by Adlercreutz (2013), Es et al. (2015), Hanefeld et al. (2009), Villeneuve et al. (2000) and textbooks (Guisán 2006; De Castro et al. 2008; Zanin and Moraes 2014).

10.3 Production of Biodiesel Catalyzed by Lipases

Biodiesel is produced by a sequence of reversible reactions: (i) from triacylglycerol (TAG) to diacylglycerol (DAG), (ii) from DAG to monoacylglycerol (MAG), and (iii) from MAG to alkyl ester (biodiesel), generating glycerol as a co-product. At each step of reaction, an alkyl ester molecule is released. The stoichiometric ratio of the reaction corresponds to 1 mol of TAG for 3 mols of alcohol. Empirically, it has been proven that alcohol excess usually ensures the equilibrium in the direction of product formation (Knothe et al. 2010). Lee JH et al. (2013) propose that the enzymatic biodiesel production consists of three steps. The first is the rate-determining step, in which interfacial reaction occurs due to the insolubility of low-chain alcohols and oils. As the reaction progresses, the products (fatty acid alkyl esters and glycerol) act the emulsifiers and the interface disappears, becoming a homogeneous phase, which increases the reaction rate. Lastly, the glycerol concentration builds up, the alcohol moves to the glycerol layer and the rate of reaction is again decreased. The lipase-mediated transesterification reaction involves the catalytic triad of the enzyme, consisted of aspartic or glutamic acids, histidine and serine amino acids. In the first stage of the reaction, the hydroxyl group (alcohol) of the serine acts as nucleophile by the action of histidine that attracts the proton of the hydroxyl forming an oxyanion. The oxyanion of the serine attacks the carbon of a carbonyl of the substrate, forming the tetrahedral intermediate 1. Then the electrons of the oxyanion are pushed back to the carbonyl carbon, and the proton in the histidine fraction is transferred to the diacylglycerol, which is subsequently released. The formed serine ester reacts with the alcohol to complete the transesterification. The histidine nitrogen removes the hydrogen from the alcohol molecule to form the alkyl oxide anion. Such structure attacks the carbonyl carbon, and the intermediate oxyanion is stabilized by a hydrogen bond (tetrahedral intermediate 2). The following step is composed by an electron push back to the carbonyl carbon, and the free fatty acid is formed. Serine oxygen recovers the hydrogen located in the histidine to reestablish the hydrogen bond network. Aspartic acid serves to extract the positive charge of histidine during the times when it is fully protonated (Jegannathan et al. 2008).

Most of the research published in the field uses methanol as acyl group acceptor. Due to the high hydrophilicity of the C1-alcohol, the reactions are usually carried out in medium containing organic solvent, in generally within high proportions (of the order of 50–90% relative to the total mass of reagents involved). From the economic point of view, methanol also stands out as one of the cheapest alcohols. With the replacement of methanol by other alcohols with, for example, ethanol, propanol and butanol, the use of solvents becomes unnecessary, which can make the biodiesel production more feasible, reducing solvent costs and distillation steps, reducing the energy consumption (Iso et al. 2001). However, in this case, the biodiesel yield may be lower and the reaction time longer (Mittelbach 1990). The use of ethanol as acyl acceptor has increased significantly within the last decades. The production of fatty acid ethyl esters through the use of bio-ethanol provides a process with little to no dependency of fossil fuels depending on the energy requirements of the process.

According to Firdaus et al. (2014), biodiesel standards in Brazil and the USA (specification according to ASTM D6751) are applicable for both fatty acid methyl esters and fatty acid ethyl esters (FAME and FAEE, respectively). The replacement of the methyl route by ethanol in Brazil is quite attractive due to the great agricultural capacity and the already consolidated ethanol industry in the country, currently the second largest producer in the world (Reis and Hu 2017). Even considering some technical disadvantages in production (slower reaction, higher alcohol consumption and greater difficulty of separation), ethyl biodiesel has slightly higher viscosity than methyl biodiesel, promoting greater lubricity in relation to methyl biodiesel. Furthermore, FAEE usually presents lower opacity and better burning qualities than FAME, as well as requiring lower combustion temperatures, potentially reducing NO_x and CO emissions (Knothe et al. 2010; Firdaus et al. 2014). The source of lipid feedstock depends greatly on the geographic scale of production. In conditions which oils are inserted in the food chain, local policies tend to lead to the search for alternative raw materials (non-edible feedstocks) for the production of biodiesel, such as perennial crops oils (Ramos et al. 2009; Perez et al. 2014) and microbial oils (Patel et al. 2017; Talebi et al. 2013).

10.4 Main Aspects of Lipase Utilization Methods for Biodiesel Production

10.4.1 Immobilized Lipases

The first report on the enzymatic production of alkyl esters was published by Mittelbach (1990) using sunflower oil and different alcohols in the presence and absence of solvent (petroleum ether). Among the tested lipases, only immobilized enzymes (Lipase SP 382, Lipozyme® RM-IM) showed satisfactory results even in the absence of solvent, while free lipases did not provide acceptable conversions. In the following years, most biodiesel production published references employed lipase in its immobilized form. However, in the last decade efforts have been directed towards the application of lipases in their free form, which will be discussed later in this chapter. Immobilized lipases can be obtained by industrial companies, for example as the most prevalent in the current market: *Candida antarctica* lipase B (Novozym® 435) and lipase from *Thermomyces lanuginosus* (Lipozyme® TL-IM), *Rhizomucor miehei* (Lipozyme® RM-IM) and *Burkholderia cepacia* (Lipase PS-IM). Table 10.1 shows some biodiesel production data using commercial immobilized lipases.

Among the first reports, an investigation on the transesterification reaction of vegetable oils and beef tallow using primary and secondary alcohols and various lipases was reported with promising results (Nelson et al. 1996). The results with highest conversion yields were obtained from the alcoholysis of tallow oil with methanol and ethanol catalyzed by Lipozyme RM-IM®. The yields obtained with hydrated ethanol were higher than that of anhydrous ethanol. Using secondary alcohols, Novozym®

Table 10.1 Examples of biodiesel production by transesterification process catalyzed by commercial lipase immobilized

Lipase	Feedstock	Acyl acceptor	Solvent	Yield (%)	Reference
Lipase SP 382, Lipozyme® RM-IM	Sunflower oil	Primary and secundary alcohols	Petroleum ether	≥90	Mittelbach (1990)
Lipozyme IM60 (Lipozyme® RM-IM)	Beef tallow	Ethanol and isobutanol	*n*-Hexane	≥98	Nelson et al. (1996)
Novozym® 435	Mixture of soybean and rapeseed oils	Methanol	Free	≈98	Shimada et al. (1999)
Novozym® 435	Soybean oil	Metyl acetate	Free	92	Du et al. (2004)
Novozym® 435, Lipozyme® RM-IM	Palm, cashew nut, papaya, rambutan oils	Methanol	Free	≥80	Winayanuwattikun et al. (2008)
Lipozyme® TL-IM	Waste cooking oil	Methanol	*tert*-Butanol	92	Wang et al. (2008)
Novozym® 435	Waste cooking palm oil	Methanol	*tert*-Butanol	≈80	Halim et al. (2009)
Novozym® 435	Soybean oil	Methanol	*tert*-Butanol	97	Zheng et al. (2009)
Lipozyme® TL-IM	Crude palm oil	Methanol	*tert*-Butanol	≈96	Sim et al. (2010)
Novozym® 435, Lipozyme® TL-IM, Lipozyme® RM-IM	Canola oil	Methanol	Free	≈93	Yücel and Demir (2012)
Novozym® 435, Lipozyme® TL-IM, Lipase PS-IM	Andiroba, babassu, jatropha, palm oils	Ethanol	Free	≈100	Tiosso et al. (2014)
Novozym® 435	Waste frying oil	Methanol	*tert*-Butanol	>80	Azócar et al. (2014)
Novozym® 435	Microbial oil from *Mucor circinelloides*	Ethanol	Isooctane	≈93	Carvalho et al. (2015b)
Lipozyme® RM-IM	Spent coffee ground oil	Ethanol	Hexane	≈92	Caetano et al. (2017)

435 was the most efficient. The optimization of the reaction of transesterification of soybean and rapeseed oils with methanol using Novozym® 435 has also been reported (Shimada et al. 1999). The Novozym® 435-catalyzed reaction was performed with three equivalents of methanol needed for each oil equivalent, but it was noticed that addition of increased methanol molar equivalent, i.e., greater than 1.5, deactivated the enzyme at the start of the process. Therefore reactions were performed with the addition of alcohol in fed batch system, yielding an overall yield of 98.4% methyl esters. Another approach to reduce the negative effect of methanol on enzyme activity has been reported to be related to the methanol replacement for methyl acetate as acyl group acceptor, obtaining yields greater than 92% on methyl esters in a 12:

1 molar ratio of acetate to oil (Du et al. 2004). The main advantage of this process was the non-formation of glycerol as a by-product, which as previously mentioned have inhibitory effects on lipase activity.

