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Review

Developments in the Use of Lipase Transesterification for Biodiesel Production from Animal Fat Waste

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Abstract: Biodiesel constitutes an attractive source of energy because it is renewable, biodegradable, and non-polluting. Up to 20% biodiesel can be blended with fossil diesel and is being produced and used in many countries. Animal fat waste represents nearly 6% of total feedstock used to produce biodiesel through alkaline catalysis transesterification after its pretreatment. Lipase transesterification has some advantages such as the need of mild conditions, absence of pretreatment, no soap formation, simple downstream purification process and generation of high quality biodiesel. A few companies are using liquid lipase formulations and, in some cases, immobilized lipases for industrial biodiesel production, but the efficiency of the process can be further improved. Recent developments on immobilization support materials such as nanoparticles and magnetic nanomaterials have demonstrated high efficiency and potential for industrial applications. This manuscript reviews the latest advances on lipase transesterification and key operational variables for an efficient biodiesel production from animal fat waste.

Keywords: biodiesel; fuel; energy generation; lipase; immobilized lipase; animal waste; lard; tallow; animal fat; transesterification

1. Introduction

Animal byproducts generated in the European Union slaughterhouses represent nearly 17 million tons per year and, from them, 5 million tons inedible byproducts result from rendering and are mostly used for energy generation like biofuels and biodiesel [1–3]. After rendering byproducts, fat is obtained from beef tallow, mutton tallow, pork lard and chicken fat [4,5]. Such fat is majorly composed of triacylglycerols with fatty acids of 16 to 18 carbons. The most abundant saturated fatty acids are palmitic (16:0) and stearic (18:0) acids; the major monounsaturated fatty acid is oleic acid (18:1) and the most abundant polyunsaturated fatty acids are linoleic (18:2) and arachidonic (20:4) acids [6,7]. Animal fat waste is also obtained from the meat processing industry and from recycled waste from the cooking business [8,9] that are classified as yellow grease if the content of free fatty acids is lower than 15% by weight and brown grease when it is higher than 15% [10]. In 2019, more than 800 thousand tons of animal fats, equivalent to 6% of total feedstock, were used to produce biodiesel in the European Union [11,12], while 8.4% of total feedstock was used in the case of the US, consisting of mainly 74 tons of poultry fat, 132 tons of tallow and 243 thousand tons of white grease [13].

Biodiesel produced from animal fats is cheaper than when made from vegetable oils. An additional advantage is that fossil CO_2 reduction is higher when using animal fat for biodiesel generation; nearly 80% CO_2 reduction may be reached for animal fat in comparison to 30% reduction when using

vegetable oil [14,15]. The bioenergy demand is continuously increasing and in 2050 it is expected to reach 30% of the fuel consumed in the world for road transport [15,16]. Research on biodiesel production is trying to maximize the yield and minimize the costs by using better catalysts that can be reused and improve the transesterification efficiency [17,18]. Furthermore, the feedstock used as raw material for biodiesel production represents up to 80% of the total cost [19] and it explains its variability in different geographic areas depending on the climate and agriculture [20].

Total biodiesel world production has been increasing progressively year by year, reaching nearly 45 million tons in 2019 [12]. The European Union has the largest biodiesel production through its 202 plants producing more than 14 million tons of biodiesel in 2019 [11,21]. More than 5.6 million tons of biodiesel were produced in the US in 2019 through its 91 plants [13,22]. Nearly 80% of new diesel vehicles are prepared for B20 use that consists of fossil diesel blended with 20% biodiesel [13].

