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Production of Biodiesel by Enzymatic Transesterification: Review

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Abstract: Problem Statement: The research on the production of biodiesel has increased significantly in recent years because of the need for an alternative fuel which endows with biodegradability, low toxicity and renewability. Plant oils, animal fats, microalgal oils and waste products such as animal rendering, fish processing waste and cooking oils have been employed as feedstocks for biodiesel production. In order to design an economically and environmentally sustainable biodiesel production process, a proper understanding of the factors affecting the process and their relative importance is necessary. **Approach:** A comprehensive review of the literature on the subject of biodiesel production was carried out. Traditionally biodiesel has been produced using either acid or base catalysts. The multi-step purification of end products, wastewater treatment and energy demand of the conventional process has lead to search for alternative option for production of biodiesel. The use the enzyme lipase as a biocatalyst for the transesterification reaction step in biodiesel production has been extensively investigated. Lipase is produced by all living organisms and can be used intracellularly or extracellularly. **Conclusion:** To date, the most popular microbes used for their lipases have been filamentous fungi and recombinant bacteria. A summary of lipases used in transesterification and their optimum operating conditions is provided. In addition to the choice of lipase employed, factors which make the transesterification process feasible and ready for commercialization are: enzyme modification, the selection of feedstock and alcohol, use of common solvents, pretreatment of the lipase, alcohol to oil molar ratio, water activity/content and reaction temperature. Optimization of these parameters is necessary in order toreduce the cost of biodiesel production. Use of no/low cost waste materials as feedstocks will have double environmental benefits by reducing the environmental pollution potential of the wastes and producing an environmentally friendly fuel.

Key words: Biodiesel, transesterification, enzymes, lipases, solvents, alcohols, environment

INTRODUCTION

Finite fossil fuel reserves, political, economic, health and environmental (ozone depletion, global warming, greenhouse gases) issues and/or concerns have promoted biodiesel as an alternative renewable and eco-friendly fuel. Biodiesel has shown its ability to meet the energy demand of the world in the transportation, agriculture, commercial and industrial sectors of the economy (Akoh *et al*., 2007; Basha *et al*., 2009; Shafiee and Topal, 2009; Robles *et al*., 2009). The annual world consumption of diesel is approximately 934 million tons, of which Canada and the United States consume 2.14 and 19.06%, respectively (Marchetti *et al*., 2008). As a green renewable and potentially unlimited, biodiesel has recently come out as the superlative alternative fuel which can be used in compression ignition engines with minor or no modifications (Xu and Wu, 2003; Vasudevan and Briggs, 2008; Robles *et al*., 2009; Leung *et al*., 2010).

The concept of biofuel is not new. Rudolph Diesel was the first to use a vegetable oil (peanut oil) in a diesel engine in 1911 (Akoh *et al*., 2007; Antczak *et al*., 2009). The use of biofuels in place of conventional fuels would slow the progression of global warming by reducing sulfur and carbon oxides and hydrocarbon emissions (Fjerbaek *et al*., 2009). Because of economic benefits and more power output, biodiesel is often blended with diesel fuel in ratios of 2, 5 and 20% (Vasudevan and Briggs, 2008). The higher the ratio of biodiesel to diesel the lower the carbon dioxide emission (Fukuda *et al*., 2001; Harding *et al*., 2007). Using a mixture containing 20% biodiesel reduces carbon dioxide net emissions by 15.66% (Fukuda *et al*., 2001) while using pure biodiesel makes the net emission of carbon dioxide zero (Vasudevan and Briggs, 2008).

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Biodiesel is a mixture of Fatty Acid Methyl Esters (FAMEs) which is produced from renewable resources Srivastava and Prasad, 2000). However, fats and oils are often used interchangeably referring to the feedstock employed in biodiesel production. The raw materials used for production of biodiesel can be either crude, refined or waste such as frying oils/fats (Marchetti *et al*., 2008). The feedstock can also be classified as plant derived, animal derived, microbial or waste materials (Akoh *et al*., 2007). Subramanian *et al*. (2005) identified more than 300 oil-bearing plants/trees that can be utilized to make biodiesel. The most popular plant derived oils used for biodiesel production are: canola, coconut, cottonseed, groundnut, jatropha, karanj, olive, palm, peanut, rapeseed, safflower, soybean and sunflower oils (Demirbas, 2003; Akoh *et al*., 2007; Robles *et al*., 2009). Waste oils and fats (beef tallow, lard and yellow grease), hemp oil, waste cooking oil, the greasy by-product from omega-3 fatty acids production from fish oils and microalgae oil are also considered as potential alternative feedstocks for biodiesel production (Demirbas, 2003; Marchetti *et al*., 2008; Ranganathan *et al*., 2008; Antczak *et al*., 2009).