Residual oils have also been employed with relative success as feedstocks in biodiesel synthesis. The transesterification of corn oil with methanol catalyzed by Lipozyme® TL-IM was conducted in the presence of *tert*-butanol to reduce inactivation of the biocatalyst resulting in conversion into methyl esters 92.0% after 12 h of reaction (Wang et al. 2008). The synthesis of methyl esters using waste or waste frying cooking palm oil was catalyzed by Novozym® 435 and the maximum conversion achieved was approximately 80% (Halim et al. 2009; Azócar et al. 2014). An extensive work involving the sorting of vegetable oils was carried out by Winayanuwattikun et al. (2008) using the methyl route and Novozym® 435, and Lipozyme® RM-IM lipases. Among the 27 oils investigated, only palm, cashew nut, and rambutan oils provided biodiesel samples with suitable properties to be used as biofuel. More recently, it has been reported yields of ethyl esters near 100% from non-edible vegetable oils in a solvent-free system. Better performances were obtained with PS-IM and Novozym® 435 lipases (Tiosso et al. 2014). It has been observed that samples from the biodiesel and jatropha babassu oils presented viscosity in accordance with those values predicted by the technical standards of ASTM D6751 (1.9–6.0 mm^2/s) (Tiosso et al. 2014).

Quality of the biodiesel obtained by enzymatic route depends on the reaction system to be used (type of oil and acyl acceptor), origin of the enzymatic preparation, immobilizing matrix, among others. In some cases, the process presents technical potential, but the product does not always meet the specifications set by ASTM D6751 and EN 14214. However, in most cases the immobilized system maintained satisfactory activity over several recycles with a slight decrease on the biodiesel yields. Some examples of biodiesel synthesis using lipase immobilized on different supports and procedures are presented in Table 10.2.

The physical properties of high tensile strengths silica carriers make them robust and resistant to breakage through mechanical shear in the reactor running, thus producing a product suitable for multiple reuses (Cazaban et al. 2017). Another type of support that has been the focus of researchers is silica xerogel, obtained by sol-gel technique involving hydrolysis and condensation of $Si(OR)_2$ in the presence of a trialkoxysilane (Pierre 2004; Kandimalla et al. 2006). In such kind of functionalization, a material of the type $xSiO_2 \cdot SiO_{3/2}–(CH_2)_n–L$ is obtained, in conditions in which it is possible to control the density of ligand anchored to the silica surface. This technique has been mainly used for the immobilization of lipase to present good retention of activity (Reetz et al. 1996; Meunier and Legge 2012). The combination of inorganic and organic components, constitute also an alternative approach to produce matrixes having specific features for a specific application or supports with properties that cannot be found in conventional materials (Samuneva et al. 2008; Pandey and Mishra 2011). For example, the biocompatibility of the silane precursor with tetraethoxysilane (TEOS) and polyvinyl alcohol has been successfully tested for the immobilization of different sources of lipase, such as porcine pancreatic (Paula et al. 2007), *Pseudomonas fluorescens* (Moreira et al. 2007) and *Burkholderia cepa-*

Table 10.2 Examples of biodiesel production by lipase immobilized on different supports and procedures

Lipase	Support	Immobilization method	Feedstock	Acyl acceptor	Solvent	Yield (%)	Reference
Pseudomonas fluorescens (Lipase AK)	Kaolinite particles (Toyonite 200-M)	Physical adsorption	Safflower oil	1-Propanol	Free	100	Iso et al. (2001)
Chromobacterium viscosum	Celite-545 particles	Physical adsorption	Jatropha oil	Ethanol	Free	92	Shah et al. (2004)
P. fluorescens (Lipase AK)	Epoxy SiO_2-PVA	Covalent	Palm oil	Ethanol	Free	≈98	Moreira et al. (2007)
Porcine pancreatic	SiO_2-PVA particles activated with glutaraldehyde	Covalent	Babassu oil	Ethanol, 1-propanol, 1-butanol	Free	75–95	Paula et al. (2007)
P. fluorescens (Lipase AK)	Macroporous polypropylene particles	Physical adsorption	Soybean oil	Methanol	Free	98	Salis et al. (2008)
Burkholderia cepacia (Lipase PS)	SiO_2-PVA particles activated with epichlorohydrin	Covalent	Andiroba, babassu, jatropha, macaw palm, palm oils, beef tallow	Ethanol	Free	92–100	Carvalho et al. (2013)
Rhizomucor miehei (Palatase®)	Liposome nanospheres covered by porous silica shell	Encapsulation	Triolein	Methanol	Free	≈90	Macario et al. (2013)

(continued)

Table 10.2 (continued)

Lipase	Support	Immobilization method	Feedstock	Acyl acceptor	Solvent	Yield (%)	Reference
Pseudomonas mendocina cells	Fe_3O_4-chitosan microspheres	Covalent	Soybean oil	Methanol	Free	≈87	Chen et al. (2016)
B. cepacian (Lipase PS)	Silicone microspheres	Encapsulation	Soybean oil	Ethanol	*n*-Hexane	≈95	Ma et al. (2016)
B. cepacia (Lipase PS) *Rhizopus oryzae*	PEI microcapsules modified by carbon nanotubes	Encapsulation	Soybean oil	Ethanol	Free	>90%	Su et al. (2016)
B. cepacian (Lipase PS)	Magnetic carbon nanotubes modified by PAMAM dendrimers	Covalent	Soybean oil	Methanol	Free	≈93	Fan et al. (2017)

SiO_2-PVA silica-polyvinyl alcohol; *PAMAM* polyamidoamine; *PEI* polyethyleneimine

cia (Carvalho et al. 2013). In these cases, conversions into biodiesel above 95% were found using non-edible oils as feedstocks.

Lipase from *Chromobacterium viscosum* immobilized on Celite-545 particles by physical adsorption was used as a biocatalyst in the ethanolysis of Jatropha oil resulting in a conversion of 92% (Shah et al. 2004). Also via physical adsorption process, lipase from *P. fluorescens* was immobilized on kaolinite particles and the biocatalyst was used in the transesterification of safflower oil with 1-propanol. The authors obtained 100% oil conversion after 10 h of reaction. Salis et al. (2008) performed immobilization of *P. fluorescens* on macroporous polypropylene particles. By using a biocatalyst for the transesterification of soybean oil with methanol, the authors obtained high FAME yields (98%) in 70 h. It has been reported that using octyl functionalized silica, glycerol was not absorbed on the support surface (Lima et al 2015). This proves to be an important advantage for batch and continuous use, since glycerol accumulation on the surface is a concern for mass transfer and enzymatic activities (Dossat et al. 1999; Xu et al. 2011; Costa-Silva et al. 2016).

The literature also points to a variety of innovative technologies for lipase immobilization to mediate the synthesis of biodiesel. With larger specific area, less diffusion limitation, and many other advantages, nanostructured materials (nanoparticles, nanotubes and nanofibrous membrane) have been used as novel and promise support (Shuai et al. 2017). The immobilization of both *R. oryzae* and *B. cepacia* lipases in polyethyleneimine (PEI) microcapsules was assessed by Su et al. (2016). The authors also evaluated the modification of the carbon nanotubes with microcapsules with the aim of improving the enzymatic activity by increasing the emulsion interface of oil and water to reduce toxicity of PEI on the biomolecule (reduction of positive charges on the polymer). The results showed a support with high stability and retention of the enzyme, which may be located mainly in the microcapsule wall. Evaluating other immobilization technique using lipase from *B. cepacia* as an enzyme model, carbon nanotubes with magnetic properties modified by polyamidoamine dendrimers were synthesized (Fan et al. 2017). The modification aimed at increasing the effective loading of the enzyme, among other factors. According to the authors, the immobilization technique enhanced 17 times the catalytic activity compared to free enzyme, and made it more stable to pH and temperature variations. The biocatalyst also presented catalytic activity retention of about 90% after 20 cycles and easy removal from the reaction medium by using a magnetic field.

The amphiphilic liposome composition also permits the formation of biocompatible structures, containing inside an aqueous microenvironment, which is suitable for the encapsulation of a variety of hydrophilic substances, including enzymes. For example, in their study, Macario et al. (2013) found that the immobilization of lipase on liposome nanospheres, the enzyme remained stable for five consecutive reaction cycles.