Transesterification through alkaline catalysis is the preferred process at industrial biodiesel production plants [23]. However, raw materials like animal fat that contain moisture and free fatty acids are troublesome for alkaline transesterification due to soap formation. Acid catalysis does not have such troubles, but the reaction is much slower than alkaline catalysis, needs a larger size reactor and requires a higher alcohol to fat molar ratio [24]. Heterogeneous catalysts are not sensitive to the presence of free fatty acids and moisture, can catalyze esterification and transesterification simultaneously, and can be separated from the reaction media. However, such solid catalysts tend to form three phases resulting in a reduced reaction rate and high energy consumption [25]. The simultaneous esterification and transesterification also occur with supercritical technology where high temperature and pressure conditions (i.e., >250 °C and 10 MPa) increase the solubility and reduce the mass transfer limitation resulting in good efficiency but with high energy consumption [26–28]. Pseudo catalytic transesterification using biochar as the porous material for the pseudo-catalytic reaction at more than 300 °C has the same advantages as supercritical transesterification, but also has high energy consumption [29,30]. Therefore, animal fats may be processed for biodiesel production through enzymatically catalyzed transesterification even though some issues, like the cost of lipase and its poor stability, can be improved through immobilization [31]. Lipases have the advantage to generate biodiesel under mild reaction conditions through the conversion of free fatty acids and triacylglycerols in the presence of an acyl acceptor [32]. This manuscript reviews and discusses the latest advances in the use of free and immobilized lipases for an efficient transesterification of animal fat waste.

2. Mechanisms of Action of Lipases

Lipases, triacylglycerol ester hydrolases (EC 3.1.1.3), are serine hydrolases with an active site containing an amino-acid triad of serine, histidine and aspartate [32]. Lipases are obtained from a variety of sources such as animal and plant tissues and microorganisms. Lipases show a wide range of pH and temperature for activity and vary from strain to strain regarding specificity and hydrolysis rate [33] Lipases exhibit good stability in non-aqueous mediums and exhibit neutral pH range; such stability is increased when the enzyme is immobilized.

Lipases can catalyze esterification, inter-esterification, and trans-esterification reactions in non-aqueous environments. Lipases catalyze the hydrolysis of triacylglycerols at the aqueous-non aqueous interface but these enzymes can also catalyze the synthesis of esters from alcohols and long chain fatty acids in low moisture environment [33]. Lipases follow a two-step mechanism for the generation of fatty acid methyl esters in transesterification reactions, usually through the Ping-Pong Bi Bi mechanism [34].

Most triacylglycerol lipases are regiospecific because they can only hydrolyze primary ester bonds at the sn-1 and sn-3 positions, external positions within the triacylglycerol, and can generate either one free fatty acid and diacylglycerol, or two free fatty acids and 2-monoacylglycerol that remain unhydrolyzed. The full process from triacylglycerols into biodiesel and glycerol as end products is shown in Figure 1. Regiospecificity is characteristic of extracellular bacterial lipases from *Bacillus* sp. [35,36].

Monoacylglycerol lipases (EC 3.1.1.23) catalyze the hydrolysis at the specific sn-2 position of 2-monoacylglycerol into free fatty acid and glycerol. Such lipases may be present in the enzyme extract and masked when measuring activity with standard activity methods like those based on triolein hydrolysis measurement. Monoacylglycerol lipases have been the object of few studies [37], although they might be present in some microbial enzyme preparations [38]. Other lipases are nonspecific and can act on any of the ester bonds of the triacylglycerol and therefore break down the triacylglycerol to release free fatty acids and glycerol as the final products. This is the case of lipases from *Staphylococcus aureus* and *hyicus* [39], *Geotrichum candidum*, *Corynebacterium acnes*, *Penicillium cyclopium* [24] and *Chromobacterium viscosu* [40]. Another alternative for the hydrolysis of monoacylglycerols is the acyl migration in the glycerol backbone from the sn-2 position to sn-1 or sn-3 positions [41].

The specificity of lipases depends on the length of fatty acids, presence of double bonds, branched groups and, consequently, reaction rates may have important variations depending on the composition of triacylglycerols present in the fat waste. Lipases are especially active against medium to long chain fatty acids, which are those more usual in animal fat waste [17].

