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Lipase Applications in Biodiesel Production

Sevil Yücel, Pınar Terzioğlu and Didem Özçimen

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1. Introduction

Because of the global warming and depletion of fossil fuels, in recent years, intensive investigations are carried on for providing the greater use of sustainable biofuels instead of fossil fuels. Biomass, which various biofuels are produced from, has an important role among other alternative energy sources including wind energy, solar energy, geothermal energy, etc.

Biodiesel is one of the important biofuels and a clean energy source as an alternative to petroleum-based diesel fuels. Biodiesel has some advantages and disadvantages. Transportability, high combustion efficiency, low sulphur and aromatic content, high cetane number and biodegradability are advantages of the biodiesel [1]. Disadvantages of biodiesel are high viscosity, lower energy content, high cloud and pour point, high nitrogen oxide emission, lower engine speed and power, injector cooking, high price and engine erosion [2].

The flash point of biodiesel is higher than diesel fuel. This feature is important for fuel storage and transportation in the way of safety. Cetane number of biodiesel (~50) is higher than diesel fuel [3]. Biodiesel does not include aromatic and sulphur content and contains oxygen at the rate of 10-11% by mass [4]. Cetane number is an important factor to determine the quality of diesel fuel, especially ignition quality of diesel fuel. In other words, it determines the ignition tendency of fuel when being injected into engine. Ignition quality of biodiesel is determined by the structure of methyl ester [5]. Viscosity is also an important factor for biodiesel. Viscosity affects mostly fuel injection equipment and the increase of fuel viscosity changes the viscosity at low temperatures. High viscosity has a negative effect on fuel spray atomization [6]. Amounts of elements and compounds in biodiesel and diesel fuel are present in Table 1 [7]. Biodiesel has more polar structure than diesel fuel because of the oxygen, which is an electronegative element present in its structure, and therefore biodiesel has higher viscosity comparing with diesel fuel. In addition, elemental oxygen content is responsible for lower heating value of biodiesel when compared with diesel fuel. [7-9]. Biodiesel

can be used in its pure form or when mixed with diesel fuel in certain proportions. Most common biodiesel blends are B2 (2 % biodiesel, 98 % diesel), B5 (5 % biodiesel, 95% diesel), B20 (20 % biodiesel, 80 % diesel) [10].

	Biodiesel Content (%)	Diesel Content (%)
Carbon	79.6	86.4
Hydrogen	10.5	13.6
Oxygen	8.6	-
Nitrogen	1.3	-
C/H	7.6	6.5
n-Aliphatics	15.2	67.4
Olephenics	84.7	3.4
Aromatics	-	20.1
Naphtens	-	9.1

Table 1. The comparison of elemental and chemical content of diesel and biodiesel [7]

The transesterification reaction can be influenced by several factors including molar ratio of alcohol, catalyst, presence of water, free fatty acid in oil samples, temperature, time and agitation speed. In this context, an understanding of the factors affecting the process is very important to make economically and environmentally biodiesel production [11].

To accelerate reaction rate, transesterification process is carried out in the presence of catalysts. So, biodiesel production is made by using chemical or enzymatic catalysts. Compared to chemical, enzymatic reaction is more attractive because of ability of make a high quality product, simplify the separation of products, mild reaction conditions, the reuse of the catalyst and especially environmental impact, although high conversion and reaction rate are obtained with chemical catalysts [11-14]. Lipase is important enzyme catalyst that catalyzes esterification and transesterification reaction to produce methyl esters (biodiesel). Figure 1 presents the enzymatic transesterification reaction [15].

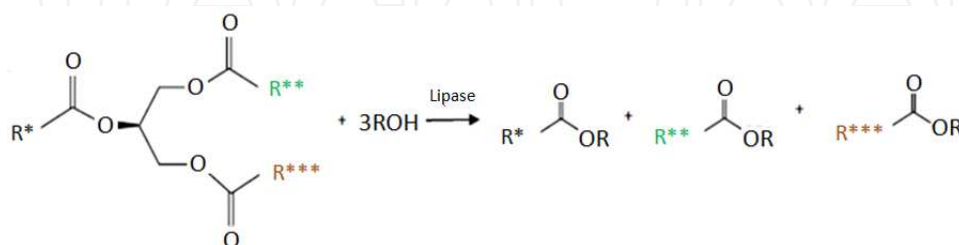


Figure 1. Enzymatic transesterification reaction [15].

In this study, enzymatic approach for biodiesel production was reviewed, and especially the usage of lipases in biodiesel production and factors affecting the effectiveness of lipase in reaction were explained in detail.

2. Lipases in biodiesel production

Biocatalyst based biotechnological applications are receiving increasing attention. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are the important biocatalysts because of their excellent biochemical and physiological properties. Lipases are the hydrolytic enzymes that can be used in various industrial applications for alcoholysis, acidolysis, aminolysis and hydrolysis reactions. Biodiesel production is one of the stunning applications of lipase. Lipase catalyzed biodiesel production was reported first by Mittelbach [16]. Lipase-catalyzed transesterification takes place in two steps, which involves hydrolysis of the ester bond and esterification with the second substrate [15]. A ping-pong bi bi mechanism generally used for kinetic studies of enzyme catalyzed transesterification.

Lipases can be isolated from many species of plants (papaya latex, oat seed lipase, and castor seed lipase), animals (pig's and human pancreatic lipases), bacteria, filamentous fungi and yeast [17-19]. For industrial enzyme production generally microorganisms are preferred because of their shortest generation time [20]. The other advantages of microorganisms can be listed as high yield of conversion of substrate into product, great versatility to environmental conditions and, simplicity in genetic manipulation and in cultivation conditions [20]. Although lipases from different sources are able to catalyze the same reaction, bacterial and fungal lipases are mostly used in biodiesel production such as *Aspergillus niger*, *Candida antarctica*, *Candida rugosa*, *Chromobacterium Viscosum*, *Mucor miehei*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Photobacterium lipolyticum*, *Rhizopus oryzae*, *Streptomyces sp.*, and *Thermomyces lanuginose* [21]. *Candida rugosa*, obtained from yeast, is the most used microorganism for lipase production [22]. Recently, *Streptomyces sp.* was investigated as a potent lipase producing microbe for biodiesel production and found applicable in the field of biodiesel [23].

Specificity of lipases has a great importance in the selection of the usage area of lipases. Lipases can be divided into three groups due to their specificity as 1,3-specific lipases, fatty acid-specific lipases and nonspecific lipases. Especially, 1,3-specific lipases which release fatty acids from positions 1 and 3 of a glyceride and hydrolyze ester bonds in these positions such as *Aspergillus niger*, *Rhizopus oryzae* and *Mucor miehei* catalyze transesterification reactions efficiently [20,24]. The study of Du et al. [25], showed that higher yield (90%) was achieved for biodiesel production by using a sn-1,3-specific lipase, *Thermomyces lanuginosa* immobilized on silica gel (Lipozyme TL IM). Thus, the use of sn-1,3-specific lipases can give rise to biodiesel yield of above 90% under appropriate conditions [24]. Substrate specificity of lipases is also a crucial factor towards the biodiesel production which acts on the choice of the proper enzyme based on the composition of raw materials by consisting in the capability of distinguishing structural features of acyl chains [20,24]. Lipases from *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Candida rugosa*, *Candida antarctica* and *Candida cylindracea* are suitable for transesterification reaction by displaying both wide substrate specificity and regioselectivity [24].

2.1. Immobilization of lipases

The immobilization of enzymes, which is attracting worldwide attention, was firstly reported in 1971 at Enzyme Engineering Conference [26]. During the past decade, chemical modification, physical modification, and gene expression techniques have been developed to obtain more economic, active, selective, or stable lipases. Immobilization is a modification method that can be defined as attaching the enzyme onto an insoluble solid support material [18]. By immobilization more operational and temperature stable lipases can be obtained and also lipases can be reused in the reactions. In addition, reusability of lipases will be a possible solution to the high cost of the enzymes and make them suitable for applications in industrial scale. The comparison of free enzymes and immobilized enzymes is given in Table 2. Methods for enzyme immobilization can be classified as adsorption, covalent bonding, entrapment, and cross-linking. The selection of method and support material is a prominent factor for obtaining an efficient lipase. The results of comparative studies revealed that the same lipase molecule can show very different catalytic activities after immobilization onto different supports [27].