Various other alternative forms of immobilization have gained increasing attention being developed to ensure better interact with the enzyme reaction medium to increase the interfacial area required for full activity of lipase and facilitate separation of the biocatalyst from the medium. When immobilized, lipase from *B. cepacia* in silicone microspheres, Ma et al. (2016) observed an excellent thermal and mechanical

stability of the biocatalyst. In addition, the biocatalyst was recycle over 15 batch runs while maintaining biodiesel yields greater than 70%. Chen et al. (2016) produced magnetic whole cell biocatalysts constructed by immobilizing *Pseudomonas mendocina* cells into Fe_3O_4-chitosan microspheres to be applied for biodiesel production. A yield of 87.32% was obtained under optimum operating conditions (biocatalyst concentration of 10 wt%, water content of 10 wt%, 35 °C, methanol-to-oil molar ratio of 4:1 and a four-step addition of methanol) for 48 h. The biocatalyst had an excellent reusability and still gave a biodiesel yield of 83.57% after 10 cycles, which was higher than that of Fe_3O_4-uncontained whole cell biocatalysts (74.1%). Moreover, the biocatalyst could be separated and be recycled easily due to their superparamagnetism.

10.4.2 Soluble Lipases

Liquid enzyme formulations are composed of a given enzyme in liquid solution with added stabilizers to prevent denaturation of the biomolecule (for example, glycerol or sorbitol), or additives to prevent microbial growth (e.g., benzoate) (Nielsen et al. 2008). With the use of biocatalyst in soluble form, and to avoid the cost of immobilization procedure on solid supports, some limitations are reduced, such as those related to mass transfer. Furthermore, the use of the enzyme in the soluble form prevents the insertion of a third phase (solid) in the reaction system and recovery (and recycle) is based on amphiphilic property of the biomolecule. Due to being active in the oil and water interface, lipase is found concentrated in the emulsified phase between the ester and glycerol phases, and the products can go through separation by centrifugation or natural gravity phase decantation (Nielsen et al. 2008, 2016; Nielsen and Rancke-Madsen 2011).

Novozymes laboratories initiated the industrial-scale innovation in the use of soluble lipase enzyme instead of immobilized for biodiesel production in 2006 (Nielsen 2014). From the following years until recently, a number of collaborative partnerships among Novozymes, the Danish Advanced Technology Foundation, universities and biodiesel producers aiming not only to develop a competitive and less demanding biocatalyst with respect to raw materials, but also to be effectively used in the production scale. As a result, lipase formulations were developed from engineered variants of *Thermomyces lanuginosus* by Novozymes with patent registration enabling the implementation of the first industrial process for enzymatic production of biodiesel in a refinery located in the United States (Fig. 10.2).

A proof-of-concept transesterification reaction was carried out with soluble lipase batch reactor, with the formation of an oil emulsion with the addition of a small amount of water and alcohol (methanol) and the enzyme under constant stirring. The remainder of the ethanol (a total of 1.4–1.5 molar equivalents of alcohol to fatty acid) was step fed in order to decrease inactivation of the biocatalyst, and the reaction occurred in the temperature range of 35–45 °C for 4–24 h, depending on the amount of enzyme added (Nielsen and Rancke-Madsen 2011; Nielsen 2014). The methyl esters

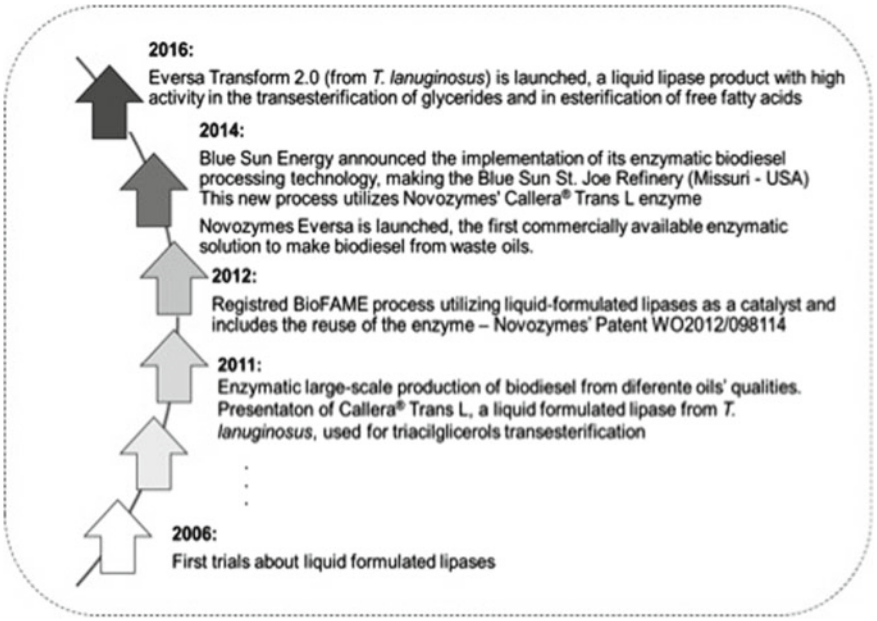

Fig. 10.2 World energy consumption by various sectors for different time period (Adapted and modified from IEO 2017, an open source article)

and glycerol phases were separated by centrifugation and the enzyme at this stage was recycled. Tests conducted in bench level allowed to verify the mode of action and the potential of this type of biocatalyst, as well as the challenges of the process, also describing ways to circumvent the limitations in order to produce a compound, which fit specifications among the specifications of existing standards. Cesarini et al. (2013) were the first group to report high yield conversion (96%) of crude feedstock (non-degummed soybean oil) into fatty acid methyl esters (FAMEs) from with stepwise addition of methanol in the presence of water (3–15%). In this study, the authors used Callera® Trans L and noted the importance of water to maintain the enzyme activity and found that this lipase has a specific mode of action. Evidence was obtained that TAGs are hydrolyzed by Callera Trans L into DAGs, MAGs and FFAs during the first 5 h of reaction for all water concentrations. This hydrolysis process was also favored by the low initial MeOH concentration, added step-wise during the reaction. The release of FAMEs during the first 5 h is probably due to a true transesterification activity of the enzyme, whereas at longer reaction times, when TAGs have almost disappeared, esterification activity is predominant and FAMEs formation derives from the FFAs generated by the complete hydrolysis of TAGs, DAGs and MAGs. This effect was particularly evident at 3–5% water concentrations, where FAMEs production by Callera Trans L was more effective.

In practice, the separation procedure at the end of the reaction typically results in a light ester phase, but the procedure may difficult to obtain a clean glycerine phase and

recovering the emulsified layer containing the enzyme may require energy-intensive separation procedures. A strategy adopted consists in removing only those esters out of the reactor, which results in lower observed losses of catalytic activity, but with progressive accumulation of glycerol, limiting the use of the enzyme only in 3 to 4 batches. With recent advances and the price reduction of the enzymes, the trend therefore is to consider the use of biocatalyst in only one-step in process conducted in a single reactor, in conditions, which ensure the product is within the required specifications (Nielsen et al. 2016). Based on this principle, Nielsen et al. (2016) proposed a process for obtaining biodiesel employing Eversa® Transform, a recent version of Callera® Trans formulation. After initial stage of transesterification, the enzyme was added into the medium containing excess methanol, and NaOH was added to saponify the remaining free fatty acids (FFA). As a result, a high yield of biodiesel to 97% was achieved, with a reduction in FFA levels (<0.25%) and MAG (0.9–0.6%). In addition, phase separation was facilitated by the lack of phase between the ester phase and the heavy phase. Yet according to the authors, this process has been expanded to production scale operation. However, up to date there are no reports of using Eversa® Transform 2.0 formulation on other scales.

10.4.3 Mycellium-Bound Lipase (Whole Cells)

The first example of using intact cells (whole cells) as biocatalyst in biodiesel production has been reported by Ban et al. (2001), who used immobilized cells of *Rhizopus oryzae* for transesterification of soybean oil with methanol. Since then, extensive studies have been reported the use of whole cells as a biocatalysts in order to reduce the cost of biodiesel production as reviewed by Fukuda et al. (2008) and Cortez et al. (2017). Whole cells have attracted considerable attention, mainly because it avoids the added costs of purifying enzymes and further separations. The whole cell acts as support, avoiding the steps of extraction, isolation and purification of enzymes, which are typically labor intensive and contribute to make the process cost-inhibitive. Moreover, the natural environment of intact cells ensures the stability of the enzyme because its optimal spatial location remains intact (Milner and Maguire 2012; Cortez et al. 2017). However, whole cells have low operational stability in solvent free systems (Li et al. 2008), a condition which can be modified with cell immobilization. In addition to promoting greater stability, immobilization facilitates the handling and the separation of the reaction medium cells (Perkins et al. 2015). The cell immobilization procedure occurs as a natural phenomenon or by artificial procedures following the widely used procedures for enzyme immobilization (adsorption, covalent binding, cross-linking and entrapment or encapsulation), but with some criteria imposed by cell morphology (Cortez et al. 2017). The cells are usually treated before or after the immobilization procedure to lose the multiplication capacity (viability), but with the assurance that the enzyme system remains stable and active.