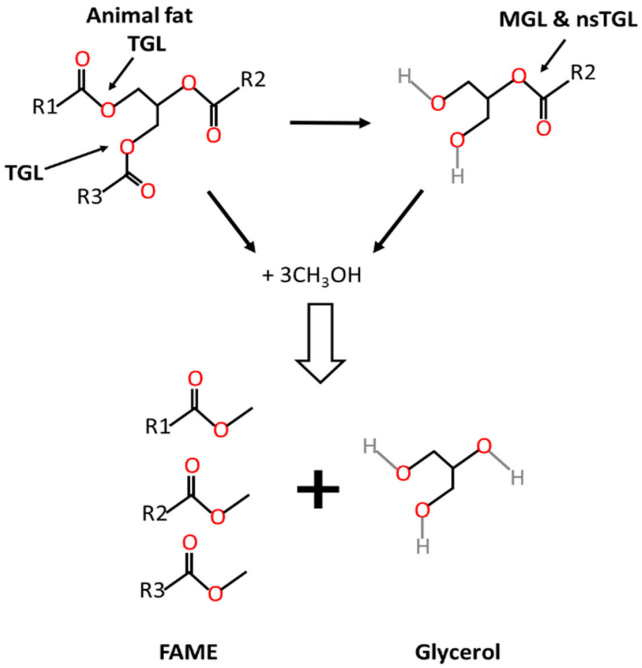

Figure 1. Transesterification of animal fat to biodiesel. TGL: triacylglycerol lipase; nsTGL: non specific triacylglycerol lipase; MGL: monoacylglycerol lipase.

3. Sources of Lipases

Most lipases originated from microorganisms and are produced in fermenters under controlled conditions (see Table 1). Lipases are produced by a variety of gram-positive and gram-negative bacterial strains, especially from the genera of *Pseudomonas* [42,43], also by filamentous fungus that are commercially important such as those belonging to the genera of *Rhizopus* sp. [44], *Aspergillus* sp. [45], *Penicillium* sp. [46], *Geotrichum* sp. [33], *Mucor* sp. [47] and *Thermomyces* sp. [48]. Lipases produced from yeasts are also relevant such as those from *Candida* sp. [49,50].

Extracellular lipases are secreted into the production medium and recovered from the microorganism broth. Then, lipases are further separated and purified but downstream processing is costly. Intracellular lipases imply the use of whole cell microorganisms and this fact reduces the costs of enzyme extraction and purification but the efficiency and biodiesel yield is low when catalyzing an oily substrate due to mass transfer limitations for substrate penetration and product release [28,51].

Some whole cell biocatalysts used to produce biodiesel are filamentous fungi like *Aspergillus* and *Rhizopus* [49].

Table 1. Bacteria, yeasts and filamentous fungi producing lipase and sources of isolation.

Lipase Origin	Reference
Pseudomonas fluorescens	[52]
Burkholderia cepacia	[42,50,53,54]
Staphylococcus haemolyticus	[55]
Chromobacterium viscosum	[56]
Phichia pastoris	[57]
Mucor miehei	[58]
Thermomyces lanuginosus	[59,60]
Aspergillus oryzae	[61]
Aspergillus niger	[62,63]
Aspergillus terreus	[64]
Rhizopus oryzae	[41,65,66]
Rhizomucor miehei	[60,67]
Geotrichum candidum	[68]
Candida antarctica	[66,69–71]
Candida cylindracea	[72]
Candida rugosa	[50,73,74]

4. Free Lipase

Lipases constitute an attractive catalyst for transesterification in those wastes containing large amounts of moisture and free fatty acids, which is the case of animal fat and is what makes it troublesome for alkaline transesterification. Table 2 shows some examples of the use of free lipases for biodiesel production from animal fat waste. The use of lipases has relevant advantages over conventional alkali catalysts. The most relevant are the absence of soap formation in the reactor, insensitivity to water content and acidity value, moderate reaction conditions, broad substrate range, good purity of biodiesel after transesterification and absence of pollutants, especially when treating cooking oil waste containing large amounts of free fatty acids [75]. On the contrary, there are also important disadvantages such as the high enzyme costs, poor enzyme stability, and the enzyme deactivation by alcohol [76] and partly by the generated glycerol [66].