However, there is a concern about using plant derived oils and fats since the crops used for biodiesel production are also needed for food, feed and oleochemical industries (Li *et al*., 2007; Jegannathan *et al*., 2008). Biodiesel factories must compete with food, cosmetic, chemical and livestock feed demands for the crops (McNeff *et al*., 2008). There is, also, an environmental concern because an increased demand for vegetable oils requires an increase in the use of fertilizers which contribute to greenhouse gas emissions. In fact, biodiesel production from heavy fertilized crops could result in a 70% increase (from current value) in greenhouse gas emission (Jegannathan *et al*., 2008).

The choice of feedstock depends on where the biodiesel is being produced and used which could meet norms of internationally accepted ASTM standards. Parameters such as saponification number, iodine value and cetane number of fatty acid methyl esters of the oil, also, play an important role in selection of feedstock for biodiesel production (Sharma and Singh, 2010). Today, the United States largely uses soybean oil, Europe uses rapeseed and sunflower oils, Southeast Asia uses palm oil and the Philippines uses coconut oil (Bhatti *et al*., 2008; Murugesan *et al*., 2009). Soybean oil has emerged as one of the more popular feedstock choices but has been shown to have a downside (Vasudevan and Briggs, 2008). The oxidative instability of soybean derived biodiesel limits its use to warmer climates, making it an impractical option for much of North America (Marchetti *et al*., 2008).

that can be either in a solid state (fats) or a liquid state (oils) (Ma and Hanna, 1999; Fjerbaek *et al*., 2009).

Another important factor in biodiesel production is the fatty acid composition of the source oil or fat. Oils containing higher levels of saturated fatty acids than unsaturated fatty acids (have one or more double bonds) may solidify and clog the fuel lines during the winter condition (Pinto *et al*., 2005; Akoh *et al*., 2007; Demirbas, 2008). Biodiesel which contains high levels of unsaturated fatty acids are less viscous and show higher pour and cloud points properties which make biodiesel suitable for warm and cold weather conditions. However, the use of these oils lower the cetane index and combustion temperature which reduce the quality of biodiesel. Biodiesel produced from oils with large chain fatty acids (greater than 18 carbons) have a high cetane index and combustion temperature but have low cloud and pour points and greater viscosity (Robles *et al*., 2009).

It can be said that the choice of feedstock is a balance between the unsaturation and the length of fatty acid chains (Robles *et al*., 2009). It has been predicted that feedstocks with a high level of oleic acid (an unsaturated fatty acid that is 18 carbons long with a single double bond) are the best suited for biodiesel production. Biodiesel produced from feed stocks containing oleic acid has characteristics that are the most similar to conventional biodiesel (Knothe, 2005; Robles *et al*., 2009). Table 1 shows the approximate fatty acid profile by percentage of many fats and oils used for biodiesel production.

MATERIALS AND METHODS

Because of its high viscosity and low volatility, the direct use of feedstock in diesel engines can cause problems including: high carbon deposits, scuffing of engine liner, injection nozzle failure, gum formation, lubricating oil thickening and high cloud and pour points (Fukuda *et al*., 2001; Murugesan *et al*., 2009). In order to avoid these problems, the feedstock is chemically modified to its derivatives which have properties more similar to conventional diesel (Fukuda *et al*., 2001). The free fatty acids and triglycerides contained in the oil are reduced to Fatty Acid Alkyl Esters (FAAEs) (Fjerbaek *et al*., 2009). The three most recognized methods of biodiesel production are: Pyrolysis, microemulsification and transesterification (Ma and Hanna, 1999; Murugesan *et al*., 2009).

Pyrolysis involves chemically reducing triglyceride molecules to FAAEs through the application of extreme

Table 1: Fatty acid profile of oils and fats used for biodiesel production (Akoh *et al*., 2007; Marchetti *et al*., 2007)

Oil/fat	Arachidic (20:0)	Behemic (22:0)	Gadoleic/Gondoic (20:1)	Lignoceric (24:0)	Linoleic (18:2)	Linolenic (18:3)	Oleic (18:1)	Palmitic (16:0)	Palmitoleic (16:1)	Stearic (18:0)	Other
Canola					22.3	8.2	64.4	3.5		0.9	0.7
Coconut							6.0	5.0		3.0	86.0
Cotton seed					57.5		13.3	28.3		0.9	
Groundnut					26.0		51.6	8.5		6.0	7.9
Jatropha	0.2				36.2		37.0	16.4	1.0	6.2	3.0
Karanj	1.6	5.4	1.2	1.4	17.7	3.6	51.8	10.2		7.0	0.1
Microalgae					2.2	0.9	1.3	15.5	17.3	0.3	62.5
Olive	0.4		0.3		8.5	0.7	74.2	11.8	1.5	2.6	
Palm Oil					10.1	0.2	40.5	42.6	0.3	4.4	1.9
Peanut	1.3	2.5		1.2	32.0	0.9	48.3	11.4		2.4	
Rapeseed					22.3	8.2	64.4	3.5		0.9	0.7
Safflower seed					77.0		13.5	7.3	0.1	1.9	0.2
Soybean	0.3				53.8	9.3	20.8	11.4		4.4	
Sunflower	0.3				62.4		25.5	7.1		4.7	
Tallow							44.5	29.0		24.5	2.0