A growing number of studies published in the literature on the use of whole cells from filamentous fungi immobilized in the production of biodiesel is found

throughout the years (Ban et al. 2001; Xiao et al. 2010; Andrade et al. 2012; Carvalho et al. 2015a; Soares et al. 2017). Despite by the fact that whole cells reduce the number of steps to obtain a purified biocatalyst, causing a reduction of the final cost of the product, some disadvantages must be taken into consideration. Cell immobilization makes the synthesis of biodiesel slower than the isolated enzyme-based process (Robles-Medina et al. 2009). In this regard, various studies are being performed aiming at enhancing the operational stability of whole cells in order to reduce the deactivation associated to mycelium-bound lipases caused by the excess methanol in the reaction medium. This, in addition to proposing new process strategies aimed at increasing conversion efficiency and productivity gains has been the key drivers of investigations in this research field. Table 10.3 shows some examples of biodiesel production catalyzed by immobilized whole cells of filamentous fungi.

It is clearly seen that the majority of published studies favors the use of whole cells of *R. oryzae* immobilized on polyurethane foam particles due to the catalytic efficiency of this lipase, with the operational simplicity of immobilization technique and support characteristics (inert material with good mechanical properties, low cost, high porosity and surface adsorption). In this case, the immobilization is by entrapment into preformed porous matrix, characterized by the diffusion of cells (hyphae) through the pores and surface adhesion (Cortez et al. 2017). Table 10.3 also presents strategies used to favor the production of biodiesel by transesterification using whole cells.

10.4.4 Fermented Solids with Lipase Activity

The use of fermented solids containing lipase is also low-cost alternative to reduce biodiesel production costs (Christopher et al. 2014). In this case, the producing organisms can be grown on low cost substrates such as agro-industrial residues maintaining low moisture content. At the end of cultivation, the fermented solid is dried and dilapidated to remove fatty compounds deriving from the fermentation and then used directly as biocatalyst (Fernandes et al. 2007; Salum et al. 2010; Aguieiras et al. 2014). The first work using this type of biocatalyst was reported by Fernandes et al. (2007). In this study, the authors described promising results using the freeze-dried fermented solid (corn bran) containing *Burkholderia cepacia* LTEB11 for transesterification of corn oil with ethanol as acylant agent and ester conversions were in the range of 83–95% after 120 h, depending on the experimental conditions. Subsequently, Salum et al. (2010) produced a solid fermented lipase from the same microorganism (*B. cepacia* LTEB11) in sugarcane bagasse and sunflower seed meal to catalyze the biodiesel synthesis in a packed-bed reactor running on substrate based on soybean oil. Their results showed a high conversion of 95% after 46 h at 50 °C, with an ethanol-to-oil molar ratio of 3:1. Another specie of *Burkholderia* genus (*B. contaminas*) was used as lipase source also in a form of fermented solid (sugarcane bagasse) to catalyze biodiesel production by solvent-free ethanolysis of palm oil (Galeano et al. 2017). By using a packed-bed reactor in batch mode with

Table 10.3 Examples of biodiesel production by whole cells immobilized of filamentous fungi (mycelium-bound lipase)

Filamentous fungi	Strategies	Results	Reference
R. oryzae	Addition of substrate-related compounds to the culture medium; Stepwise additions of methanol, in the presence of 15% water	Olive oil was as the most suitable compound to enhance intracellular methanolysis activity Methyl esters (MEs) content in the reaction mixture reached 90%	Ban et al. (2001)
R. oryzae	Addition of ionic liquid to the reaction medium; Reuse of biocatalyst;	Yields higher than 90% and decrease to 60% from the second recycle	Arai et al. (2010)
R. oryzae	Oil degumming and use of hexane as solvent	Yield of 78% after 73 h of reaction	Ganesan et al. (2012)
R. oryzae, *M. circinelloides*, *P. citrinum*	Screening of lipase-producing fungus isolated from different sources and immobilization and utilization of the whole cells for biodiesel production by transesterification process	The highest performance was attained by *M. circinelloides* immobilized on polyurethane foam particles, giving 83.22 ± 3.68% ester yield in less than 96 h reaction	Andrade et al. (2012)
A. nominus	Addition of solvent (*tert*-butanol) in the reaction medium; Reuse of the biocatalyst	Addition of the solvent descrease the enzyme inactivation and yield of 95.3% in esters was obtained after 40 h reaction	Talukder et al. (2013)
R. oryzae	Packed bed reactor running on batch mode with substrate recycle with periodic feeding of methanol	Conversions were maintaining in 80% for four consecutive cycles for 200 h	Kyeong and Yeom (2014)
R. oryzae	Two steps reaction: previous hydrolysis of the vegetable oil following the esterification of the free fatty acids	Yields in methyl esters 88.6%, after 42 h with 79% retention of the activity after six recycles.	Zhou et al. (2015)

(continued)

Table 10.3 (continued)

Filamentous fungi	Strategies	Results	Reference
M. circinelloides	Screening of non-edible vegetable oils	High yields were obtained with coconut oil (97%) and macaw palm oil (95%)	Carvalho et al. (2015a)
M. circinelloides	Assessment of polyurethane foams synthetized with different types of polyol to be used as a low-cost support to immobilize whole cells	The type of polyol influenced the the pore diameter, water sorption and solvent absorption; Hydrophobic and hydrophilic character of the matrix influenced the attachment of the microorganism and substrate transfers;Ethyl esters varied from 60 to 90% at 120 h	Souza et al. (2017)

recirculation in a closed-loop system and stepwise ethanol addition (alcohol: oil ratio of 5.5:1), a conversion of 89% was attained after 30 h.

Using a fermented solid composed by *Rhizopus microsporus* in a mixture of sugarcane bagasse and sunflower seed meal, Zago et al. (2014) reported 91% conversion of corn oil into ethyl esters in the presence of *n*-heptane, at 48 h in shake flasks. More recently, a promising simultaneous esterification/transesterification method for FAEE production from macaw palm oil containing high acid level was proposed by Aguieiras et al. 2017 using fermented solid *Rhizomucor miehei* on dry babassu cake. The biocatalyst was able to convert oils with different acidities into ethyl esters (biodiesel) in a single reaction step. FAEE content above 85% was achieved at 96 h of reaction with enzyme loading of 13 U per g of oil, 120 mmol of hydrous ethanol (95% ethanol and 5% water)/ 20 mmol of oil (molar ratio ethanol:oil of 6:1), at 40 °C. After two consecutive enzymatic reactions, 90.8 wt% FAEE content was obtained. Although the composition of the final product did not meet the required quality to be used as a fuel, according to the authors the process has potential to decrease the costs of enzymatic biodiesel production.

10.5 Lipase-Catalyzed Production of Biodiesel in Continuous Operations

The transesterification of vegetable oils is substantially faster and more economically viable in continuous reactors than in batch reactors, even considering the higher ini-

Table 10.4 Enzymatic transesterification for biodiesel production under continuous flow

Lipase	Feedstock	Acyl acceptor	Solvent	Reference
Novozym® 435	Residual oil	Methanol	Free	Shimada et al. (2002)
B. cepacian	Residual oil	Ethanol	Free	Hsu et al. (2004)
Immobilized Candida sp. in cotton membrane	Vegetable oil Residual oil	Methanol	Éter de petróleo/água	Nie et al. (2006)
Novozym® 435	Cotton oil	Methanol	*tert*-Butanol	Royon et al. (2007)
Novozym® 435	Soybean oil	Methanol	*n*-Hexane *tert*-Butanol	Shaw et al. (2008)
Novozym® 435	Waste cooking palm oil	Methanol	*tert*-Butanol	Halim et al. (2009)
Novozym® 435	Sunflower oil	Methanol	Free	Ognjanovic et al. (2009)
Novozym® 435	Soybean oil	Iso-propanol	Free	Chang et al. (2009)
Novozym® 435	Soybean oil	Methanol	*tert*-Butanol	Chen et al. (2011)
Lipase *P. fluorescens* imobilizada em SiO_2-PVA	Palm oil	Ethanol	*tert*-Butanol	Dors et al. (2012)
Lipase *B. cepacia* imobilizada em SiO_2-PVA	Macaw palm oil	Ethanol	Free	Costa-Silva et al. (2014)
Lipase *B. cepacia* imobilizada em SiO_2-PVA	Babassu oil	Ethanol	Free	Simões et al. (2015)
Whole cells of *M. circinelloides* immobilized	Coconut oil	Ethanol	*tert*-Butanol	Carvalho et al. (2015a)

tial capital expenditures (Chisti 2006; Zanin and Moraes 2014; Poppe et al. 2015a). In this sense, the use of continuous reactors has been widely reported in the literature due to its advantages, such as cost and volumetric productivity, which can be adjusted according to the operating levels. In addition, greater amount of biodiesel per unit volume can be obtained and easier control of reaction conditions in terms of optimizing the product quality (Poppe et al. 2015a; Christopher et al. 2014; Meunier et al. 2017). Table 10.4 lists some published references on the production of biodiesel reactors operating in continuous flow.