As said, lipases are sensitive to the alcohol, in most cases methanol, used for biodiesel production and this fact increases the operational costs. There are some alternatives to avoid enzyme damage by methanol: stepwise addition of methanol to reaction mixtures avoiding a high concentration [65], the use of co-solvents like hydrophilic tert-butanol that dissolve glycerol and methanol and therefore allow high transesterification yields and rates [77], also the addition of longer-chain alcohols [78], or methyl or ethyl acetate as acyl acceptors [79,80]. Another solution is the use of novel lipases that can support one-step addition of high methanol concentration [81]. In this sense, another solution is the use of tools like protein engineering, recombinant methods and metabolic engineering that are used to improve thermostability as well as stability in organic solvents [80]. Glycerol may be extracted with organic solvents although the enzyme activity may be affected [81].

Lipase transesterification requires an extended time of reaction, and has a slow conversion rate as shown in Table 2. The recovery of the enzyme is rather difficult and the enzyme stability is poorer at high temperature and pH [81–84]. All these troubles have hindered its adoption at an industrial scale and therefore, transesterification with alkaline catalysis is still preferred at biodiesel-producing industrial plants [23]. However, such troubles experienced with enzymes can be partly overcome through its immobilization on a solid material that acts as enzyme carrier and increases its stability and efficiency [81,85,86]. Immobilization also allows an easy downstream separation from the product and decreases cost [82]. However, recyclability is an issue because lipases tend to lose activity after continuous operation [86]. In any case, it was reported that the use of soluble lipases might be more

competitive if the commercial enzymes would have a price 50 times lower than the immobilized lipase [25,87]

Table 2. Biodiesel production with various free soluble lipases.

Lipase Source	Feedstock	Conditions (T, alcohol:oil, t)	Yield (%)	References
A. niger	Waste cooking oil	45 °C, 4.2:1, 30 h	90 [a]	[63]
T. lanoginosus	Beef tallow	35 °C, 4.5:1, 6 h	84.6 [a]	[88]
C. antarctica	Lard	30 °C, 1:1, 72 h	74 [a]	[89]
C. antarctica	Lard	50 °C, 5:1, 20 h	97.2 [b]	[90]
Candida sp	Lard	40 °C, 3:1, 30 h	87.4 [b]	[91]
T. lanoginosus	Chicken fat	-	89.04 [b]	[92]
C. antarctica	Chicken fat	32 °C, 3:1, 24 h	96 [a]	[71]

[a] Biodiesel yield (wt/wt.%) was determined as the methyl esters amount produced by the lipases in the reaction process divided by the initial amount of esters; or [b] by the amount of oil.

5. Immobilized Lipase

5.1. Types of Supports and Immobilization Procedures

The immobilization of lipases consists of the retention of the enzyme at the surface of the support material. In this way, immobilized lipases show an improved efficiency and reduced costs, with longer enzyme stability and better resistance to denaturation by alcohol. There are many available supports of organic, synthetic and inorganic nature for lipase immobilization. Such materials may vary in characteristics such as particle dimensions, shape, pore volume, hydrophobicity, and density and must be stable to physical, chemical, and microbial degradation [93]. Porous supports with controlled pore distribution are very interesting for lipase immobilization because they offer an extensive surface area and therefore, higher enzyme loading. However, caution must be observed if pores are too small because they could get blocked by the enzyme, reducing its efficiency.

There is a large variety of immobilization procedures (see Figure 2) such as adsorption, covalent binding, cross-linking, entrapment, or encapsulation that have been developed to enhance the catalytic activity, and its stability, and make possible the reutilization of the enzyme in relation to the soluble lipase [94]. Some methods like cross-linking enzyme aggregates are not considered in this review because even though they are inexpensive, highly efficient, and do not need support for immobilization, they have rather poor mechanical stability [95].