Characteristics	Free Enzyme	Immobilized Enzyme
Price	High	Low
Efficiency	Low	High
Activity	Unstable	Stable
Reusability and recovery	Not possible	Possible
Tolerance to temperature, pH, etc.	Low	High
To separate from the substrate	Difficult	Easy
To separate from the product	Difficult	Easy

Table 2. The comparison of free enzyme and immobilized enzyme [19]

2.1.1. Adsorption technique

Adsorption is the adhesion of lipase on the surface of the adsorbent by weak forces, such as van der Waals, ionic and hydrophobic interactions, or dispersion forces [28]. Immobilization via adsorption method is the simply mixing of an aqueous solution of enzyme with the carrier material for a period and washing away the excess enzyme from the immobilized enzyme on the carrier after a time [29]. The level of adsorption is strictly related to the pH, temperature and ionic strength. Adsorption is the most widely employed method besides

other methods because of its special commercial advantages and simplicity. Adsorption is the only reversible enzyme immobilization method. The advantages of adsorption is mild and easy preparing conditions, low cost, no need for chemical additives, the carrier can be recovered for repeated use, and high activity [30].

Various types of carriers used in immobilization of lipases. Acrylic resin, celite, polypropylene and textile membrane are broadly used carriers. Some of the reported results of adsorption technique based immobilized enzymes used in biodiesel production are summarized in Table 3. As can be seen from table generally the biodiesel yields using the enzymes obtained by adsorption method are higher than 85%. Novozym 435 is a commercial lipase, which is obtained by immobilization of *Candida antarctica* lipase on acrylic resin and is a good catalyst that provides biodiesel yield higher than 90% with vegetable oil or waste cooking oil as feedstock [31]. The other commercialized lipase is known as *Candida sp.* 99–125 lipase immobilized on textile membrane, which can catalyze lard, waste oil and vegetable oils with higher yields that is more than 87% [31]. Besides many advantages of immobilization by adsorption method, the main disadvantage is that desorption of the lipase from the carrier occurs because of the weak interactions between the enzyme and support.

2.1.2. Covalent binding technique

Another approach is covalent binding technique, which is the formation of covalent bonds between the aldehyde groups of support surface and active amino acid residues on the surface of the enzyme [29]. A variety of supports have been used such inorganic materials, natural polymers (agarose, chitin and chitosan), synthetic polymers (hydrophobic polypeptides, nylon fibers) and Eupergit® (made by copolymerization of N,N'-methylenebis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide) for immobilization of lipases by covalent binding [56]. The main advantage of covalent binding method is obtaining thermal and operational stable enzymes because of strong interactions between the lipase and the carrier [31]. The comparison of biodiesel production performance using immobilized lipase via covalent binding method is summarized in Table 4. Chitosan is a promising carrier as a natural polymer due to its membrane forming and adhesion ability, high mechanical strength and facility of forming insoluble in water thermally and chemically inert films [57]. Xie and Wang [58], reported a technique for immobilization of *Candida rugosa* lipase on magnetic chitosan microspheres for transesterification of soybean oil. The immobilized enzyme was determined as an effective biocatalyst for the transesterification reaction due to giving a good conversion of soybean oil and retaining its activity during the four cycles [58].

Using two immobilized lipases with complementary position specificity instead of one lipase is a new approach to produce a cost effective biodiesel [19]. Lipase from *Rhizopus orizae* and *Candida rugosa* was covalently bound to the silica, which was used to produce biodiesel from crude canola oil. Under optimum conditions, the conversion rate of degummed crude canola oil to fatty acid methyl esters was 88.9%, which is higher than the conversion obtained by free enzyme mixture (84.25%) [59].

Lipase Source	Carrier	Acid/Oil Source	Alcohol	Maximum Performance (%)	Reference
<i>Burkholderia sp. C20</i>	Alkyl-functionalized Fe ₃ O ₄ -SiO ₂	Olive	Methanol	92 (conversion)	[32]
<i>Candida antarctica</i>	Acrylic resin	Soybean	Methanol	92(yield)	[33]
<i>Candida antarctica</i>	Acrylic resin	Soybean and rapeseed	Methanol	98.4 (conversion)	[34]
<i>Candida antarctica</i>	Acrylic resin	Soybean and rapeseed	Methanol	"/>95 (conversion)	[35]
<i>Candida antarctica B</i>	Granular activated carbon	Palm	Isobutanol	100 (conversion)	[36]
<i>Candida sp. 99-125</i>	Textile membrane	Lard	Methanol	87.4 (yield)	[37]
<i>Candida sp. 99-125</i>	Textile (cotton) membrane	Salad	Methanol	96 (conversion)	[38]
<i>Candida sp. 99-125</i>	Textile membrane	Crude rice bran	Methanol	87.4 (yield)	[39]
<i>Candida rugosa and Pseudomonas fluorescens</i>	Acurel	Palm	Ethanol	89 (yield)	[40]
<i>Chromoacterlum viscosum</i>	Celite-545	Jatropha	Ethanol	92 (yield)	[41]
<i>Geobacillus thermocatenulatus</i>	Poly-hydroxybutyrate beads	Babassu	Ethanol	100 (yield)	[42]
<i>Pseudomonas aeruginosa</i>	Celite	Soybean	Methanol	80(yield)	[43]
<i>Pseudomonas cepacia</i>	Celite	Jatropha	Ethanol	98 (yield)	[44]
<i>Pseudomonas cepacia</i>	Electrospun polyacrylonitrile fibers	Rapeseed	n-butanol	94 (conversion)	[45]
<i>Pseudomonas cepacia</i>	Polystyrene	Sapium sebiferum	Methanol	96.22 (yield)	[46]
<i>Pseudomonas cepacia</i>	Ceramic beads	Waste cooking	Methanol	40 (yield)	[47]
<i>Pseudomonas fluorescens</i>	Porous kaolinite particle	Triglyceride triolein	1-propanol	"/>90 (conversion)	[48]
<i>Pseudomonas fluorescens and Pseudomonas cepacia</i>	Polypropylene powder	Soybean	Methanol	58 37 (yield)	[49]
<i>Penicillium expansum</i>	Resin D4020	Waste	Methanol	92.8 (yield)	[50]
<i>Rhizomucor miehei</i>	Hydrophilic resins	Olive husk	Ethanol	-	[51]
<i>Rhizomucor miehei</i>	Silica	Waste cooking	Methanol	91.08 (yield)	[52]
<i>Rhizopus oryzae</i>	Macroporous resin HPD-400	Pistacia chinensis bge seed	Methanol	94 (yield)	[53]
<i>Saccharomyces cerevisiae</i>	Mg-Al hydrotalcite	Rape	Methanol	96 (conversion)	[54]
<i>Thermomyces lanuginosus (Lipozyme TL IM)</i>	Hydrotalcite	Waste cooking	Methanol	95 (yield)	[55]

Table 3. Comparison of biodiesel production performance using immobilized lipase via adsorption method

Lipase Source	Carrier	Acid/Oil Source	Alcohol	Maximum Performance (%)	Reference
<i>Burkholderia cepacia</i>	Niobium Oxide (Nb ₂ O ₅)	Babassu	Ethanol	74.13 (yield)	[60]
<i>Burkholderia cepacia</i>	Polysiloxane–Polyvinyl Alcohol (SiO ₂ –Pva)	Babassu Beef Tallow	Ethanol	100 89.7 (yield)	[60]
<i>Candida rugosa</i>	Chitosan Microspheres	Soybean	Methanol	87 (conversion)	[58]
<i>Candida rugosa</i>	Chitosan Powder	Rapeseed Soapstock	Methanol	95 (conversion)	[61]
<i>Enterobacter aerogenes</i>	Silica	Jatropha	Methanol	94 (yield)	[62]
<i>Porcine pancreatic</i>	Chitosan Beads	Salicornia	Methanol	55 (conversion)	[63]
<i>Pseudomonas fluorescens</i>	Toyopearl Af-Amino-650m Resin	Babassu	Ethanol	94.9 (yield)	[64]
<i>Rhizopus oryzae</i>	Resin Amberlite Ira-93	Pistacia Chinensis Bge Seed	Methanol	92 (yield)	[63]
<i>Rhizopus oryzae</i>	Polystyrene Polymer(Amberlite Ira-93)	Soybean	Methanol	90.05 (yield)	[65]
<i>Rhizopus Orizae</i> + <i>Candida rugosa</i>	Silica	-	Methanol	">98 (conversion)	[66]
<i>Rhizopus oryzae</i> + <i>Candida rugosa</i>	Silica	Crude Canola	Methanol	88.9 (conversion)	[59]
<i>Thermomyces lanuginosus</i>	Olive Pomace	Pomace	Methanol	93 (yield)	[67]
<i>Thermomyces lanuginosus</i>	Polyglutaraldehyde Activated Styrene-Divinylbenzene Copolymer	Canola	Methanol	97 (yield)	[68]
<i>Thermomyces lanuginosus</i>	Toyopearl Af-Amino-650m Resin	Palm	Ethanol	100 (yield)	[64]
<i>Thermomyces lanuginosus</i>	Polyurethane Foam	Canola	Methanol	90 (yield)	[69]
<i>Thermomyces lanuginosus</i>	Aldehyde-Lewatit	Soybean	Ethanol	100 (conversion)	[70]
<i>Thermomyces lanuginosus</i>	Magnetic Fe ₃ O ₄ Nano-Particles	Soybean	Methanol	90 (conversion)	[71]