Nie et al. (2006) conducted experiments aimed at optimizing the methanolysis of vegetable oils and residual oil mediate by *Candida* sp. 99–125 lipase immobilized on cotton membrane under continuous runs. A three-step transesterification reactions

were carried out by using reactors in series, in which each reactor was fed with methanol and at the same time an apparatus facility (hydrocyclone) was used to on-line separate the formed byproduct (glycerol). The conversion of the continuous process was 90% for vegetable oil and 92% for the residual oil. The operational stability of the immobilized lipase was reported to be close to optimum values for over 20 days.

Royon et al. (2007) reported the production of biodiesel through methanolysis of cottonseed oil using Novozym® 435 catalyst and *tert*–butanol as solvent. Yields as high as 95% were obtained using a continuous packed bed reactor (6 × 180 mm) with a flow rate of 9.6 mL h^{-1}. The system operated continuously for over 500 h without showing significant reduction in the yield of esters.

The continuous process of methanolysis of soybean oil catalyzed by Novozym® 435 in the presence of co-solvent (mixture of *n*-hexane: *tert*-butanol) was investigated by Shaw et al. (2008). The packed bed reactor consisted of a stainless-steel tube (25 × 250 mm) packed with 1 g of enzyme. Response surface methodology was used to evaluate the effects of parameters on the reaction conversion. According to the authors, the best conditions were: temperature 52 °C, flow 0.1 mL min^{-1} and molar ratio of methanol to soybean oil = 4.3:1. The predicted value for the conversion was 74.2% and the experimental value of 75.2%. Halim et al. (2009) studied the methanolysis of residual palm oil catalyzed by Novozym® 435 to determine an optimal continuous procedure in a packed bed reactor to investigate the possibility of larger scale production. The two columns with dimensions of 180 mm × 10 mm were operated in an up-flow regime. Two important process variables were analyzed, height of the bed and substrate flow rate. The optimum conditions for transesterification were as follows: 105.3 mm bed height and volumetric flow rate 0.57 mL·min^{-1} of substrate, which promoted FAME yield of 79.0%, having a predicted value of 80.3% through statistical design of experiments.

Ognjanovic et al. (2009) demonstrated the possibility of using Novozym® 435 in biodiesel synthesis from sunflower oil and methanol in a solvent free system in a packed bed reactor, obtaining a conversion of 93.6% for a special-time of 8 h.

The production of biodiesel in a packed bed reactor (stainless-steel, 250 mm × 46 mm) catalyzed by Novozym® 435 lipase using soybean oil with isopropanol in a solvent-free system was investigated by Chang et al. (2009). To determine the optimal conditions, a statistical experiment design was used, and under optimized conditions (flow rate = 0.10 mL min^{-1}, temperature = 51.5 °C and molar ratio of 1:4.14 (oil: alcohol), 1.7 g of lipase) concentrations as high as 75% isopropyl esters were obtained. The lipase showed excellent operational stability allowing operating the system for seven days without reduction in the concentrations of isopropyl esters. Chen et al. (2011) also employed the statistical design as a tool to establish the conditions for biodiesel synthesis from soybean oil and methanol in the presence of *tert*-butanol (32.5 wt% relative to oil) mediated by Novozym® 435. The analysis of results was made by response surface methodology, investigating the influence of the independent variables (temperature of reaction, volumetric flow rate and molar ratio of the substrate) in the response variable (mol conversion). The optimal conditions for a packed bed reactor operating in continuous mode provided maximum conversion

of 83.31% with a volumetric flow rate of 0.1 mL min^{-1}, 52.1 °C and molar ratio of 4:1 (ethanol:oil). The bed reactor operated for more than 30 days without significant loss in the substrate conversion.

Dors et al. (2012) evaluated the continuous alcoholysis reaction mediated by lipase of palm oil with ethanol in presence and absence of solvent (*tert*-butanol), using *P. fluorescens* immobilized in a hybrid matrix of polysiloxane-polyvinyl alcohol (SiO_2-PVA) in a packed bed reactor. The best performance was found for the reactor running in the presence of *tert*-butanol, which resulted in a stable operating system and an average yield of 87.6 ± 2.5%. This strategy also gave high biocatalyst operational stability, revealing a half-life of 48 days and an inactivation constant of 0.6×10^{-3} h^{-1}. Simões et al. (2015) assessed the transesterification reaction of babassu oil with ethanol mediated by *Burkholderia cepacia* lipase immobilized on SiO_2-PVA composite in a packed bed reactor running in the continuous mode. Experiments were performed in a solvent-free system at 50 °C. The performance of the reactor (14 mm × 210 mm) was evaluated using babassu oil and ethanol at two molar ratios of 1:7 and 1:12, respectively, and operational limits in terms of substrate flow rate were determined. Based on the results obtained, the best reactor performance was achieved for runs in which the oil to alcohol molar ratio of 1:12 was used. Under such condition, and at space time greater than or equal to 11 h, an average transesterification yield of 96.0 ± 0.9% and a productivity of 41.1 ± 1.6 $mg_{ester}\ g^{-1}_{catalyst}\ h^{-1}$ were achieved. This also resulted in biodiesel samples with viscosity values (average 4.3 ± 0.7 $mm^2\ s^{-1}$) complying with the international standard for biodiesel viscosity i.e. ASTM 6751-02 (1.0 < kinematic viscosity of B100 < 6.0 $mm^2\ s^{-1}$).

It is important to highlight that in processes conducted in a continuous flow, the effect of the glycerol, byproduct of the reaction, is more pronounced on the efficiency of the process than in the batch process. Glycerol makes it difficult for the substrate to diffuse onto the lipase molecule, thus reducing reaction efficiency (Shimada et al. 2002). This is due to glycerol being a hydrophilic viscous liquid that easily adsorbs onto the surface of the immobilized enzyme, forming a hydrophilic layer, limiting the enzyme performance in hydrophobic substrates, and consequently impairing the transesterification yield (Hama et al. 2011; Xu et al. 2011). Thus, from the standpoint of planning and operation of an industrial plant, the efficient removal of glycerol may be an obstacle in the implementation of continuously operating reactors (Hama et al. 2011). In order to minimize the negative effects of glycerol different strategies have been investigated (Table 10.5).

Dossat et al. (1999) used silica gel and other adsorbent substances to extract glycerol. Watanabe et al. (2000) developed a continuous methanolysis system in a three columns (15 mm × 80 mm) packed with the immobilized enzyme (Novozym® 435 at 30 °C and flow rate of 6 mL h^{-1}), in which the addition of methanol was made in three steps, one third molar equivalent added to each column. Each column had their effluent treated to remove glycerol, and the subsequent column was fed. The conversions of the vegetable oil into FAMES obtained in each reactor were respectively 33, 66 and 93% and the immobilized lipase was reported to be used by more than 100 days without activity reduction. Li et al. (2006) and Royon et al. (2007) investigated the

Table 10.5 Removal glycerol strategies in processes carried out under continuous flow

Lipase	Glycerol removal Strategy	Results	Reference
Lipozyme® RM-IM	Use of silica gel to adsorb the produced glycerol	In a continuous plug flow reactor the addition of silica resulted in a partitioning of glycerol between silica and support. About 0.05 g/L of glycerol was removed from the reaction medium and this extended the biocatalyst half-life	Dossat et al. (1999)
Novozym® 435	Residual oil methanol	Addition of methanol was made in three steps, one third molar equivalent added to each column. Each column had their effluent treated to remove glycerol, and the subsequent column was fed.	Watanabe et al. (2000)
Novozym® 435	Fluidized bed reactor coupling with a column packed with Lewatit GF202 for continuous glycerol removal	The best performance was obtained by running the reactor with biocatalyst loading of 12% and a space-time of 8 h, attaining an average yield of 98.1% and productivity of 9.9 $mol_{ester}\ g_{cat}^{-1}\ min^{-1}$ System under stable conditions for 30 days without any loss of biocatalyst activity	Fidalgo et al. (2016)
Burkholderia cepacia immobilized on SiO_2-PVA	Two-stage packed-bed reactor incorporating a column with Lewatit GF 202 to remove the glycerol	At space-time of 14 h, a FAEE content of 58.5 ± 0.87 wt% was achieved, corresponding FAEE yields of 97.3 ± 1.9% and productivities of 41.6 ± 1.0 $mg_{ester}\ g_{medium}^{-1}\ h^{-1}$. The immobilized lipase was found to be stable, showing half-life time ($t_{1/2}$) ~ 1540 h	Costa-Silva et al. (2016)
Burkholderia sp. lipase immobilized on alkyl-celite	Series of three packed-bed reactors integrated with glycerol removal devices	In the first column, TAG was converted to FAME, glycerol and intermediate products. The second and third columns continuously converted intermediate products to FAME with the supply of methanol and without the accumulation of glycerol. A biodiesel yield of 85% was achieved	Tran et al. (2016)