Immobilization by adsorption on materials such as water-absorbing polymer, hydrophobic macroporous polypropylene particles or silica gel is simple, but can result in an undesirable leakage making it necessary to assure the retention of the enzyme by additional ionic or covalent bonds. This can be an ion exchange resin or cross-linking with glutaraldehyde [68]; performance was improved by crosslinking with glutaraldehyde. The stability in acid pH was improved as well as thermostability at 45–50 °C. It retained 80% of relative biodiesel production after 5 consecutive batches [96]. Adsorption of lipase from *Burkholderia cepacia* was compared to covalent immobilization on epoxy acrylic resin. The adsorbed enzyme gave a higher conversion than the covalent one after a three-step addition of ethanol, 68% vs 47% [97]. The covalently immobilized enzyme showed lower affinity towards diglycerides and monoglycerides; this was attributed to blockage of the active groups by the covalent bonds to the support material, which resulted in enzyme rigidity [98]. Mesoporous materials are attractive because they have high surface area, larger pore volume, absence of toxicity, and good stability [99]. Examples of reported immobilized lipases used in recent studies are shown in Table 3.

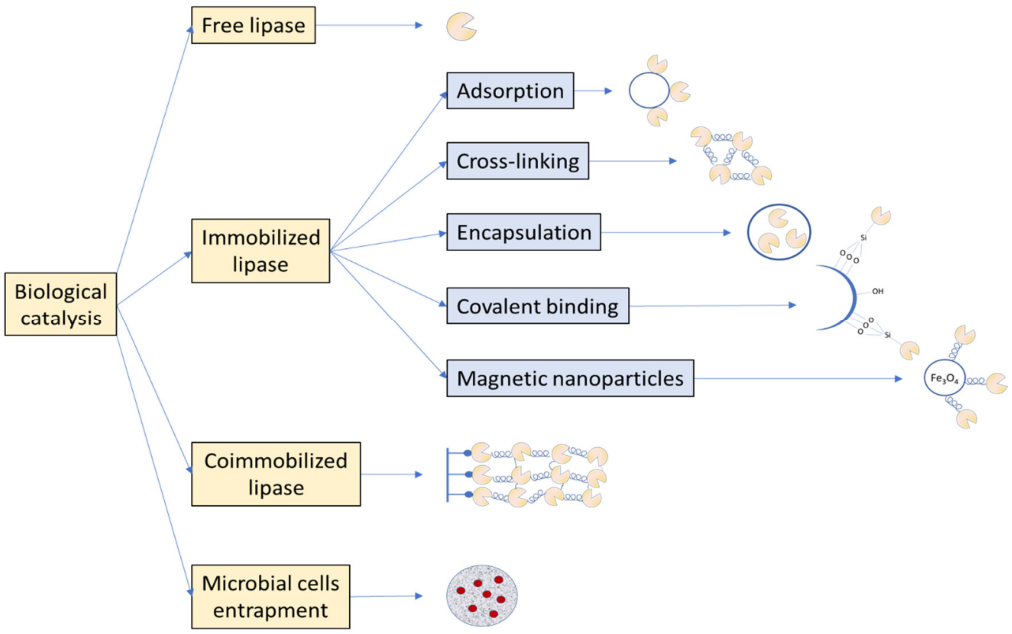

Figure 2. Major types of biological catalysis for biodiesel generation.

Table 3. Biodiesel production with various immobilized lipases.

Lipase Source	Feedstock	Immobilization	Yield [a] (%)	No. Cycles	References
A. niger	Sardine oil	Activated carbon	94.5 [a]	5	[62]
T. lanoginosus	Lard	Silica gel	97.6 [a]	20	[90]
C. antarctica	Waste cooking oil	Silica nanoflower pickering emulsion	98.5 [a]	15	[70]
B. cepacia	Castor oil	Polyvinylalcohol alginate	75 [b]	6	[85]
R. miehei and C. antarctica	Waste cooking oil	Epoxy functionalized silica	91.5 [a]	14	[100]
Streptomyces sp	Waste cooking oil	XAD 1180 resin	95.45 [a]	4	[79]
B. cepacia	Beef tallow	Polysiloxane–polyvinyl alcohol (SiO2–PVA) hybrid composite	89 [a]	-	[101]
C. antarctica	Waste fish oil	Acrylic resin	95 [a]	4	[102]
C. antarctica	Waste fish oil	Acrylic resin	75 [a]	10	[103]

[a] Biodiesel yield (wt/wt.%) was determined as the methyl esters amount produced by the lipases in the reaction process divided by the initial amount of esters, or [b] by the amount of oil.