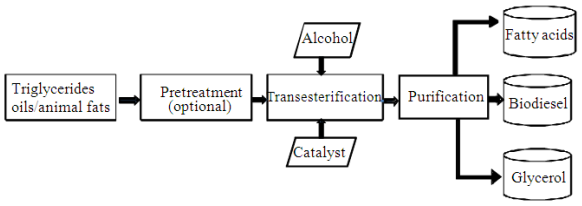
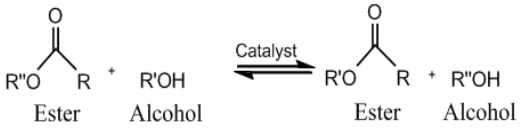


Fig. 1: Sequence of transesterification process (Pierre, 2008)

heat. Microemulsification involves the use of solvents to physically reduce the viscosity of the feedstock. Transesterification is the exchange of the alcohol moiety of an ester (contained in the feedstock) with another alcohol moiety, often from another alcohol (Ma and Hanna, 1999; Ranganathan *et al*., 2008). The sequence of the process is shown in Fig. 1.

Transesterification has been demonstrated as the simplest and most efficient route for biodiesel production in large quantities, against less ecofriendly, costly and eventual low yield methods of pyrolysis and microemulsification. Therefore, transesterification has become popular and the production method of choice (Ma and Hanna, 1999; Akoh *et al*., 2007; Robles *et al*., 2009; Ranganathan *et al*., 2008). One of the classic organic reactions (transesterification) is the step wise reversible reactions of a triglyceride (fat/oil) with an alcohol to form esters and glycerol. Little excess of alcohol is used to shift the equilibrium towards the formation of esters. A general equation for transesterification (where group R is a fatty acid, R' is the length of the acyl acceptor and R" is the rest of the triglycercide molecule) is as follows:

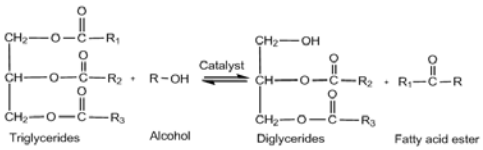


(1)

Acyl-acceptors of the transesterification reaction can be carboxylic acids (acidolysis), alcohols (alcoholysis), or another ester (interesterification). Only the latter two produce the FAAEs that make up biodiesel (Robles *et al*., 2009). Alcohols, the most frequently used acyl-acceptors, that can be used for transesterification include: methanol, ethanol, propanol, butanol, amyl alcohol, octanol and branched alcohols (Fukuda *et al*., 2001).

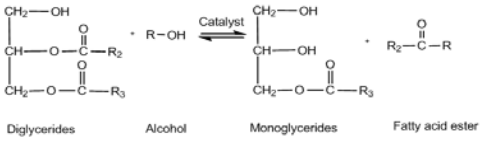
Transesterification using an alcohol is a sequence of three reversible consecutive steps. In the first step, triglycerides are converted to diglycerides. In the second step, diglycerides are converted to monoglycerides. In the third step, monoglycerides are converted to glycerin molecules (Freedman *et al*., 1984; Noureddini and Zhu, 1997; Marchetti *et al*., 2008). Each conversion step yields one FAAE molecule, giving a total of three FAAEs per triglyceride molecule as described by the following equations (Murugesan *et al*., 2009):

1. Conversion of triglycerides to diglycerides



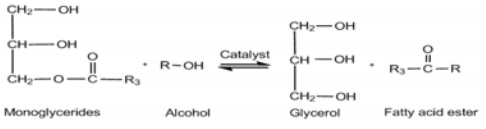
(2)

2. Conversion of diglycerides to monoglycerides



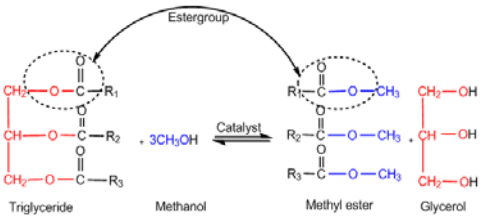
(3)

3. Conversion of monoglycerides tto glycerin molecules



(4)

Methanol is the most popular alcohol used in the transesterification process because of its relatively cheaper price compared to other alcohols. When methanol is used in the process, the reaction is known as methanolysis as shown in the following equation:



(5)

Figure 2 represents a typical methanolysis reaction of sunflower oil (sunflower oil: Methanol = 3:1 mol mol^{-1}; KOH = 0.5%; T = 25°C) in which the feed concentration of triglycerides is declining and the expected product (methyl esters) is increasing, with low concentrations of partial mono- and diglycerides (Mittelbach and Trathnigg, 2006). The use of an alcohol results in the desired FAAEs and a glycerol byproduct which can be utilized in other industries (Bacovsky *et al*., 2007). Feedstock with higher concentration of Free Fatty Acids (FFA's) may pose a problem of soap formation and lead to under reacted material, thus affecting yield. A free fatty acid is one that has already been separated from the glycerol molecule when the feedstock has been in repeated use (Leung *et al*., 2010; ISTC, 2007).