Table 4. Comparison of biodiesel production performance using immobilized lipase via covalent binding method

2.1.3. Entrapment technique

Entrapment method is based on capturing of the lipase within a polymer network that retains the enzyme but allows the substrate and products to pass through [72]. This method can be simply defined as mixing an enzyme with a polymer solution and then crosslinking the polymer to form a lattice structure that captures the enzyme [29]. Entrapment is often used for industrial applications because the method is fast, cheap and can be carried out under mild conditions [73]. Entrapment can be divided into three categories such as gel or fiber entrapment and microencapsulation [74]. A number of supports have been investigated such as alginate, celite, carrageenan, resins, acrylic polymers etc. Some carriers used for entrapment and the biodiesel production yields obtained by these enzymes are displayed in Table 5. A disadvantage of entrapment method is the mass transfer problem due to the act of support as a barrier, so the lipase became effective only for low molecular weight substrates [19,75].

Lipase Source	Carrier	Acid/Oil Source	Alcohol	Maximum Performance (%)	Reference
<i>Burkholderia cepacia</i>	K-Carrageenan	Palm	Methanol	100 (conversion)	[76]
<i>Burkholderia cepacia</i>	Phyllosilicate Sol-Gel	Tallow and Grease	Ethanol	94 (yield)	[77]
<i>Burkholderia cepacia</i>	Mtms-Based Silica Monolith Coated With Butyl-Substituted Silicates	Jatropha	Methanol	95 (yield)	[78]
<i>Candida antarctica</i>	Celite®	Triolein	Methanol	60 (conversion)	[79]
<i>Candida rugosa</i>	Calcium Alginate Matrix	Palm	Ethanol	83 (yield)	[80]
<i>Candida rugosa</i>	Activated Carbon	Palm	Ethanol	85 (conversion)	[81]
<i>Pseudomonas cepacia</i>	Hydrophobic Sol-Gel	Soybean	Methanol	67 (conversion)	[82]
<i>Pseudomonas fluorescens</i> Mtcc 103	Alginate	Jatropha	Methanol	72 (yield)	[83]
Via Encapsulation Method					
<i>Burkholderia cepacia</i>	Silica Aerogels	Sunflower Seed	-	56 (conversion)	[84]
<i>Burkholderia cepacia</i>	K-Carrageenan	Palm	Methanol	100 (conversion)	[85]
<i>Candida antarctica</i>	Silica Aerogels	Sunflower Seed	Methanol	90 (conversion)	[86]

Table 5. Comparison of biodiesel production performance using immobilized lipase via entrapment method

2.1.4. Cross linking technique

Cross-linking is another method for immobilization that can be defined as the interaction of a three dimensional network within enzyme, coupling reagent, and carrier [19]. The advantage of cross-linking is obtaining stable lipases due to the strong interaction between the lipase and the carrier. On the other hand, the cross-linking conditions are intense and the immobilized lipase shows lower activity [31].

The high free fatty acid content of waste cooking oil form water by esterification with alcohol which cause agglomeration of lipase and lowering biocatalysis efficiency [87]. Hence, free *Geotrichum sp.* lipase was not a suitable enzyme catalyst for transesterification of waste cooking oil. Yan et al. [87], report a modification procedure for preparation of cross-linked *Geotrichum sp.* The obtained lipase exhibited improved pH and thermostable stability compared to free lipase. The relative biodiesel yield was 85% for transesterification of waste cooking oil with methanol.

Kumari et al. [88] studied the preparation of *Pseudomonas cepacia* lipase cross-linked enzyme aggregates. It was shown that cross linked lipases has a greater stability than free enzymes to the denaturing conditions. The enzyme also used to catalyze madhuca indica oil, which's transesterification is difficult by chemical routes due to its high free fatty acid content. As a result, 92% conversion was obtained after 2.5 h.

Immobilization of *Candida rugosa* lipase on fine powder of *Scirpus grossus* L.f. by glutaraldehyde by cross linked technique for biodiesel production from palm oil, as already investigated by Kensingh et al. [89]. It was concluded that immobilized lipase yielded higher conversion of biodiesel than that of free lipase.

Lorena et al. [90] investigated the immobilization of the *Alcaligenes spp.* lipase on polyethylenimine agarose, glutaraldehyde agarose, octyl agarose, glyoxyl agarose, Sepabeads® by the aggregation and crosslinking method. The transesterification of canola oil was achieved with a yield 80% using a six-step addition of methanol and lipase immobilized on Sepabeads® by the aggregation method.

All these methods are shown schematically in Figure 2.

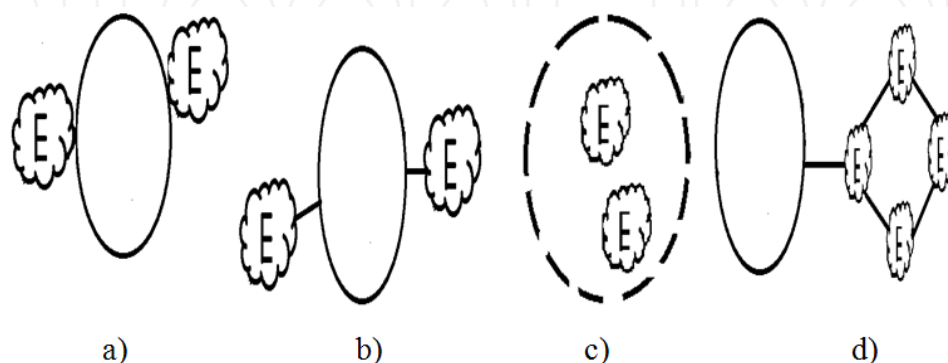


Figure 2. Schematic diagram of enzyme immobilization methods: a) Adsorption method b) Covalent binding method c) Entrapment method d) Crosslinking method

2.1.5. Whole cell immobilization

The applicability of lipases for the bulk production of fuels was limited significantly by the high cost of lipases [91]. Utilizing microbial cells such as fungi, bacteria, and yeasts cells containing intracellular lipase instead of extracellular lipases (free and immobilized lipase) is an easier and a cost effective way of enzymatic biodiesel production. Compared to conventional enzymatic processes, the use of whole cells provides excellent operational stability and avoids the complex procedures of isolation, purification and immobilization [91,92]. The general preparation steps for immobilized extracellular enzymes and whole cell enzymes showed in Figure 3. Biomass support particles have been used for immobilization of whole cells.

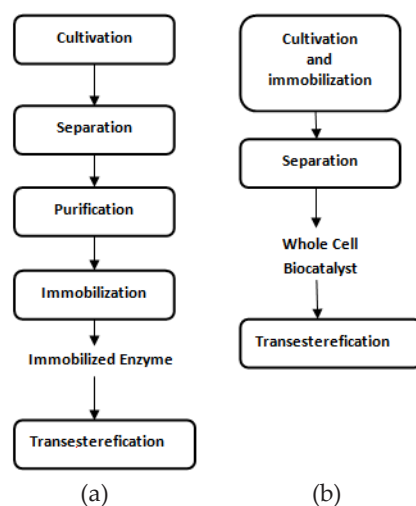


Figure 3. The preparation steps of a) immobilized extracellular lipase and b) whole cell biocatalyst

Aspergillus and *Rhizopus* have been most widely used as whole cell biocatalyst. Ban et al. [93], used first a whole cell biocatalyst, immobilized *Rhizopus oryzae* IFO4697 (a 1,3-positional specificity lipase) cells within biomass support particles, for the production of biodiesel and 91.1% methyl ester content was attained which was a similar result as that using the extracellular lipase. Many researchers have experimented on the use of whole cells to catalyze transesterification reaction summarized in Table 6.