There is a good affinity for immobilization of lipases on hydrophobic supports [104], giving a fast and good attachment by hydrophobic adsorption [105]. Transesterification of waste lard was tested with immobilized lipase B from *Candida antarctica* with the assistance of ultrasound for improving the dispersion and collision of the reagent molecules. Ultrasonic wave amplitude, ultrasonic cycles, and reaction parameters were optimized and a kinetic model was developed [69]. Pulsed ultrasound irradiation increased by about 3 times the synthesis rate of fatty acid ethyl ester by lipases immobilized on hydrophobic carriers like octadecyl-sepabeads [106].

Porous silica nanoflowers have center-radial pore structure that allows the load of lipase inside the structure to have good mass transfer for substrates and products [107]. Dichlorodimethylsilane was used to modify the silica nanoflowers for the adsorption of *Candida antarctica* lipase B and the biocatalytic pickering emulsion was constructed [94]. Pickering emulsion stabilized by hybrid nanoparticles [108], solid particles [109], or carbon nanotubes crosslinked with lipase [110], have been constructed and successfully used [111,112]. In this way, this emulsion facilitates biphasic reactions and simplifies the recovery of lipase that remains in its microenvironment [113].

Metal-organic frameworks provide advantages for immobilization: they can be easily separated, they offer an extended surface area that can be tuned, they have adequate pore size, have structural and functional diversity and good stability. Immobilization may be through adsorption, encapsulation,

and coprecipitation [114]. Lipase is strongly adsorbed by metal-chelating affinity immobilization that is a simple technique with the advantage that support may be reused [54]. Desorption agents may cause the desorption of the enzyme that can be also achieved by changing the pH value [115]. The compound n-hexane could reduce the deactivation of AGMNP-CO_2^+-PFL from methanol. It was reported that biodiesel production from oil transesterification was higher with n-hexane than using tert-butanol [70,94].

Encapsulation immobilization entraps lipase by a co-precipitation method and crosslinking agents like glutaraldehyde are used to interconnect the enzymes. However, there is a high mass transfer resistance that reduces its efficiency.

The entrapment of cells having lipase activity appears to be a simple and attractive technique. Lipase immobilized in silicon granules or calcium alginate beads, with glucose supplementation for cells maintenance, achieved an increased number of cycles, 28 instead of 23, while keeping 90% activity [33]. Whole cell, recombinant methods and protein and metabolic engineering are promising options to increase lipase applications [82].

5.2. Magnetic Nanocarriers

Materials like magnetite (Fe_3O_4) are used as support for immobilization because they allow a rapid separation from the reaction medium when an external magnetic field is applied [116]. The development of magnetic nanoparticles (MNPs) as a support for enzymes immobilization has been recently reviewed [117]. Typical magnetic nanomaterials include iron oxide (Fe_3O_4 and γ-Fe_2O_3), alloy-based ($CoPt_3$ and FePt), pure metal (Fe and Co), and spinel-type ferromagnet ($MgFe_2O_4$, $MnFe_2O_4$, and $CoFe_2O_4$) [117]. Examples of lipase immobilized in various types of magnetic nanoparticles are shown in Table 4.

Table 4. Biodiesel production with various immobilized lipases using magnetic nanoparticles as carriers.