(continued)

Table 10.5 (continued)

Lipase	Glycerol removal Strategy	Results	Reference
Burkholderia cepacia immobilized on SiO_2-PVA	Two-stage packed-bed reactor incorporating a column with Lewatit GF 202 to remove the glycerol	Reactors with different height-to-diameter ratios (l/d) were used for continuous runs carried out using an oil-to-ethanol molar ratio of 1:12 at a fixed space-time (14 h). The best performance was attained by using reactor with an l/d of 15, which was further used to perform runs in a two-stage PBR by incorporating a column with cationic resin. The system operation for a space-time of 16 h resulted in a productivity of 37.9 ± 2.4 m_{gester} g_{medium}^{-1} h^{-1} (biodiesel yield = $96.3 \pm 2.1\%$)	Ramos et al. (2017)

addition of organic solvents (*n*-hexane or *tert*-butanol) to ensure homogeneity of the reaction mixture, thereby reducing the viscosity of the reaction medium. Glycerol extracting columns packing with Lewatit GF 202 have been evaluated aiming at maintaining the catalytic activity of the immobilized lipases (commercial available -Novozym 435 or homemade-*B. cepacia* immobilized on SiO_2-PVA) during the continuous ethanolysis of lauric oils for longer periods. In both cases, yields in biodiesel higher than 95% were reached and greater biocatalyst stabilities were verified.

10.6 Strategies for Enzymatic Production of Biodiesel

The importance of biodiesel as a renewable energy source makes its production, especially by enzymatic route, to be constantly challenged to obtain technologies that result in processes that are more efficient. In this respect, several strategies have been and continue to be developed in order to extend the biocatalyst half-lives. Some examples are shown in Table 10.6. The addition of a co-solvent to the reaction medium can assist in reducing the oil viscosity, the dissolution of the formed glycerol, and a significant improve in the mass transfer coefficients. Moreover, the co-solvent may assist in enzyme protection against the inhibitory effects of acyl acceptor and glycerol itself. The major drawbacks are related to process economics. Although the addition of co-solvent (e.g. *tert*-butanol) can contribute to increase the half-life time of the enzyme, the operational cost of this system is high due to the recovery of solvent. In this sense, a solvent-free system is often regards as a better choice for the enzymatic production of biodiesel. As an alternative to the high costs involved in solvents, there are increasing reports on the replacement of organic

solvents with other types of technology that act in a similar fashion (Hama et al. 2013). A common example is the case of ionic liquids. Ha et al. (2007) described the process of methanolysis of soybean oil catalyzed by Novozym® 435. This study gave 80% of conversion after 12 h of reaction at 50 °C, in addition to other benefits derived from the supplementation of the [Emin][TfO] into the reaction medium. Gamba et al. (2008) demonstrated that *B. cepacia* lipase supported in 1-n-butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide ionic liquid (BMI-NTf_2) can be a green alternative method for the production of biodiesel from the alcoholysis of soybean oil. The ionic liquid provides the ideal medium for the stabilization of the enzyme and for the removal of by-product glycerol, with increased biodiesel yields. The transesterification can be performed at room temperature, in the presence of water and without the use of organic solvents. It is also compatible with various alcohols. The biodiesel is separated by simple decantation and the recovered ionic liquid/enzyme catalytic system can be reused at least four times without loss of catalytic activity and selectivity. Another option is to perform the reactions under supercritical or near-critical conditions (Lee et al. 2009; Lee M et al. 2013). Using near-critical CO_2, Lee M et al. (2013) achieved yields close to 100% in short period of time, and reported that the enzyme maintained 90% of its original activity after 20 recycles.

The association of lipases is another approach to improve the biodiesel production process. The synergistic effect based on the specificity of each enzyme has been evaluated by a number of researchers (Hama et al. 2009; Guan et al. 2010; Tongboriboon et al. 2010; Adachi et al. 2011; Lee et al. 2011; Yücel and Demir 2012; Poppe et al. 2015b; Su et al. 2015; Amoah et al. 2016), which inferred that a combination of different types of lipases allows attaining high biodiesel quality (low contents of mono and diacylglycerols). Hama et al. (2009) described a decrease in the amount of accumulated intermediate with the use of mono- and diacylglycerol lipase from *A. oryzae* mixed with 1,3-regiospecific lipase. A biodiesel synthesis from palm oil with ethanol in solvent free medium was carried out in two-stage packed bed reactor using a mixture of two non-specific lipases (*Pseudomonas fluorescens*-AK and *Candida rugosa*-AY) was proposed by Tongboriboon et al. (2010). The highest biodiesel yield (>80%) was achieved using the combination of 50% of each lipase under the following conditions: 2% of water content, 10% enzyme dosage and 1/3 molar ratio of palm oil to ethanol. The mixed lipases could be repeatedly used under the optimal conditions for 15 times with a relative activity higher than 50%.

In another study, 1,3-specific *R. miehei* lipase and mono- and diacylglycerol lipase from *Penicillium cyclopium* were separately expressed in *Pichia pastoris* (Guan et al. 2010). The authors used the free enzymes (extract without purification) for the transesterification of soybean oil with methanol. When used in combination, conversion to biodiesel achieved yields greater than 95%. Adachi et al. (2011) developed an immobilized recombinant *A. oryzae* co-expressing triglyceride and partial glyceride lipases that attained methyl ester yields of 98% with low contents of residual glycerides.

The use of raw materials with a high concentration of free fatty acids usually requires modification of the traditional process. Based on previous studies, Watanabe

Table 10.6 Examples of combined process for the enzymatic production of biodiesel

Strategy	Technology	Lipase	Main characteristics of process and Results	Reference
Co –solvent	Ionic liquid	Novozym® 435	Methanolysis of soybean oil, using [Emim](TfO) ionic liquid as cosolvent. Production yield (80%), eight times higher than conventional solvent-free system and ≈15% higher than system using *tert*-butanol	Ha et al. (2007)
		B. cepacia	The use of lipase supported in BMI·NTf_2 ionic liquid was used to produce biodiesel from soybean oil. The best conversion (96%, 48 h) was obtained using 0.6 g lipase in 8.2 mmol BMI·NTf_2, 70:30 41.2 mmol methanol:water, 3.4 mmol oil, 30 °C	Gamba et al. (2008)
	Supercritical carbon dioxide	*Candida antarctica* lipase B	Methanolysis of olive oil under CO_2 environment, with stepwise addition of alcohol. Biodiesel conversion of ≈99% after 6 h. Mass and thermal transfer was increased, with a faster reaction rate than can occur at atmosphere pressure	Lee et al. (2009)
	Near-critical carbon dioxide	Lipozyme® TL-IM	Methanolysis of canola oil under CO_2 environment, with stepwise addition of alcohol. Conversion of ≈99.9% after 4.5 h. Biodiesel conformed to the fuel standard (EU) even without additional downstream processing, other than glycerol separation and drying	Lee M et al. (2013)
Combined lipases	Lipases with different specificities	*P. fluorescens* lipase and *C. rugosa* lipase	Ethanolysis of palm oil by continuous process on a packed-bed reactor. The mixed lipases could be used in 15 replicates with retained relative activity >50%. In a continuous system using mixed lipases packed in bed reactor, >67% of biodiesel was achieved	Tongboriboon et al. (2010)
		R. oryzae lipase and *C. rugosa* lipase	Methanolysis of soybean oil under CO_2 environment, with stepwise addition of alcohol. Yield conversion of ≈100% at 2 h, and yield of 85% after 20 reuses	Lee et al. (2011)
		Novozym® 435 and Lipozyme® RM-IM	Methanolysis of canola oil, with stepwise addition of alcohol. Ester yields of 97.2%	Yücel and Demir (2012)
		R. oryzae lipase immobilized and Novozym® 435	Ethanolysis of soybean oil, with stepwise addition of alcohol. Yield > 98.3%, with reaction time shortened from 60 to 21 h. Yield retained (≈80%) after 20 cycles in a solvent-free system	Su et al. (2015)
		Novozym® 435 and Lipozyme® TL-IM and Lipozyme® RM-IM	Ethanolysis of olive oil by combi-lipase (mixture of three immobilized lipases). Conversion efficiency of 95% in 18 h, up from 50% for Novozym®435. Biocatalyst systems could be used for at least seven cycles keeping higher than 80% of their initial activities	Poppe et al. (2015b)

(continued)

Table 10.6 (continued)