A technique using glutaraldehyde cross-linking treatment on whole cell catalyst for methanolysis of soybean oil was developed by Sun et al. [94]. The glutaraldehyde cross linking treatment resulted in higher methanol tolerance and high catalytic activity (with the ratio of methanol to oil reaching 3). Also, a novel methanol addition strategy was proposed as stepwise addition of different amounts of methanol (1.0, 1.2, 1.5, and 2.0M equivalent of oil) every 24 h. It was found that the highest methyl ester yield could reach 94.1% after 24 h reaction by 1.2 mol, 1.5 M and 1.2 mol methanol additions at 0, 8, and 14 h. In general, the whole cell catalyzed process is slower than extracellular lipase catalyzed process. Sun et al. [94], also reported that the reaction time could be shortened by this way. It is clear that significant reduction in the cost of biodiesel production can be achieved by combining the whole cell biocatalyst process with stepwise addition of methanol.

Lipase Source	Carrier	Acid/Oil Source	Alcohol	Maximum Performance (%)	Reference
<i>Aspergillus niger</i>	BSPs ^a	Waste Cooking	Methanol	86.4 (yield)	[96]
<i>Aspergillus niger</i>	Polyurethane BSPs ^a	Palm	Methanol	>90 (yield)	[97]
<i>Aspergillus niger</i>	BSPs ^a	Palm	Methanol	87 (yield)	[98]
<i>Aspergillus oryzae</i> NS4	BSPs ^a	Soybean	Methanol	98 (conversion)	[99]
<i>A. oryzae</i> carrying r-CALB ^b	BSPs ^a	Palm Soybean	Methanol	85 90 (conversion)	[100]
<i>Aspergillus oryzae</i> expressing r-FHL ^c	BSPs ^a	Rapeseed	Methanol Ethanol 1-Propanol 1- Butanol	96 (yield) 94 (yield) 96 (yield) 97 (yield)	[101]
<i>Escherichia coli</i> BL21	-	Rapeseed	Methanol	97.7 (conversion)	[102]
<i>Rhizopus chinensis</i> CCTCC M201021	-	Soybean	Methanol	>86 (yield)	[103]
<i>Rhizomucor miehei</i> displaying <i>Pichia pastoris</i>	-	Soybean	Methanol	83.14 (yield)	[104]
<i>Rhizopus oryzae</i> IFO 4697	BSPs ^a	Refined Rapeseed Crude Rapeseed, Acidified Rapeseed	Methanol	~60(yield) ~60(yield) ~70(yield)	[105]
<i>Rhizopus oryzae</i> IFO 4697	BSPs ^a	Soybean	Methanol	~90(yield)	[106]
<i>Rhizopus oryzae</i> IFO 4697	BSPs ^a	Soybean	Methanol	~85(yield)	[107]
<i>Rhizopus oryzae</i> IFO4697	-	Soybean	Methanol	71 (conversion)	[108]
<i>Rhizopus oryzae</i> IFO 4697 and <i>Aspergillus oryzae</i> niaD300 (combined use)	BSPs ^a	Soybean	Methanol	~100 (conversion)	[109]

Lipase Source	Carrier	Acid/Oil Source	Alcohol	Maximum Performance (%)	Reference
<i>Rhizopus oryzae</i> ATCC 24563	-	Soybean (Free Fatty Acid Content 5.5%)	Methanol	97 (conversion)	[110]
<i>Rhizopus oryzae</i> IFO 4697	BSPs ^a	Soybean	Methanol	72 (yield)	[111]
<i>Rhizopus oryzae</i>	Polyurethane foam BSPs ^a	Soybean	Methanol	90 (conversion)	[112]
<i>Rhizopus oryzae</i>	BSPs ^a	Jatropha Curcas	Methanol	80 (conversion)	[113]
<i>Rhizopus oryzae</i> IFO 4697	-	Soybean	Methanol	86 (yield)	[114]
<i>Rhizopus oryzae</i>	BSPs ^a	Rapeseed	Methanol, Ethanol, 1-Propanol, 1- Butanol	83, 79, 93, 69 (yield)	[101]
<i>Serratia marcescens</i> YXJ-1002	-	Grease	Methanol	97 (yield)	[115]

^aBSPs: Biomass support particles

^br-CALB: *Candida antarctica* lipase B

^cr-FHL: *Fusarium heterosporum* lipase

Table 6. Comparison of biodiesel production performance using whole cell biocatalysts

Whole cell biocatalysts will be a way to industrialization of biodiesel production but the limited mass transfer efficiency of product and substrate is a hurdle to further investigations [95].

3. Feedstocks

The main aim of researches is to obtain a biodiesel, which will have a competitive price compared to other conventional sources of energy [116]. At this point, selecting the feedstock, represents more than 75-80% of the overall biodiesel production cost, is a vital step to ensure a cost effective biodiesel production. Different kinds of feedstock with varied range of edible and inedible vegetable oil, animal fats, waste oil, microbial oil and microalgae oil can be used for enzyme catalyzed transesterification [117].

3.1. Vegetable oils

Vegetable oils are candidates as alternative fuels for diesel engines with their high heat content [118]. But, direct use of vegetable oils is not possible because of the high kinematics viscosity of them which varies in the range of 30–40 cSt at 38 °C and are about 10 times higher than of diesel fuel (Grade No. 2D) leads to many problems [118,119]. Therefore, modification of vegetable oil is necessary and the valuable product of this modification is named “biodiesel”. The edible vegetable oils such as soybean [120,121], sunflower [122-124], palm [81,125], corn [126], cottonseed [127], canola [68,69,128] and olive [129,130] oils have been widely used in enzymatic transesterification. In developed countries, edible constitute more than 95% of biodiesel production feedstock because the produced biodiesel from these oils have properties very similar to petroleum-based diesel [131]. Also, the country and its climate, the oil percentage and the yield per hectare are effective parameters in selecting the potential renewable feedstock of fuel [118,132]. For example, while rapeseed oil prevailing the EU production, soybean oil prevailing the US and Latin American production, and palm oil mainly being used in Asia [133].

Inedible oils do not find a place in human consumption due to including toxic components. Therefore, inedible oils do not compete with food crops. Thus, inedible vegetable oils are an alternative feedstock for biodiesel production. Babassu (*Orbinya martiana*), *Jatropha curcas* (Linnaeus), neem (*Azadiracta indica*), polanga (*Calophyllum inophyllum*), karanja (*Pongamia pinnata*), rubber seed tree (*Hevea brasiliensis*), mahua (*Madhuca indica* and *Madhuca longifolia*), tobacco (*Nicotina tabacum*), silk cotton tree, etc. are promising inedible vegetable oil sources. *Jatropha curcas* is an attractive feedstock between various oil bearing seeds as it has been developed scientifically and found to give better biodiesel yield and productivity [134]. Crude *Jatropha* oil contains about 14% of free fatty acid that is too high for alkaline catalyzed biodiesel production [118]. However, high free acid content is not a problem in the production process of biodiesel via using enzyme catalysts. Besides *Jatropha curcas*, 26 species of fatty acid methyl ester of oils of including *Azadirachta indica*, *Calophyllum inophyllum*, and *Pongamia pinnata* were found most suitable for use as biodiesel, which adjust to the major specification of biodiesel standards of European Standard Organization, Germany, and USA [135]. Modi et al. reported conversion of crude oils of *Pongamia pinnata* (karanj), *Jatropha curcas* (*jatropha*) via immobilized Novozym 435 to biodiesel fuel with yield 90, and 92.7%, respectively [136].

3.2. Animal oils/fats

Animal fats are another group of feedstock for biodiesel production. Animal fats used to produce biodiesel via enzymatic route include lard [137], lamb meet [138] and beef tallow [139]. Animal fats are economically feasible feedstocks compared to vegetable oils. Animal fat methyl ester also has many favorably properties such as non-corrosive, high cetane number, and renewable [140,141]. However, animal fats saturated compounds lead to a tendency to oxidation and crystallization unacceptably at high temperatures [142].