Lipase Source	Carrier	Immobilization	Yield [a] (%)	No. Cycles	References
B. cepacia	Silica coated hydroxyapatite and glutaraldehyde	Encapsulation	98	4	[118]
A. terreus	Iron oxide polydopamine	Covalent bonding	92	7	[64]
T. lanuginosis & *C. antarctica*	Core-shell structured iron oxide	Covalent bonding	99	11	[119]
P. fluorescens	Co^{2+} chelated, with 3-glycidoxypropyltrimethoxysylane	Adsorption	95	10	[70]
R. miehei and T. lanuginosa	Silica core shell iron oxide with tryethylamine	Covalent bonding	93.1	5	[60]
C. antarctica	Poly(urea-urethane) encapsulated magnetite	Encapsulation	95	8	[120]
C. antarctica	Magnetic iron oxide with 1-Butyl-3-methylimidazolium tetrafluoroborate & 3-aminopropyltriethoxysilane	Covalent bonding	89.4	5	[121]
B. cepacia	Polysiloxane–polyvinyl alcohol hybrid magnetic-polymer composite	Covalent bonding	96.5	-	[122]

[a] Biodiesel yield (wt/wt.%) was determined as the methyl esters amount produced by the lipases in the reaction process divided by the initial amount of esters.

Magnetic nanoparticles (MNPs) have good biocompatibility and non-toxicity but tend to aggregate and oxidize, so they need to be functionalized on the surface and use a cross-linking agent to bind the enzyme. One way is by using silica coating where a silica shell is formed on the surface by using amino-functional reagents like 3-aminopropyl triethoxysilane (APTES). Fe_3O_4 particles were encapsulated with mesoporous silicon and modified with APTES or 3-mercaptopropyl trimethoxysilane (MPTMS) followed by binding of the lipase with glutaraldehyde. APTES-modified Fe_3O_4 particles were reported to give better yield of biodiesel (90%) than MPTMS particles [75]. *Burkholderria* sp. lipase on Fe_3O_4 MNPs also achieved 90% conversion [123].

Another way to protect MNPs is with organic polymers, including synthetic polymers and biopolymers. The polymer can be either incorporated into the precursor solution to form Fe_3O_4 MNPs

or externally to create the core shell [117]. The polymer surface provides numerous functional groups that facilitate the enzyme binding. Magnetic chitosan binds the lipase by covalent attachment [124].

Separation of nanobiocatalysts is difficult in an oily system [119] but the magnetic properties of MNPs can facilitate the separation of enzyme from reaction media. In this way, the reaction may be immediately finished as well as using the enzyme for further uses [117].

5.3. Coimmobilization

Some works have proposed to use coimmobilization of enzymes. The advantage is that the first enzyme releases the product that is transferred to the next coimmobilized enzyme with a short diffusional distance. This is especially relevant for lipases due to their specificity for triacylglycerols ester bonds. The mixture of 1,3-specific lipase and a non-specific lipase enhances the global activity because it removes the limiting acyl-migration step. Several coimmobilized systems have been studied for biodiesel production like *Candida rugosa* lipase and *Rhyzopus oryzae* lipase simultaneously on silica gel [74], *Candida antarctica* lipase B and *Thermomyces lanuginosus* lipase, on the surface of the *Phichia pastoris* cell [57], and *Rhizomucor miehei* and *Candida antarctica* lipases on epoxy-functionalized silica [67,100].

Lipases are coimmobilized on the same material surface in order to get better global activity and improved enzyme specificity and selectivity for hydrolysis of triacylglycerols as well as those generated diacylglycerols and monoacylglycerols that must be further hydrolyzed [96]. However, the most active enzyme may get some loss of activity through this procedure [125]. A different coimmobilization strategy was proposed by immobilizing several lipases layer-by-layer using abcoating with polyethylenimine [126]. Other authors have used coating with PEI/glutaraldehyde to form 5 enzyme layers of lipases A and B from *Candida antarctica*, lipases from *Rhizomocur miehei*, and *Themomyces lanuginosus* and phospholipase Lecitase Ultra [125]. Although it gives an innovative way for fats hydrolysis some problems might arise either from inhibition by coating agents used, high costs of different lipases used or steric hindrance for accessibility of triacylglycerols to the active site of lipases immobilized in the inner layers.