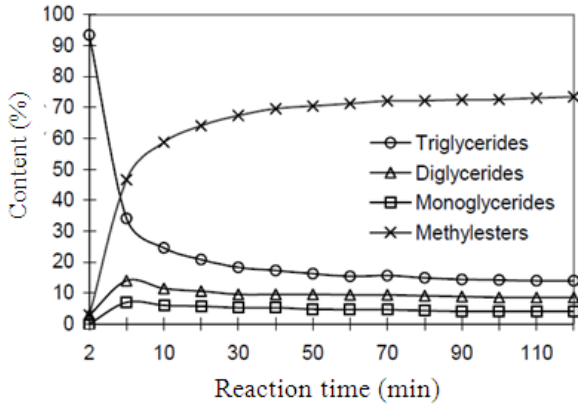


Fig. 2: Methanolysis reaction of sunflower oil (Mittelbach and Trathnigg, 2006)

Thus, there is a need to pretreat FFA's before the transesterification using one of the following methods: (a) acid esterification (b) ion exchange resins and (c) extraction with alcohol (Turkay and Civelekoglu, 1991; Ozbay *et al*., 2008; Banerjee and Chakraborty, 2009). Allowable FFA's content in the feedstock is lower than 2.5% wt. and the pretreatment step becomes necessary before the transesterification process when the FFA content is higher than 2.5% wt. (ISTC, 2007).

Transesterification can generally proceed by the simple mixing of the reactants. However, in order for the transesterification reaction to be applicable for biodiesel production, the process must be accelerated by the use of catalyst which may be alkaline, acids or enzymes (Bacovsky *et al*., 2007; Murugesan *et al*., 2009; Leung *et al*., 2010). The catalyst employed directly effects the purity of the feedstock required, the reaction rate and the extent of post reaction processing needed (McNeff *et al*., 2008). To speed up the reaction, heat is also applied. However, this process is very energy intensive and inefficient since FAAE yield below 350°C is very low and temperatures above 400°C degrade the ester bonds (Ranganathan *et al*., 2008). Generally, the reaction mix is kept just above the boiling point of the alcohol (71-72°C) to speed up the reaction. The variables known to affect the reaction are: temperature, alcohol to oil molar ratio, catalyst concentration and mixing intensity (Marchetti *et al*., 2007).

Transesterification catalysts: The transesterification process is catalyzed by alkalis, acids or enzymes. However, the use of alkali catalysts is 100% in commercial sector. The most common alkaline catalysts are sodium hydroxide (NaOH) and potassium hydroxide (KOH) (Schuchardt *et al*., 1998; Marchetti *et al*., 2008; Robles *et al*., 2009). Other alkaline catalysts include carbonates, methoxide, sodium ethoxide, sodium propoxide and sodium butoxide (Fukuda *et al*., 2001). These chemicals proved to be the most economic because of higher conversion rate of esters under a low temperature and pressure environment and short reaction time (Bacovsky *et al*., 2007; Leung *et al*., 2010). The main drawback of the technology is the sensitivity of alkaline catalysts with respects to feedstock purity. The presence of free fatty acids and water in the feedstock has a significant impact on the transesterification reaction (Leung and Guo, 2006; Marchetti *et al*., 2008). Representation of alkali transesterification process is shown in Fig. 3. Besides the multi step purification of end products, alkaline transesterification requires treatment for the waste water that is produced from the process.

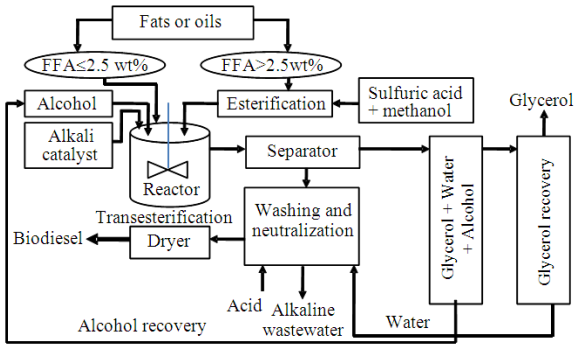


Fig. 3: Process flow schematic for production of biodiesel by alkali process (Bacovsky *et al*., 2007; Leung *et al*., 2010)