3.3. Waste oils/fats

In general, around the world only half of the discharged edible oils recycled as animal feed or as raw material for lubricant and paint and the remainder is discharged into the environment [143]. Hence, the use of waste oils/fats for biodiesel production is very important to reduce and recycle the waste oil [143], to eliminate the environment and human health risk caused by waste oils [144] and to lower the biodiesel production cost. Waste cooking oil, animal fats, yellow grease, brown grease obtained from highly oxidized yellow grease or recovered waste grease from plumbing trap and waste sludge or soap-stock from the vegetable oil refining process were the major sources of waste oil have been used for biodiesel production [145]. The selection of a catalyst to be used for the production of biodiesel fuel is mostly influenced by the amount of free fatty acid content in various feedstocks [146]. The lipase-catalyzed reaction is a promising method for converting waste oils which contains high percentage of free fatty acids and high water content, into biodiesel with high yield [145]. It has been reported that Novozym 435 is capable of converting the used olive oils [129].

3.4. Algae oils

There is a considerable interest in the use of algae (micro and macro) oils for synthesis of biodiesel. Because these oils are cheap raw materials besides animal fats and have rapid growth rate and productivity when compared to conventional forestry, agricultural crops, high lipid content, tolerance for poor quality water, smaller land usage up to 49 or 132 times less when compared to rapeseed or soybean crops [142,147]. The smaller land usage brings the advantage of reducing the competition for arable soil with other crops, in particular for human consumption [147]. However, there are still some drawbacks for utilization of algae for biodiesel production. A considerable investment in technological development and technical expertise is needed to optimize the microalgae harvesting and oil extraction processes, to use cheap sources of CO₂ for culture enrichment [147]. Algae oils contain about 20-40% oil [148]. Several researchers have been experimented on microalgal oils as raw material for biodiesel production. Tran et al. [130], investigated the conversion of microalgal oil from *Chlorella vulgaris* ESP-31 to biodiesel by using immobilized Burkholderia lipase and a high fatty acid methyl esters conversion efficiency of 97.25 wt% oil (or 58.35 wt % biomass) was obtained for 48 h reaction. It is proposed that microalgal oil has good potential for application in the commercial production of biodiesel. The enzymatic conversion of microalgal oils to biodiesel in ionic liquids was firstly studied by Lai et al. [149]. Four microalgae two strains of *Botryococcus braunii* (BB763 and BB764), *Chlorella vulgaris*, and *Chlorella pyrenoidosa* have been catalyzed by two immobilized lipases, *Penicillium expansum* lipase and *Candida antarctica* lipase B (Novozym 435), in two solvent systems: an ionic liquid (1-butyl-3-methylimidazolium hexafluorophosphate, [BMIm][PF6]) and an organic solvent (tert-butanol). *Penicillium expansum* lipase was found more efficient for this application and the ionic liquid [BMIm][PF6] showed a greater conversion yield (90.7% and 86.2%) obtained relative to the one obtained in the commonly used organic solvent tert-butanol (48.6% and 44.4%).

4. The effect of reaction parameter on enzymatic transesterification

4.1. The effect of temperature on enzymatic transesterification

Enzymatic transesterification takes place at low temperatures varying from 25 to 60°C. In general, initially the rate of reaction increases with rise in reaction temperature, because of an increase in rate constants with temperature and less mass transfer limitations [150,151]. Nevertheless, increased temperature after the optimum temperature promotes to denaturation and higher thermal deactivation of the enzyme, since it decreased the catalytic activity [152].

Various researches have been carried out to find out the effect of temperature on biodiesel production with immobilized enzymes. It is clear that immobilization provide more temperature resistance compared to free enzymes due to supplying a more rigid external backbone for lipase molecule [150,151]. However, optimum temperature is specific for each production. The studies about the effect of temperature for enzymatic transesterification are shown in Table 7.

Lipase	Oil Source	Alcohol	Performed Temperatures In The Range (°C)	Optimum Temperature (°C)	Reference
Immobilized <i>Aspergillus niger</i>	Palm	Methanol	25-50	40	[153]
Immobilized <i>Aspergillus niger</i>	Waste Cooking	Methanol	25-50	30	[154]
Immobilized <i>Burkholderia cepacia</i>	Babassu	Ethanol	39-56	39	[155]
<i>Candida antarctica</i>	Cotton Seed	T-Butanol	30-50	50	[156]
<i>Candida antarctica</i>	Acid	Methanol	30-50	30	[157]
Immobilized <i>Candida</i> Sp. 99-125	Salad	Methanol	27-50	40	[158]
<i>Candida</i> Sp. 99-125	Waste Cooking	Methanol	35-50	40-50	[159]
Immobilized <i>Enterobacter aerogenes</i>	Jatropha	T-Butanol	30-55	55	[160]
Immobilized <i>Enterobacter aerogenes</i>	Crude Rapeseed	Ethanol	25-50	35	[161]
Lipozyme RM IM	Soybean	Butanol	20-50	30	[162]
Lipozyme RM IM	Soybean	Methanol and Ethanol	40-60	50	[163]
Lipozyme RM IM	Soybean Oil Deodorizer Distillate	Ethanol	45-78	50	[164]
Lipozyme TL IM	Rapeseed	N-Butanol	30-60	40	[165]
Lipozyme TL IM	Soybean	Ethanol	20-50	35	[162]

Lipase	Oil Source	Alcohol	Performed Temperatures In The Range (°C)	Optimum Temperature (°C)	Reference
Lipozyme TL IM	Palm	Ethanol	30-78	50	[166]
Novozyme 435	Rapeseed	Methanol	25-55	40	[167]
Novozyme 435	Tung and Palm	Methanol and Ethanol	45-55	55	[168]
Novozym 435	Cottonseed	-(Dimethyl Carbonate As Organic Solvent)	30-55	50	[169]
Novozym 435	Canola	Methanol	25-65	38	[170]
Novozym 435	Olive	Methanol	30-70	40	[129]
Novozym 435	Soybean	T-Amyl	30-60	40	[171]
Novozym 435	Sunflower	Methanol	25-65	45	[172]
Novozym 435	Stillingia	Methanol	30-60	40	[173]
Novozym 435	Cotton Seed	Methanol	30-70	50	[174]
Novozym 435, Lipozyme TL IM and Lipozyme RM IM	Soybean	Ethanol	25-60	25	[175]
Immobilized Penicillium expansum	Waste	T-Amyl	25-55	35	[176]
Immobilized Pseudomonas cepacia	Soybean	Methanol and Ethanol	25-60	35	[177]
Pseudomonas cepacia	Soybean	Methanol	20-60	30	[178]
Immobilized Pseudomonas fluorescens	Triolein	1-Propanol	40-70	60	[48]
Pseudomonas fluorescens	Soybean	Methanol	30-60	40	[49]
Rhizopus chinensis CCTCC M201021	Soybean	Methanol	30-40	30	[179]
Thermomyces lanuginosus	Canola	Methanol	30-70	40	[69]

Table 7. Data on optimum temperature for enzymatic biodiesel production

4.2. The effect of water content on enzymatic transesterification

Water content is one of the key factors for enzymatic transesterification reaction that have a strong effect on lipase's active three-dimensional conformational state [21,180]. Biocatalysts, needs a small amount of water to retain their activities [181]. Lipase has an unique feature on the water-oil interface, and the lipase activity depends on this interface. The presence of an oil-water interface required because it provides a suitable environment for enzyme activation which occurs due to the unmasking and restructuring the active site through confor-

mational changes of the lipase molecule [182,183]. When the addition of water increased, the amount of water available for oil to form oil–water droplets also increases, hence increasing the available interfacial area [182]. Thus, enzymatic activity can not be possible in a water free media. However, excess water cause reverse reaction of hydrolysis. The amount of required water, to provide an optimum enzyme activity, differs according to the type of enzyme and reaction medium composition. Enzymes, substrates, organic solvent and also immobilized support have a crucial role on optimal water activity for lipase [184]. Optimum water content not only provides keeping the hydrolysis of ester linkages at the minimum level, but also ensures the highest degree of transesterification [24]. Thus, a better control of water content is very important for enzymatic process.

Water activity (a_w) is defined as free (boundness) water in the system, which is a ratio of vapor pressure over the given system versus that over pure water [24]. Thermodynamic water activity is the best predictor of reaction rate that can be determined in any phase by different kinds of sensors such as holographic sensor, Weiss LiCl humidity sensor [180,185]. Also, several methods have been developed for control of water activity, for example, equilibration with saturated salt solutions [186], addition of salt hydrate pairs [187,188] and introduction of air or nitrogen into the reactor [189]. Recently, Peterson et. al. developed a practical way for control of water activity in large-scale enzymatic reactions by using a programmable logic controller. On the other hand, percentage water content is another expression which is used widely in transesterification, generally assayed by Karl-Fischer coulometer.