Strategy	Technology	Lipase	Main characteristics of process and Results	Reference
		Callera® Trans L. and *C. rugosa* lipase	Methanolysis of soybean oil added with phospholipid, with stepwise addition of alcohol. Methyl esters yield more than 95% at 6h. The lipase system could be useful for the conversion of unrefined oils	Amoah et al. (2016)
		Whole cells immobilized of *R. oryzae* and *Aspergillus oryzae*	Methanolysis of soybean oil in packed-bed reactor (PBR) system, with stepwise addition of alcohol. Ten repeated-batch methanolysis cycles in the PBR maintained methyl ester content >90%, with MAG and DAG at 0.08–0.69 and 0.22 1.45%, respectively	Hama et al. (2009)
		Rhizomucor miehei lipase and *Penicillium cyclopium* lipase	Methanolysis of soybean oil, with stepwise addition of alcohol. Conversion of 99.7% after 24 h	Guan et al. (2010)
		Wholes cells immobilized of *A. oryzae*	Methanolysis of soybean oil, with stepwise addition of alcohol. By using recombinant whole-cells, the methyl ester content (98%) was superior to the attained with lipase-mixing and two-step reactions	Adachi et al. (2011)
Two stages reactions	Enzymatic hydro-esterification	*C. rugosa* lipase and Novozym® 435	Hydrolysis of high acid oil followed by esterification of resulting FFA with methanol. After hydrolysis the resulting oil composition was 91.5 wt% FFA, 0.8 wt% TAG, 0.4 wt% DAG. The total esterification reached 99% after 24 h	Watanabe et al. (2007)
		C. rugosa lipase and Novozym® 435	Crude palm oil was first hydrolyzed in the presence of isooctane and the FFA esterified with stoichiometric excess of methanol. Higher biodiesel yield (98%) was attained the single-step Novozym® 435 catalyzed methanolysis (92%) and the solvent-free system with three successive additions of methanol (92%)	Talukder et al. (2010a)
		Vegetable lipase (dormant castor seeds) and fermented solid (*Rhizomucor miehei* lipase)	Hydrolysis of macaw palm oil followed by esterification of released FFA with ethanol. Hydrolysis produced 99.6% of FFA after 6 h. Esterification yielded 91% after 8 h in a solvent-free system	Aguieiras et al. (2014)
	Enzymatic hydrolysis following by chemical esterification	*C. rugosa* lipase immobilized and sulfuric acid	Hydrolysis of soybean oil followed by esterification with methanol. Biodiesel conversion of 99% was obtained after 12 h. The product met the ASTM standard	Ting et al. (2008)
		Physic nut (*Jatropha curcas* L.) lipase niobic acid	Hydrolysis of the physic nut oil, and subsequent esterification of the generated FFA with methanol. The resulting biodiesel was of excellent quality: viscosity (5.5 $mm^2.s^{-1}$), ester content (97.1%), total glycerol (0.09 % w/w), max. methanol (0.05 % w/w)	De Souza et al. (2010)

(continued)

Table 10.6 (continued)

Strategy	Technology	Lipase	Main characteristics of process and Results	Reference
		C. rugosa lipase and Amberlyst® 15	Hydrolysis of waste cooking oil followed by esterification of FA with methanol, in the presence of isooctane. The activity of *C. rugosa* lipase slightly decreased with recycling, and FA yield after five cycles was 92%. Amberlyst 15 was repeatedly used for 100 cycles without loosing its activity	Talukder et al. (2010b)
Two stages reactions	Enzymatic esterification following by alkaline transesterification	Novozym® 435 and alkaline catalyst	Esterification of palm fatty acid distillate with methanol in packed-bed reactor, using a two steps process: first with small excess of methanol and after, with water removal. The resulting product is followed to typical alkaline transesterification step. Both reaction steps in the esterification process are relatively fast, resulting in 15% FFA after column 1 and 5% FFA after column 2. The product can then typically be blended with the deodorized oil and continue through to alkaline transesterification with <0.3% total FFA	Brask et al. (2011)
	Transesterification and esterification	Experimental immobilized lipase (NS 88001) and Novozym® 435	Transesterification of rapessed oil with ethanol, with stepwise addition of alcohol, followed by esterification of resultant product with ethanol (polishing the biodiesel). Separation of the glycerol and water between passes. By using a packed bed reactor system, the second stage brought the biodiesel composition to 'in-spec' levels according to the European specifications. The overall productivity of the proposed two-stage process was 1.56 kg FAEE (kg catalyst)$^{-1}$ h^{-1}	Xu et al. (2012)
	Two stages of transesterification	Lipozyme® RM IM	Butanolysis of low quality rapeseed oil in reaction carried out in two steps: first, glycerol/enzyme is removed and the product washed; second, product and enzyme are returned to the system with addition of more biocatalyst and alcohol. Strategy resulted in a 96.6% butyl ester yield from oil rich in FFA with longer-chain alcohol	Sendzikiene et al. (2016)
Unconventional heating	Reaction assisted by ultrasound	*C. rugosa*	Methanolysis of canola oil, with ultrasonic horn inserted in the reaction mixture to provide sonication. Ultrasonic assisted reaction resulted in complete conversion in 90 min reaction while in its absence, the biodiesel yield was close to 99% after about 24 h. Enhanced mass transfer as a result of cavitation bubble collapse increases the transesterification reaction rates by increasing the collision frequency between reactants	Bhangu et al. (2017)
		Lipozyme RM® IM	Ethanolysis of soybean oil using an ultrasonic water bath equipped with a transducer having longitudinal vibrations. Reaction yield (≈90%) was obtained in a relatively short reaction time (4 h) at mild irradiation power supply (~100 W) and 60°C. The repeated use of the enzyme resulted in a decay in both enzyme activity and product conversion after two cycles	Batistella et al. (2012)

(continued)

Table 10.6 (continued)

Strategy	Technology	Lipase	Main characteristics of process and Results	Reference
		Novozym® 435	Ethanolysis solvent-free of macaw palm oil, using an ultrasonic water bath equipped with a transducer having longitudinal vibrations. Reaction yields ≈70 wt% were obtained at mild irradiation power supply (~132 W) and 65 °C within 30 min. Reutilization of enzyme showed that it may be advantageously employed up to 5 reuse cycles	Michelin et al. (2015)
	Transesterification assisted by microwave	Immobilized *B. cepacia* lipase	Ethanolysis of babassu oil, in reaction performed in a microwave reactor. Under optimal conditions, full conversion was attained at 10 h. The purified product contained no glycerol-bound and properties, such as specific gravity and viscosity, are in accordance with standard fuel specifications	Da Rós et al. (2014)
		Lipase PS immobilized	Ethanolysis of Jatropha oil in a solvent-free system, in reaction performed in a microwave reactor. Ethyl ester yields 98.3% were attained at 72 h of reaction. The operational stability of immobilized lipase PS was determined in repeated batch runs under conventional and microwave heating systems, revealing half-life times of 430.4 and 23.5 h, respectively	Souza et al. (2016)
		Novozym® 435	Ethanolysis of algal oil with isooctane as solvent, in reaction performed in a microwave reactor. The viscosity values for the microbial oil (51.9 $mm^2\ s^{-1}$) sharply decreased to 10.7 $mm^2\ s^{-1}$, upon the progress of transesterification reaction. The maximum FAEE yield (80%) achieved was due to the presence of non-lipid compounds, which may have affected the biocatalyst activity	Da Rós et al. (2017)
Other technologies	In situ transesterification of of microbial biomass bearing oil	Novozym® 435	Direct enzymatic transesterification of microalgae biomass using methanol (added at stepwise) and tert-butanol as solvent. Maximum FAME conversion of 99.5% (56 h). FAME conversion decreased by 40% when a lipase batch was used to catalyze three successive reactions	López et al. (2016)
		Novozym® 435	Enzymatic biodiesel production (ethanolysis) from microalgae biomass using propane as pressurized fluid. Conversion of 75.8% was achieved using (20 wt.%) of the enzyme with mild temperature demonstrating that microalgae biomass could be potentially used in biodiesel production	Marcon et al. (2017)
	Blend of alcohols (methanol + ethanol) as acyl acceptors	Novozym® 435	Transesterification of soybean oil with blended alcohols by lipase Among six proportions tested, 0 (100 mol% ethanol), 20, 40, and 60 mol% methanol in the blended alcohols exhibited high yields of biodiesel. For the optimum temperature (30 °C), the highest yield of biodiesel (>95 wt%) was obtained at an enzyme loading of 5–10 wt%	Zhao et al. (2014)

et al. (2007) proposed an enzymatically hydroesterification using different lipases at each stage: (i) hydrolysis of acylglycerols by *C. rugosa* lipase, (ii) followed by esterification of resulting oil fatty acid with immobilized *C. antarctica* lipase. The methyl esterification of fatty acids proceeds on a much higher rate than the triglyceride methanolysis. Other process combinations have been proposed, as chemoenzymatic hydroesterification (Ting et al. 2008; De Souza et al. 2010; Talukder et al. 2010a; Brask et al. 2011), which is consisted on an enzymatic transesterification followed by esterification of the resultant product to improve the biodiesel yield by converting FFA and partial glycerides to FAEE (Xu et al. 2012). These also consider two steps of transesterification for more efficient conversion of low quality oils using higher chain alcohols (Sendzikiene et al. 2016). In addition to high conversion into biodiesel in a short time reaction, these technologies allow low-risk conditions of inactivation of the biocatalyst, obtaining a final product within normative specifications.