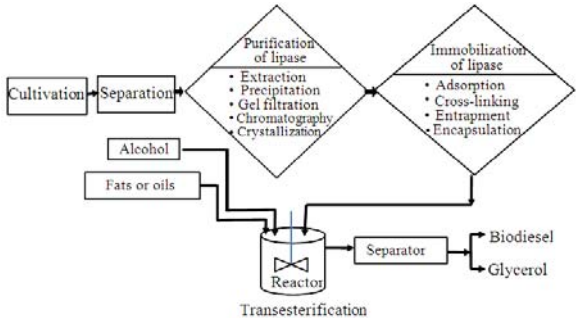


Fig. 4: Enzymatic production of biodiesel with immobilized lipase (Du *et al*., 2008; Fukuda *et al*., 2001)

The amount of waste water produced is approximately 0.2 ton per ton biodiesel produced. The need for extensive downstream processing makes alkaline transesterification expensive and not environmentally friendly (Fjerbaek *et al*., 2009).

The second commercially used catalysts are acid-catalysts. The most commonly employed acids are: Sulfuric acid, hydrochloric acid and sulfonic acid. Despite the fact that yield is very high and no soap formations, the corrosive nature of acid, very slow reaction rate and higher temperature conditions limit the use of the technology for esterification reactions (Freedman *et al*., 1984; Bacovsky *et al*., 2007).

The acid and alkali Transesterification processes are energy intensive and require extensive downstream processing (Xu and Wu, 2003). Post treatments are required after the completion of transesterification reaction as the end products are a mixture of esters, glycerol, mono-and diacylglycerols, pigments, unreacted alcohol, catalyst and tri, di and monoglycerides. These post treatment include a multi-step purification of end products which include: (a) separation of glycerol by gravitational settling or centrifugation, (b) neutralization of the catalyst, (c) deodorization and (d) removal of pigments (Antczak *et al*., 2009; Banerjee and Chakraborty, 2009).

Enzymatic transesterification is, therefore, an attractive method for biodiesel production over chemical methods because of the reduced feedstock limitations, downstream processing and environmental impact (Jegannathan *et al*., 2008). The use of enzyme catalysts eliminates these problems associated with acid and alkali catalysts as well as presents other production benefits.

Unlike the alkaline catalysts, enzymes do not form soaps so there is no restriction on free fatty acid content (Harding *et al*., 2007; Fjerbaek *et al*., 2009). Unlike the acid catalysts, enzymes are not severely inhibited by water, so there is little concern about water production (Dizge and Keskinler, 2008). Since the enzymes are capable of completely converting free fatty acids to FAAEs, low cost feedstocks such as waste oils and lard can be used (Fukuda *et al*., 2001). The enzymes are most often immobilized when used, which simplifies the separation of products, produces a high quality glycerol and allows for the reuse of the catalyst (Akoh *et al*., 2007; Robles *et al*., 2009).

Enzymatic transestrification of triglycerols: Enzymes are biological catalysts which allow many chemical reactions to occur within the homeostasis constraints of a living system. Enzymes have enormous potential for reducing energy requirements and environmental problems in the chemicals and pharmaceutical industries. Over the last two decades, substantial research has been performed on the use of enzymes in the synthesis of various organics (Roberts, 1989; Arnold, 1998). Large scale applications of enzymes have been reported in the production detergents, drinks and textiles, starch hydrolysis and fructose production, genetic engineering, semisynthetic penicillins, rare sugars, leather, pulp and study, baking and lipase based reactions (Kudli-Shrinivas, 2007). Enzyme catalyzed transesterification reactions have been extensively used in production of drug intermediates, biosurfactants and designer fats (Shah *et al*., 2003).

Enzymatic approach for production of biodiesel has been extensively reported, although this technology has not received much commercial attention except in china where the first industrial scale for biodiesel production in the world (with lipase as the catalyst at a capacity of 20,000 tons year^{-1}) is in operation (Du *et al*., 2008). Presentation of enzymatic production of biodiesel with immobilized lipase is shown in Fig. 4.

Table 2: Comparison of alkali catalyst and biocatalyst transesterification (Shah *et al*., 2003, Fukuda *et al*., 2001)

Major factors	Alkali catalyst transesterification	Biocatalyst transesterification
Temperature	60-80°C	20-60°C
Presence of FFA's in feed stock	Soap formation	Completely conversion into the methyl ester
Presence of water	Towards for more soap formation as hydrolysis of the oil may takes place	No effect on final product
Yield of biodiesel production	High, nearly 99%	Comparatively lower than alkali catalyst, around 90%
Down stream processing	Multi-step purification of end products	None
Biodiesel production cost	Cheap, as catalysts are comparatively cost less	Really expensive as biocatalyst are expensive
Commercialization	100% commercialized	Not exactly
Waste water generation	Saline and alkaline effluents needs treatment before discharge	No waste water generation