In general, lipases show higher activity with higher water activities in solvent free systems instead of *Candida antarctica* lipase (Novozym 435) [184]. For *Candida* sp. 99–125 lipase, the optimum water content is 10–20% based on the oil weight to maintain the highest transesterification activity [31].

Salis et al., investigated production of oleic acid alkyl esters by using *Pseudomonas cepacia* and determined that a_w in the range 0.4–0.6, 1-butanol:triolein 3:1 – were the best conditions to reach maximum enzymatic activity. It was also found that at the higher values of water activity, no hydrolysis reaction was occurred [190].

Noureddini and Philkana [82] tested immobilized *Pseudomonas cepacia* for the transesterification of soybean oil with methanol and ethanol and observed that increased addition of water provide a considerable increase in the ester yield. The optimal conditions were determined for processing 10 g of soybean oil by 475 mg lipase in 1 h as 1:7.5 oil/methanol molar ratio, 0.5 g water in the presence of methanol that resulted in 67 % yield and 1:15.2 oil/ethanol molar ratio, 0.3 g water in the presence of ethanol that resulted in 65% yield.

Al-Zuhair et al. studied the esterification of n-butyric acid with methanol in the presence of *Mucor miehei* lipase, and found similar results with literature [191] that higher water content, makes lipase more efficient [182].

Shah and Gupta used immobilized *Pseudomonas cepacia* lipase for ethanolysis of *Jatropha* oil and noted that the best yield 98% gained by in the presence of 4–5% (w/w) water in 8 h. The yield was only 70% in absence of water [44].

Kawakami et al. determined the effect of water content for transesterification of *Jatropha* oil and methanol to characterize *Burkholderia cepacia* lipase immobilized in an n-butyl-substituted hydrophobic silica monolith. The authors reported that biodiesel yield reached 90% with water content of 0.6% (w/w) after 12 h using a stoichiometric mixture of methanol and oil (3:1) [78].

Chen et al. investigated the effect of water content for production of biodiesel with oleic acid with methanol catalyzed by soluble lipase NS81020, produced by modification of *Aspergillus oryzae* microorganism, in the biphasic aqueous-oil systems and found that the esterification yield is low if the water was scant. The higher reaction rate and fatty acid methyl ester yield was obtained with 10 wt % water by oleic acid weight [192].

It is clear that during the past decade numerous investigations have been made to determine the optimal water content for transesterification. As a result, the necessary amount of water content is an important factor to create an interfacial surface between oil and water and to ensure optimal enzymatic activity. Also, water has a strong influence on structural integrity, active site polarity, and protein stability of lipase [21,193]. However, it differs from enzyme to reaction conditions.

4.3. The effect of acyl acceptors on enzymatic transesterification

Methanol, short chain alcohol, usually used as an acyl acceptor due to its low price and availability. Insoluble and a relatively high amount of methanol with respect to oil, have a negative influence on the stability of lipases and could be solved by a stepwise addition of the alcohols [15, 194]. To eliminate inhibitory effects of methanol some co-solvents are added to the reaction mixture. Tert-butanol is one of the important co-solvents which is added to enzymatic reaction. Usage of tert-butanol, a polar solvent, is also a possible solution for eliminating the inhibitory effects of methanol and glycerol (both of them soluble in tert-butanol) and suggested instead of using butanol [195]. Liu et al. [196], transesterified waste baked duck oil by three different commercial immobilized lipases (Novozym 435, Lipozyme TLIM and Lipozyme RMIM) with different monohydric alcohols (methanol, ethanol, propanol, isopropanol, isobutanol, isoamyl alcohol) and fusel oil-like alcohol mixture (containing 15% isobutanol, 80% isoamyl alcohol, 5% methanol) in solvent-free and tert-butanol systems. It was reported that each lipase presented a different kinetic pattern depending on the monohydric alcohols. The results showed that Lipozyme TL IM and Novozym 435 gave high conversion rate with isobutanol and isoamyl alcohol either in solvent-free or in tert-butanol system. Thus, the combined use of lipases, Novozym 435 and Lipozyme TLIM, as catalyst and fusel oil-like mixture as raw material for biodiesel synthesis was found effective in view of cost saving of biodiesel production [195].

Recently, novel acyl acceptors were investigated such as ethyl acetate, methyl acetate, butyl acetate, vinyl acetate [197], dimethyl carbonate [198]. Du and coworkers demonstrated the positive effect of methyl acetate, on enzymatic activity of Novozym 435 and found that lipase could be reused directly without any additional treatment [199]. The advantage of using methyl acetate is that the cost of the catalyst can be reduced dramatically due to the longer operational life and reusability of lipase. The byproduct of the system is triacetylgly-

cerol, which does not have any negative effect on the fuel property, and also no glycerol produced [200]. Hence, these advantages will provide industrial implementation of enzymatic biodiesel production. Dimethyl carbonate is another promising alternative acyl acceptor, which is eco-friendly, odorless, cheap, non-corrosive, and non-toxic [200]. The transesterification reaction is irreversible, because carbonic acid monoacyl ester, the intermediate compound, immediately decomposes to carbon dioxide and alcohol [200]. The fatty acid methyl ester yield is higher for lipase-catalyzed transesterification of vegetable oils with dimethyl carbonate besides conventional acyl acceptors (methanol and methyl acetate) [200]. Only, the higher price of acyl acceptor besides alcohols is a disadvantage [194].

4.4. Effects of the solvent on enzymatic transesterification reaction

In enzymatic transesterification reaction, excess of alcohol increases reaction efficiency, but if alcohol doesn't dissolve in reaction medium it can disrupt the enzyme activity. Methanol and vegetable oil in the values close to 1:1 molar ratio forms a solution in 40°C. Solvent is added into the reaction medium to increase the solubility of alcohol and thus it allows first step enzymatic transesterification by blocking degradation lipase catalytic activity [24]. To overcome deactivation of lipase activity and improve the lipase activity, various organic solvents have been used for enzymatic biodiesel synthesis. These solvents have been listed in Table 8. Cyclohexane, n-hexane, tert-butanol, petroleum ether, isooctane and 1,4-dioxane are mainly studied hydrophilic and hydrophobic organic solvents in enzymatic biodiesel production. In organic solvent medium, overall alcohol is added at the beginning of the reaction. In solvent free reaction medium, alcohol is added in several portions to prevent enzyme activity with high alcohol concentration [24].

Hexane is generally preferred because of its low cost and easily availability in the market. Some studies were performed in hexane solvent systems with soybean and tallow oil using monohydric alcohols [70,201, 202]. Nelson et al. performed transesterification of tallow with monohydric alcohols by Lipozyme IM 60 (*M. miehei*) and Novozyme SP435 (*C. antarctica*) in hexane and a solvent-free system. They compared the transesterification yields of two different systems. The yields with higher than 95% were obtained with methanol, ethanol and butanol with Lipozyme IM 60 lipase under hexane system (Table 8) while reaction yields under solvent-free system were 19% for methanol, 65.5% for ethanol, and 97.4% for isobutanol [201]. Similar results were found by Rodrigues et al. [70]. They compared the yields of transesterification of soybean with ethanol by Lipozyme TL IM. In the presence of n-hexane with 7.5:1 molar ratio of ethanol:soybean oil, the transesterification conversion was found to be as 100% while in solvent-free system the yield was 75%. At stoichiometric molar ratio, the yield was 70% conversion after 10 h of reaction in both systems. Transesterification conversion was obtained as 80% by three stepwise addition of ethanol, while a two step ethanolysis produced 100% conversion after 10 h of reaction in both solvent and solvent-free systems.

In enzyme catalyzed reaction, both alcohol amount and low glycerol solubility in biodiesel have negative effects on enzyme activity. Deposit of glycerol coating the immobilized catalyst is formed during the process, which reduces the enzymes activity [203]. The solubility of

methanol and glycerol in hydrophobic solvents is low. For this reason, this problem may occur in hydrophobic solvent system.