6. Industrial Applications of Lipase-Catalyzed Biodiesel

Even though transesterification through alkaline catalysis is the preferred process in the majority of industrial biodiesel production plants [23], a few lipase-based processes have already been implemented to plant-scale operation. The collaboration of Novozymes (Bagsvaerd, Denmark) with Piedmont Biofuels (Pittsboro, NC, USA) resulted in a patent application to produce fatty acid alkyl esters, by a lipolytic enzyme in a solution containing triacylglycerol, alcohol, water, and glycerol [93,127]. Viesel Fuel (Terrac Stuart, FL, USA) upgraded in 2013 its facility through an enzymatic process developed by Novozymes (Denmark) to use brown grease and waste cooking oil to produce up to 11 million gallons biodiesel per year using Eversa Transform® lipase from Novozymes, a soluble lipase produced by a genetically modified strain of *Aspergillus oryzae* [128], and an ion exchange resin system for removal of remaining free fatty acids during crude biodiesel refining [129,130]. Viesel Fuel, Novozymes and Tactical Fabrication also collaborated with Buster Biofuels to upgrade its facility in San Diego (CA, USA) to produce up to 5 million gallons per year [131]. Lvming and Environmental Protection Technology Co. Ltd. (Shanghai, China) used lipase of *Candida* sp. to produce 10,000 tons per year from waste frying oil [95]. A plant in Sumaré (Sao Paulo, Brazil) produces biodiesel from mixed beef tallow and soybean oil using Callera® Trans L lipase in a batch reactor [132]. These companies are using liquid lipase formulations but the efficiency of the process can be improved further by using recent developments in immobilized lipases. So, Hunan Rivers Bioengineering Co. Ltd. (Hunan, China) was reported to use Novozym 435® lipase in a stirred tank reactor to produce 20,000 tons of biodiesel per year. The enzyme is a lipase B from *Candida antarctica* immobilized on a resin consisting of macroporous support formed by poly(methyl methacrylate) crosslinked with divinylbenzene [133]. New technology protected with patents [134] has been provided by EnzymoCore, a leading global

producer company founded in 2007 in Israel and with several active biodiesel plants around the world. This company has developed modified-immobilized enzymes, supported on solid organic resins, with high resistance to methanol and able to produce biodiesel from any type of oil or fat, even those cheap and with very large content of free fatty acids and polar lipids [135].

7. Conclusions

Animal fat waste, usually resulting from slaughterhouses, the meat processing industry, and cooking facilities, is being increasingly used for biodiesel production. Transesterification through alkaline catalysis is the preferred process at industrial biodiesel production plants. Transesterification with lipases has not been attractive for industry yet because of the higher operative costs in comparison to alkaline catalysis; transesterification with lipases has problems including poor enzyme stability, difficulties in reusability, and denaturation by alcohol although they are not affected by water and free fatty acids typically found in animal fats. However, a few companies could solve such troubles since they are running liquid lipase formulations for producing biodiesel from cooking oil waste at industrial scale. However, the efficiency of the process can be further improved. Recent developments in immobilized lipases and availability of different types of supports such as mesoporus materials, silica nanoflowers, pickering emulsion, and metal-organic frameworks demonstrate improved efficiency and reduced costs. Immobilization of the enzyme in such materials increases its stability and makes it more resistant to denaturation by alcohol. Magnetic nanomaterials constitute an even better support for enzyme immobilization because they can be recovered when an external magnetic field is applied. These nanoparticles are functionalized on the surface by coating with silica or organic polymers that enhance the efficiency of the process. The entrapment of whole cells with lipase activity, appears to be simple and efficient although more research is needed. Coimmobilization of lipases is an innovative process, but not so attractive for industrial application. It needs further research because of the need for different lipases that increases the costs and the efficiency affected by steric difficulties for enzymes to hydrolyze triacylglycerols.

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