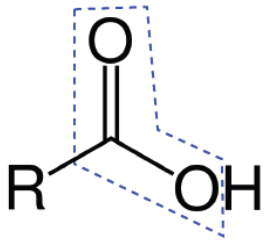


Fig. 5: The dotted blue line outlines the ester group of the carboxylic acid (Joseph *et al*., 2008)

The benefits of using enzymes as catalyst over the acid and alkali catalysts are: (a) no soap formation (b) have ability to esterify both FFA's and triglycerides in one step without the need of a washing step (c) capitulate a higher quality glycerol (d) ability to handle large variation in raw material quality (e) a second generation raw materials like waste cooking oils, animal fat and similar waste fractions, with high FFA and water content, can be catalyzed with complete conversion to alkyl esters with significantly condensed amount of wastewater and (f) works under milder conditions (which lead to less energy consumption) with lower alcohol to oil ratio than chemical catalysts (Narasimharao *et al*., 2007; Tamalampudi *et al*., 2008; Fjerbaek *et al*., 2009). A comparison of alkali catalyst transesterification versus biocatalyst transesterification is presented in Table 2.

However, enzymatic transesterification has several drawbacks: (a) longer reaction time. (b) higher catalyst concentration is required to completion of reaction, (c) high cost of production (enzymes cost $1000 US per kg whereas sodium hydroxide is only $0.62 US per kg), (d) although repeated use of lipase becomes possible after immobilization of lipase on carrier, it loses its activity in 100 days of application (Bacovsky *et al*., 2007; Jeong and Park, 2008; Fjerbaek *et al*., 2009).

Lipases as biocatalysts: Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) constitute a diverse and ubiquitous family of enzymes which are produced by animals, plants and microorganisms. The animal lipase most commonly used is the pancreatic lipase. Plant lipases include papaya latex, oat seed lipase and castor seed lipase (Akoh *et al*., 2007). Microbs have been found to produce high yields of lipases compare to the animal and plants. Because their bulk production is easier, commercialization of microbial lipases and their involvement in enzymatic biodiesel production are more common than animal and plant ones (Hasan *et al*., 2006; Akoh *et al*., 2007; Antczak *et al*., 2009). Lipases from microorganisms (bacterial and fungal) are the most used as biocatalysts in biotechnological applications and organic chemistry.

The physical and biochemical properties vary among lipases. As such, each industrial application requires lipases with specific properties. Therefore, there is always interest in new lipases that could be used in new applications (Aires-Barros *et al*., 1994; Abramic *et al*., 1999). Lipases have been successfully used in novel biotechnological applications for the synthesis of biopolymers and the production of enantiopure pharmaceuticals, flavor compounds, agrochemicals and biodiesel (Jaeger and Eggert, 2002).

Lipases are considered hydrolases which naturally hydrolyse triacylglycerols (Salis *et al*., 2005) and are capable of catalyzing other unnatural reactions such as the alcoholysis of 15 triglycerides (Jaeger and Reetz, 1998; Joseph *et al*., 2008). They act on the ester bonds of carboxylic acids (Fig. 5) allowing them to carry out their primary reaction of hydrolyzing fats (Joseph *et al*., 2008). Many lipases are limited because they are fatty acid chain is length specific, substrate specific and regioselective. However, the majorities of lipases are capable of converting triglycerides, diglycerides, monoglycerides and free fatty acids to FAAEs in addition to fat hydrolysis (Akoh *et al*., 2007; Joseph *et al*., 2008). It is the stability of lipases that allows them to catalyze the unnatural reaction of transesterification (Jegannathan *et al*., 2008).

The advantages of using lipases in biodiesel production are: (a) ability to work in very different media which include biphasic systems, monophasic system (in the presence of hydrophilic or hydrophobic

solvents), (b) they are robust and versatile enzymes that can be produce in bulk because of their extracellular nature in most producing system, (c) many lipases show considerable activity to catalyze transesterification with long or branched chain alcohols, which can hardly be converted to fatty acid esters in the presence of conventional alkaline catalysts, (d) products and byproduct separation in down stream process are extremely easier, (e) the immobilization of lipases on a carrier has facilitated the repeated use of enzymes after removal from the reaction mixture and when the lipase is in a packed bed reactor, no separation is necessary after transesterification and (f) higher thermostability and short-chain alcohol-tolerant capabilities of lipase make it very convenient for use in biodiesel production (Bacovsky *et al*., 2007; Kato *et al*., 2007; Robles *et al*., 2009).

The limitations of using lipases in biodiesel production include: (a) significant cost, (b) the risk that glycerol inhibits the lipase by covering it, due to its accumulation in the reaction mixture; (c) initial activity may be lost because of volume of the oil molecule (Marchetti *et al*., 2008; Robles *et al*., 2009). However, more research is needed in order to be able to use modified lipase on a large scale.