The enzymatic alcoholysis of triglyceride also was studied with petroleum ether, isooctane, cyclohexane, 1,4-dioxane (Table 4-2) [16,48,204]. Iso et al. [48], reported that when methanol and ethanol were used as alcohol in enzymatic transesterification, the reactions need an appropriate organic solvent [48]. On the other hand, the reaction could be performed without solvent when 1-propanol and 1-butanol was used. They also used, benzene, chloroform and tetrahydrofuran as solvent and immobilized *P. fluorescens* lipase as catalyst at 50°C to compare the results that of the 1,4 dioxane. The highest enzymatic activity was observed with 1,4-dioxane. The enzymatic activity increased with the high amount of 1,4-dioxane. But high conversion of oil (app.90%) to biodiesel was obtained with high proportion of 1,4 dioxane(90%). Although usage of high amount of solvents is not preferable in industry solvents can be recovered together with methanol after transesterification reaction.

Hydrophilic organic solvents can interact with water molecule in enzyme and this may affect the catalytic activity of enzyme. However, as shown in Table 8 high performance was ensured with hydrophilic solvents such as 1,4-dioxane and tert-butanol [48,156, 205-208]. Some studies were performed in the presence of t-butanol solvent because of positive effects on enzymatic catalyzed reaction. T-butanol has moderate polarity so methanol and glycerol are easily soluble in tertiary butanol. Solubility of methanol prevents enzyme inhibition and solubility of glycerol prevents accumulation on the enzyme carrier material. Another advantage of this solvent is steric hindrance. Due to this property, tert-butanol is not accepted by the lipase. High yield and conversions were obtained in the presence of t-butanol with various vegetable oils and immobilized lipases shown in Table 4-2. For example, Liu et al., [196] studied biodiesel synthesis by immobilized lipases in solvent-free and tert-butanol media. Each lipase showed a different conversion depending on the monohydric alcohols and immobilized lipase in solvent-free medium and tert-butanol system. For methanolysis, regardless of the lipase type, the conversion rate is higher in tert-butanol than that in solvent-free medium. Novozym 435 showed higher conversion rate with straight monoalcohols in tert-butanol medium. Lipozym RM IM and Lipozyme TL IM showed lower conversion with straight and branched monoalcohols (except methanol) in solvent free system. Similar results were obtained by Halim and Kamaruddin [208], in transesterification of waste cooking palm oil using various commercial lipases (Lipozyme RM IM, Lipozyme TL IM and Novozyme 435) in tert-butanol as reaction medium. Novozyme 435 was found to be more effective in catalyzing the transesterification with methanol in in-tert-butanol medium. It was also been demonstrated that even 3:1 methanol to oil molar ratio didn't inhibit the Novozyme 435 in tert-butanol system. Du et al. [209], showed that Lipozyme TL IM could be used without loss of lipase activity for 200 batches in tert-butanol system. Li et al. [210], used acetonitrile and tert-butanol mixture as co-solvent in transesterification of stilingia oil with methanol. The highest biodiesel yield (90.57%) was obtained in co-solvent with 40% tert-butanol and 60% acetonitrile (v/v) with co-solvent. They also reported that co-solvent (as a mixture) enhance the tolerance of lipase to the methanol than the pure tert-butanol.

Solvent	Oil	Alcohol	Lipase	Temp/ Time	Reaction mixture	Performance (%)	Ref.
Tert-butanol	Cotton seed	Methanol	Novozyme 435 (<i>Candida antarctica</i>)	50 °C / 24h	13.5% meth., 54% oil 32.5% tert-butanol, Lipase:1.7% (wt of oil)	97 (yield)	[156]
Tert-butanol	Cotton seed	Methanol	Pancreatic lipase	37 °C / 4 h	Methanol :oil mol ratio:1:15 Lipase:0.5% enzyme (wt of oil) water conc.5% (wt of oil)	75–80 (conversion)	[205]
Tert-butanol	Rapeseed	Methanol	Novozyme435 & Lipozyme TL IM	35 °C / 12 h	Methanol: oil mol. ratio 4:1 tert-butanol/oil vol. 1:1 Lipase: 3% Lipozyme TL IM 1% Novozym 435 (wt of oil)	95 (conversion)	[206]
Tert-butanol	Soybean and deodorizer distillate	Methanol	Lipozyme TL IM Novozym 435	40°C/ 12 h	Methanol:oil molar ratio 3.6:1 Lipase :3% Lipozyme TL IM 2% Novozym 435 tert-butanol: 80% (wt of oil)	84 (yield)	[207]
Tert-butanol	Waste cooking palm	Methanol	Novozyme 435	40°C / 12 h	Methanol:oil mol. ratio 4:1, Lipase:4% (wt of oil)	88(yield)	[208]
Tert-butanol	Waste baked duck	Methanol	Novozym 435 Lipozyme TL IM	45 °C / 20 h	Methanol:oil mol. ratio 4:1, Lipase: 5 wt%(wt of oil)	85.4, 78.5, (conversion)	[196]
Hexane	Tallow	Methanol Ethanol Propanol	Lipozyme IM 60	45 C/ 5 h	0.34 M tallow in hexane (8 mL), Lipase: 10 (wt of oil) 200rpm	94.8, 98.0, 98.5 (conversion)	[201]

Solvent	Oil	Alcohol	Lipase	Temp/ Time	Reaction mixture	Performance (%)	Ref.
Hexane	Soybean	Methanol	Lipozyme IM 77	36.5°C/ 3h	Methanol :oil mol ratio:3.4:1 Lipase:0.9BAUN*of lipase; water 5.8% (wt% of oil)	92.2 (yield)	[202]
Hexane	Soybean	Ethanol	Lipozyme TL IM	30 °C/ 10 h	Ethanol:oil mol.ratio:7.5:1 Lipase: 15 %(wt of oil). 4% water	100 (conversion)	[70]
Cyclohexane	Sunflower	Methanol	Lipase AK Lipozyme TL IM Lipozyme RM IM	40°C/ 24 h	Volume of organic solvent/ oil: 2 ml/0.2 mmol Lipase: 10% (wt of oil)	65, 75, 35 (conversion)	[204]
Acetonitrile 60% and 40% t-butanol (v/v)	Stillingia	Methanol	Novozym 435 and Lipozyme TL IM	40°C/ 24h	Methanol:oil mol ratio: 6.4:1 Lipase: 4% (w/w) of multiple-lipase (1.96% Novozym 435+2.04% Lipozyme TL IM)	90.57 (yield)	[210]
Petroleum ether	Sunflower	Ethanol	Lipozyme IM Lipase AK	45°C / 5h	Ethanol:oil mol. ratio:11:1 Lipase:20% (wt of oil)	82, 99, (yield)	[16]
l-octane	Sunflower	Methanol	Lipase AK Lipozyme TL, IM Lipozyme RM,IM	40 °C	Methanol: oil mol ratio::3:1 Vol. of organic solvent/oil: 2 ml/0.2 mmol	80, 65, 60, (yield)	[204]
1,4-dioxane	Triolein	Methanol	Lipase AK	50°C / 80h	Methanol:oil mol. ratio: 3:1 90% solvent	~70 (conversion)	[48]

*BAUN:Batch Acidolysis Units Novo

Table 8. Effect of the solvent on the performance of enzymatic transesterification reaction

Although positive effects of the usage of the solvents on the transesterification reaction, some drawbacks has also been known) such as; extra reactor volume, solvent toxicity and emissions, solvent recovery and loss cost [133].

4.5. The effect of molar ratio of alcohol to oil on enzymatic transesterification

Biodiesel yield always increased due to the molar excess of alcohol over fatty acids in triglycerides in traditional transesterification system [15]. The transesterification reaction is reversible and so, an increase in the amount of one of the reactants will result in higher ester yield and minimally 3 molar equivalents of methanol are required for the complete conversion of methyl ester [174]. Conversely, for enzyme catalyzed transesterification, insoluble excess methanol which exists as fine droplets demonstrates negative effects on enzyme activity and also decrease the production yield [211]. The reaction medium is an important factor during the determination of the optimum molar of alcohol to oil. The inactivation of lipases occurs by contact with insoluble alcohol because the highly hydrophilic alcohol eliminates the layer of essential water from the enzymes [212]. Thus, stepwise addition of alcohol is a potential approach for ratio optimizing the molar ratio in solvent free systems [15]. Whilst, higher reaction rates could be obtained with a slight excess of alcohol in organic solvent systems [15].