The benefit of ultrasound and microwave irradiation on biodiesel production catalyzed by lipase has also been reported (Batistella et al. 2012; Da Rós et al. 2014; Michelin et al. 2015; Souza et al. 2016; Bhangu et al. 2017). Among the major benefits of using this type of technology, include the use of solvent-free systems and reduction of the reaction time. The theory of ultrasonication and its application in many reacting systems has been widely reported as an important factor in endothermic reactions, as the transesterification reaction to produce biodiesel. The reason of using ultrasound in reaction systems is due to the mechanical energy applied to the system, which induces mixing effects needed to initiate reaction (Koh and Ghazi 2011). In transesterification specifically, sonication causes cavitation bubbles near the boundary between the alcohol and oil phases leading to intensive mixing of the system. The cavitation leads to a localized increase in temperature, and due to the formation of micro-jets, neither heating nor agitation are required (Santos et al. 2009). As reviewed by Koh and Ghazi (2011), ultrasonication increases the chemical reaction speed, the efficient molar ratio of methanol-to-oil, and the yield of transesterification of vegetable oils and animal fats into biodiesel. Such method clearly works with lower energy consumption compared to the conventional mechanical stirring method. Studies have shown that increased biodiesel yields under ultrasonic irradiation are mostly attributable to the efficiency of cavitation irradiation, which is dependent on frequency. This enhances the mass transfer between the reacting mixtures, thereby increasing the reaction rate (Aransiola et al. 2014; Lerin et al. 2014).

Ultrasound irradiations have been investigated towards the enzymatic methanolysis by Lerin et al. (2014) and the conversion to fatty acid methyl esters was greater than 85% within 4 h reaction. The major drawback associated with this method, however, is the possibility of fragmenting the immobilizing support (Rufino et al. 2010).

On the other hand, the use of microwave irradiation can overcome the low speed of the enzymatic reaction with conservation of the morphological properties of the immobilized enzyme (Souza et al. 2017). Microwaves, electromagnetic waves with frequencies ranging from 300 MHz to 300 GHz, induce molecular rotation of dipolar species accompanied by intermolecular friction and energy dissipation, resulting in

volumetric heating without affecting the molecular structure. Microwave heating is a process of direct energy absorption by the irradiated material (liquid) with a uniformly distributed heat sources which prevents convection due to thermal gradients, a common phenomenon in conventional heating (Da Rós et al. 2013). The effect of overheating, i.e. by heating a given substance above its boiling point, is assigned as the major factor for accelerating reactions heated by microwave (Lidström et al. 2001). The Arrhenius equation ($k = Ae^{\frac{-\Delta G}{RT}}$, in which k = rate constant A = pre-exponential constant AG = free energy of activation, R = ideal gas constant, T = temperature) describes the rate constant for any system. Thereby, microwaves may act in three ways to increase the rate constant of a reaction: (i) by increasing the vibration frequency, thereby increasing the molecular mobility that is related to the pre-exponential factor A (dependent on the vibrational frequency), (ii) by changing the exponential factor which would cause changes of activation energy, (iii) by heat generated into the system, which causes are more generally applicable to the increased speed of reactions in a microwave. The rapid heating and power distribution cannot be achieved by conventional heating, since the latter can change the selectivity of reagents (Lidström et al. 2001).

The direct use of microbial biomass containing lipid feedstock (López et al. 2016; Marcon et al. 2017) to obtain biodiesel by transesterification have been discussed in order to develop more streamlined processes and lower operating costs. Using *Nannchloropsis gaditana* microalgal biomass, López et al. (2016) obtained a conversion of 99.5% FAME by direct transesterification employing the enzyme Novozym® 435.

Methanol and ethanol, which are the most accepted acyl acceptors for synthesis of biodiesel, have their own advantages and disadvantages. Thus, using a blended alcohol (methanol and ethanol) as an acyl acceptor for lipase-catalyzed transesterification could be an innovative strategy for overcoming the drawbacks of each alcohol (Zhao et al. 2014). According to the authors, the use of blended alcohols engendered the successful results, although proportions of methanol higher than 60 mol% in the alcohol blended adversely affected the biodiesel yield. On the other hand, the reactivity of methanol in the transesterification was higher than ethanol. The results importantly indicate that higher methanol consumption results in more ethanol remaining in the reaction mixture and show possible extension of lipase activity. It also increases the solubility of the oil in the alcohol, yielding faster reaction rate. Thus, the employment of the blended alcohols of methanol and ethanol as an acyl acceptor for the transesterification has positive effect on the enzymatic biodiesel production.

10.7 Challenges and Opportunities for Lipases in the Realm of Biodiesel Production

Despite the innumerous advances in the field of lipase-catalyzed biodiesel, the commercialization of biotechnological approaches towards a competitive market is still a challenge to be overcome. There have been significant novel approaches towards

manufacturing and optimizing lipases including the production of liquid lipases, innovative immobilization materials, and the utilization of fermentation broth as an enzymatic-active material. The recent interest of adding value to wastewater from both agricultural and food industries via cultivation of microorganisms have lead to low-cost alternatives for production of many different metabolites, including enzymes (Reis and Hu 2017). The utilization of waste materials regarded as nutrient sources for cultivation of enzyme-producing microorganisms may eventually lead to further cost cuts in the lipase production industry. It is unlikely a biodiesel plant to operate its own factory with lipases that inhibits a costly-effective process. However, the bio-valorization of "waste" via lipase-producing microorganisms promotes simultaneously the partial or full treatment of such material and the production of a valuable resource, i.e., lipases. The world overall interest in biodiesel has not maintained steady over the past few years for a number of reasons, (i): one of the major energy users and producers in the world, China, has strict policies regarding the utilization of food crops towards energy production. As China is now one of the most influential countries when it comes to innovation in the biotechnological industry, its own policies often reflect on the degree of innovation that it exports to the world. Despite being home to ambitious programs in green and sustainable energy development and security in the world, China has shifted much of its focus towards the production of other forms of renewable energy, as wind and solar. (ii): the price of fossil fuels has significantly dropped within the last decade, especially those of natural gas. The production of biodiesel became, in many parts of the world, not feasible techno-economically and has been ever since a forgotten alternative to liquid fossil fuels. (iii): life cycle assessment of first-generation biodiesel has shown that its emissions and environmental impacts may often be higher than initially expected. It has been suggested by (Hill et al. 2006) that lowering greenhouse gas emissions and water usage, as well as other environmental impact indicators, is required in order to place biodiesel as a green alternative towards the world energy grid again. The production of biodiesel spans a full generation of research, and has a key driver in developing economies as a novel source of income and energy security. Such efforts, though now several seen as over dated, should not be discarded, nor should biodiesel.

Lipases, as well as other enzymes, have been explored to their capacity and have been on their way to become a commodity "chemical", instead of the past and current label of specialty catalyst. As reviewed in this chapter, the utilization of enzymes not only is preferable to chemical reactions due to lower energy requirements and higher specificity and formation of by-products, but also due to its reduction of waste and other added resources that do not add value to the final product. Furthermore, the utilization of lipases is a direct application of several of the 12 principles of green chemistry (Anastas and Eghbali 2010), and should be directly reflected in companies and countries that are driven towards sustainable development. Therefore, instead of crediting biodiesel a label of alternative fuel, it should be seen as a feasible way of adding value to waste lipid resources, from restaurant-waste (Canakci 2007) to scum in wastewater treatment plants (Bi et al. 2015). The utilization of lipases should not be seen as the cost-inhibitive step in the process, as many still credit its own use today, but rather an alternative to add value to agricultural wastes. The decades of research

in the field of biodiesel and on lipases have advanced many of the technical and optimization steps necessary towards the development of an efficient process, and the previously developed technology should now be transferred to current platform feedstocks in order to make them economically feasible. Factors that were problematic decades ago, as resilience, solvent use, and reusability now have been overcome by the development of high-efficiency immobilization techniques, green solvents and supercritical fluids, and robust immobilization or liquid enzymes, respectively. Thus, lipases may the answer to part of the complicated energy situation in the world, and the steps required to their full implementation not only rely on the implementation of policies, but also to "connecting-the-dots" on all the impressive research done over the past decades in the field of lipases for biodiesel production.

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