Microbial lipases: Microbial lipases come from a variety of sources. Gupta *et al*. (2004) referenced 38 distinct bacterial sources from which common lipase are derived. The microbes that have been suggested for biodiesel production include: *Aspergillus niger, Bacillus thermoleovorans, Burkholderia cepacia, Candida antarctica, Candida cylindracea, Candida rugosa, Chromobacterium viscosum, Fusarium heterosporum, Fusarium oxysporum, Getrichum candidum Humicola lanuginose, Oospora lactis, Penicillium cyclopium, Penicillium roqueforti, Pseudomonas aeruginosa, Pseudomonas cepacia, Pseudomonas fluorescens, Pseudomonas putida, Rhizomucor miehei, Rhizopus arrhizus, Rhizopus chinensis Rhizopus circinans, Rhizopus delemr, Rhizopus fusiformis, Rhizopus japonicus* NR400, *Rhizopus oryzae, Rhizopus stolonifer* NRRL1478, *Rhodotorula rubra, Saccharomyces cerevisiae, Staphylococcus hyicus, Thermomyces lanuginose* (Akoh *et al*., 2007; Fjerbaek *et al*., 2009).

Of these microorganisms, *Candida antarctica, Candida rugosa, Pseudomonas cepacia, Pseudomonas fluorescens, Rhizomucor miehei, Rhizopus chinensis, Rhizopus oryzae* and *Thermomyces lanuginosa* have produced the most effective lipases for transesterification (Vasudevan and Briggs, 2008). *Candida antarctica* displayed high activity in methanolysis and ethanolysis but showed a lower conversion yield for other alcohols. Methanolysis using *Candida antarctica* in solvent free environment resulted in a 90% conversion in majority of studies. Ethanolysis using *Candida antarctica* in a solvent free medium resulted in 82% conversion (Mittelbach, 1990). Rodrigues *et al*. (2008) reports that the conversion yield decreases proportionally to the increase in carbon length of the alcohol. *Candida antarctica* gave a 90% conversion in methanolysis involving a tert-butanol solvent but only a 45% conversion in butanolysis in a solvent free medium (Salis *et al*., 2005; Li *et al*., 2006).

Methanolysis and ethanolysis in the absence of a solvent using *Pseudomonas cepacia* gave a 67% and 65% conversion respectively (Noureddini *et al*., 2005). Butanolysis of *Pseudomonas cepacia* in a solvent free medium gave a conversion yield of 100% (Salis *et al*., 2005). According to Rodrigues *et al*. (2008) *Rhizomucor miehei* presented the highest conversion yield in butanolysis over shorter chained alcohols. In a solvent free medium the butanolysis resulting in a 99% conversion (Salis *et al*., 2005). *Thermomyces lanuginosa* showed the highest conversion in methanolysis. Reactions with ethanol, propanol and butanol showed no significant variations (Rodrigues *et al*., 2008). Methanolysis in a tert-butanol solvent gave an 85% conversion (Li *et al*., 2006). The combination of two or more lipases has also been suggested in order to lower cost and optimize conversion. Li *et al*. (2006) used a combination of *Candida antarctica* and *Thermomyces lanuginosa* and obtained a 95% conversion in methanolysis using a *tert-butanol* solvent. Lee *et al*. (2002) was successful in using a combination of *Rhizopus oryzae* and *Candida rugosa*.

The lipases produced by organisms can be used in various application sectors in different form: extracellular or intracellular (immobilized and regiospecific). Extracellular lipase refers to the use of the enzyme that has been previously extracted from the producing organism and purified. Intracellular lipase refers to the use of the enzyme while it is still contained in the producing organism (Robles *et al*., 2009). Both extracellular and intracellular lipase can be immobilized on a solid support (Jegannathan *et al*., 2008). Lipases can also be regiospecific which means they only act on specific bonds of the triglyceride molecule (Robles *et al*., 2009).

Extracellular lipase: Microbial lipases are mostly intracellular which can be produced by submerged fermentation or solid state fermentation. The

fermentation process is followed by purification steps as a certain degree of purity simplifies their successful usage as biocatalysts (Balaji and Ebenezer, 2008; Barberis *et al*., 2008). The important purification step for producing extracellular lipase is a complex process and it depends on the origin and structure of the lipase (Palekar *et al*., 2000; Saxena *et al*., 2003). The large scale production of extracellular lipases should be economical, fast, easy and efficient. Unfortunately, the cost of novel purification technologies is higher (Bandmann *et al*., 2000; Joseph *et al*., 2008). The majority of immobilized lipases that are commercially available are extracellular (Robles *et al*., 2009). The most commonly used ones are: Novozym 435 which is lipase from *Candida antarctica*, Lipzyme RM IM which is lipase from *Rhizomucor miehei* and Lipozyme TL IM which is lipase from *Thermomyces lanuginosus* (Robles *et al*., 2009).