The two-step reaction system was reported to avoid the inactivation of the lipase by addition of excess amounts of methanol in the first-step reaction, and by addition of vegetable oil and glycerol in the second-step reaction [213]. Watanabe et al. [213], used a two-step reaction system for methyl esterification of free fatty acids and methanolysis of triacylglycerols using immobilized *Candida antarctica* lipase. The first step reaction was methyl esterification of free fatty acids that was performed by treating a mixture of 66 wt % acid oil and 34 wt % methanol with 1 wt % immobilized lipase. The second step reaction was conducted to convert triacylglycerols to fatty acid methyl esters. In this step, a mixture of 52.3 wt % dehydrated first-step product, 42.2 wt% rapeseed oil, and 5.5 wt% methanol using 6 wt% immobilized lipase in the presence of additional 10 wt % glycerol was treated. The contents of fatty acid methyl esters was 91.1wt.% after the second step reaction was repeated by the use of immobilized lipase for 50 cycles using recovered glycerol.

Moreno-Pirajan and Giraldo [81], added different amounts of alcohol varied from 2.7 to 13.7 molar equivalents for methanol and from 5.7 to 26.7 molar equivalents for ethanol, based on the moles of triglycerides toward the transesterification of palm oil catalyzed by *Candida rugosa* lipase and 10.4 molar ratio for all alcohols to palm oil was determined as optimal alcohol requirement resulted in 85 mol% of methyl esters yield with n-butanol.

Lipase catalyzed esterification of palmitic acid with ethanol in the presence of Lipozyme IM 20 in a solvent free medium was investigated by Vieira et al. [212]. Different acid/alcohol molar ratios were tried as 0.16, 0.5, 1.0, 1.5, and 1.84. The best result was obtained with 0.5 acid/alcohol molar ratio.

Zaidi et al. [214], explained the correlation existing between the kinetic parameters and the chain-length of the substrates in esterification of oleic acid using nylon-immobilized lipase in n-hexane. It is observed that the inhibition coefficient of the alcohol increased from 0.034

to 0.42 mol l^{-1} , when the number of carbon atoms increased from 1(methanol) to 18 (oleyl alcohol), respectively.

Dizge and Keskinler [69], used immobilized *Thermomyces lanuginosus* lipase to produce biodiesel with canola oil with methanol and investigated the role of substrate molar ratio. The biodiesel production was conducted at 1:1, 1:2,1:3,1:4,1:5;1:6 and 1:10 oil/alcohol molar ratios at 40°C . The highest methyl ester yield (85.8%) was obtained at the oil/methanol molar ratio of 1:6. Two important result from this study can be concluded as (i) an increase in the number of moles of methanol resulted in an increase in the ester production, (ii) when the formation of esters reached a maximum level the further increases in the methanol concentrations cause a decrease in the formation of esters due to enzyme inactivation.

Thus, the actual amount of alcohol needed varies significantly depending on the origin of the lipase and fat.

5. Reactors for enzymatic transesterification

Through the industrialization of enzymatic biodiesel production, it is necessary to show the applicability of enzymes in reactor systems. Various reactors, including batch reactors, packed bed reactors and supercritical reactors have been investigated by researchers. Most of the investigations on enzymatic synthesis of biodiesel have been performed in batch reactors and packed bed reactors.

Batch reactors are simple designs used in the laboratory. In batch reactors, methanol shows a good dispersion in the oil phase. But the physical agitation caused by shear stress from the stirring would disrupt the enzyme carrier which shortens the enzymes life [31]. On the other hand, batch operation is labor intensive, and not suitable for automation [215]. Packed bed reactors are alternative of batch reactors which are substantially faster and more economical continuous reactors [216]. A packed-bed reactor system is most widely used in biotechnology, as it is easy to operate and scale up these systems. In addition, these systems have high bed volume. The most important advantage of these systems is that it is lowering shear stress on immobilized enzymes which leads to long-term enzyme stability [217]. Furthermore, stepwise addition of alcohol can be performed to reduce the inactivation of the enzyme caused by excess alcohol. One of the encountered problems with an immobilized lipase is the inhibition of the enzyme due to the clogage of the catalyst by accumulation of the glycerol by-product inside the reactor [218]. Also, the separation of glycerol which remains in the bottom of the reactor can be achieved in a simple way by using more than one column. Recently, a packed-bed reactor system, in which a reactant solution is pumped through a column containing biomass support particles immobilized recombinant *Aspergillus oryzae* and the effluent from the column is recycled into the same column with a stepwise addition of methanol was developed by Yoshida et al. [219]. In this system, lipase retains its activity for five batch cycles and 96.1% methyl ester content was obtained with a residence time of 140 min per pass and stepwise addition of 4.25 molar equivalents of methanol to oil for 6 passes. The methanolysis of soybean oil in packed bed reactor system using

Rhizopus oryzae whole cell was studied by Hama et al. [112]. The final methyl ester content was over 90% at a flow rate of 25 l/h in the first cycle and also, after 10 cycles approximately 80% conversion was achieved. Wang et al. [216], developed *Pseudomonas cepacia* lipase – Fe₃O₄ nanoparticle biocomposite based packed bed reactors. A single-packed-bed reactor and the four-packed-bed reactor were used to produce biodiesel by using refined soybean oil. A high conversion rate (over 88%, 192 h) and great stability was achieved with the four-packed-bed reactor compared to single-packed-bed reactor. It is considered that the four-packed-bed reactor supplied a longer residence time of the reaction mixture in the reactor and lowered the inhibition of the lipase by products [216]. By this way, the reaction efficiency was improved. Additionally, the cost of biodiesel production can be reduced by the effective recycling of the enzyme catalysts [184].

Supercritical reactors also have been investigated by researchers for enzymatic biodiesel production. D. Oliveira and J. V. Oliveira [220], produced biodiesel from palm kernel oil in the presence of Novozym 435 and Lipozyme IM in supercritical carbon dioxide in the temperature range of 40–70 °C and from 60 to 200 bar using a water concentration of 0–10 wt % and oil/ethanol molar ratios from 1:3 to 1:10. Lipozyme IM showed better results and the highest reaction conversion was obtained as 77.5 %. It was observed that lipase structure changed at pressures beyond 200 bar. Madras et al. [221], synthesized biodiesel from sunflower oil in supercritical carbon dioxide catalyzed by Novozym. However, the obtained conversions, when the reaction was conducted in supercritical methanol and ethanol at the optimum conditions, were 23 and 27%, respectively [221]. Enzymatic transesterification of lamb meat fat in supercritical carbon dioxide was investigated by Taher et al. [222]. The maximum conversion (49.2%) was obtained at 50°C, with 50% Novozym 435 loading, 4:1 molar ratio, within 25 h reaction. Supercritical reactors could not be commercialized according to the low conversion rate and cost of the system.

Consequently, packed bed reactor systems seem to be a practical transesterification reactor system with high transesterification efficiency. These systems will bring industrial scale up enzymatic biodiesel production in an economic way.

6. Conclusion

Today, the growing energy necessity and environmental pollution problem requires the use of renewable alternative energy sources to become less dependent on fossil resources. As known, biodiesel is an important alternative energy resource and seems to be the fuel of future because it is an environmentally friendly, nontoxic, renewable, and biodegradable fuel.

Conventionally, biodiesel production is achieved by mainly alkaline or acid catalysts. The interest in the use of biocatalyst for biodiesel production has been an increasing trend due to its many advantages.

Biodiesel have been shown to be effectively produced by enzymatic catalyst and also, numerous researches have been performed to obtain highly active lipases and to optimize

process conditions for biodiesel production. Besides many advantages, to produce biodiesel by enzyme catalysts on an industrial scale, it is necessary to reduce the high cost of enzymes and obtain lipases with better features. The immobilization of lipases and genetic engineering methods seems to be an attractive way to obtain more active, stable, and reusable lipases in organic solvents and alcohols. Also, selection of alternative acyl-acceptors is an option for eliminating the negative effects of methanol on lipase activity.

It can be concluded that in enzyme catalyzed biodiesel production significant progresses have been made but further improvements such as novel reactor design should be addressed and emphasized in the future research in order to ensure industrial enzymatic biodiesel production. By making novel improvements, much attention will be focused on enzyme usage in biodiesel production, and especially lipase reactions will be applied much more in this area.

Author details

Sevil Yücel^{1*}, Pınar Terzioğlu^{1,2} and Didem Özçimen¹

*Address all correspondence to: yuce.sevil@gmail.com syucel@yildiz.edu.tr

1 Yıldız Technical University, Faculty of Chemical and Metallurgical Engineering, Bioengineering Department, Istanbul, Turkey

2 Mugla Sıtkı Koçman University, Faculty of Sciences, Chemistry Department, Mugla, Turkey

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