Intracellular lipase: The biggest issue with enzymatic biodiesel production is the cost of enzymes. Thus, eliminating the costly step (the purification needed for extracellular lipases) has led to using whole cells as biocatalysts. Direct use of compact cells for intracellular production of lipases or fungal cells immobilized within porous biomass support particles as a whole biocatalyst represents an attractive process for bulk production of biodiesel and polyesters (Iftikhar *et al*., 2008). The utilization of lipase while still contained in the cells is referred to as intracellular lipase (Robles *et al*., 2009). Some microorganisms are able to be spontaneously immobilized on certain supports. This eliminates the costly purification step and the need for an extended immobilization process, which is necessary with extracellular lipase (Fukuda *et al*., 2001). Using intracellular lipases as opposed to extracellular lipasess slows down the transesterification process (Robles *et al*., 2009), although their use increases the conversion efficiency since the lipase is relatively stable (Klibanov, 1983; Ranganathan *et al*., 2008). Only a handful of microorganisms have been used as whole cell biocatalysts: *Candida antarctica*, *Rhizopus chinensis*, *Rhizopus oryzae* and *Saccharomyces cerevisiae*, with the latter being the least popular option (Fukuda *et al*., 2008; 2009; Robles *et al*., 2009). It has been shows that *Rhizopus oryzae* whole cells can efficiently catalyze the methanolysis of vegetable oils and *Rhizopus chinensis* whole cells are efficient in transesterification of short chain fatty acids (Qin *et al*., 2008). In comparison with *Candida antarctica*, *Candida rugosa*, *porcine pancreas* and *Pseudomonas cepacia*, *Rhizopus chinensis* showed the highest catalytic ability in the transesterification of soybean bean in a solvent free system (Qin *et al*., 2008).

Immobilization of lipase: Immobilization of lipase is the attachment of the enzyme onto a solid support or the confinement of the enzyme in a region of space (Jegannathan *et al*., 2008). Immobilization can, also, be seen as the transformation of a mobile enzyme to an immobile one which overcomes the longer reaction time and/or the lower enantioselectivity (Klibanov, 1983; Kamori *et al*., 2000). Proper strategy for the development of lipase immobilization technology provides a number of important benefits including: (a) enzyme reuse, (b) easy of separation of product from enzyme and (c) the potential to run continuous processes via packed-bed reactors (Peilow and Misbah, 2001). In some cases, the activity and stability in terms of thermal, chemical and mechanical properties of the enzyme are, also, improved, thereby allowing their applications under harsher environmental conditions such as pH, temperature and organic solvents (Awang *et al*., 2007; Bhushan *et al*., 2008).

In the specific example of transesterification for biodiesel production, the lipase can be easily separated from the triglyceride molecules, free fatty acids, glycerol and FAAEs which makes the biodiesel production economical feasible (Vasudevan and Briggs, 2008). Salah *et al*. (2007) found that the butanolysis of acetic acid gave a conversion of only 3% with free lipase and a conversion of 25% with immobilized lipase. It is, therefore, thought that immobilization helps overcome the inhibition of the acylacceptor.

The cost of lipase makes up 90% of the total cost of enzymatic biodiesel production. A significant portion of that is associated with the use of expensive carrier or support materials. The chosen support system should be low cost and allows for sufficient mass transfer to optimize reaction efficiency (Dizge *et al*., 2009a). Search for cheaper support materials has been ongoing in order to reduce the overall cost of enzymatic biodiesel production (Robles *et al*., 2009). However, the choice of carrier molecule is not only dependent on its cost, but also its mechanical strength, microbial resistance, thermal stability, chemical durability, chemical functionality, hydrophobic/hydrophilic character and loading capacity (Malcata *et al*., 1990; Jegannathan *et al*., 2008).

Both whole cells and extracellular lipases should be immobilized so that they resemble ordinary solid-phase catalysts that are conventionally used in chemical reactions (Fukuda *et al*., 2001). Although there are over 100 specific immobilization techniques, all can be classified under four general techniques: (a) adsorption,

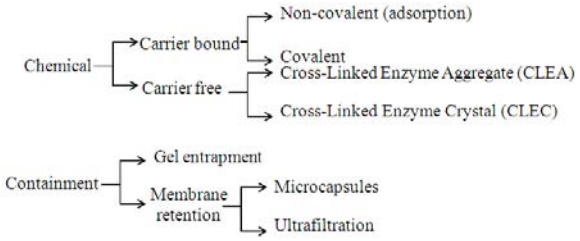


Fig. 6: Methods of enzyme immobilization (Illanes *et al*., 2008)

(b) cross linking, (c) entrapment and (d)encapsulation (Klibanov, 1983). Immobilization techniques commonly employed can also be either chemical or containment which involves the interaction of enzyme with a matrix through a chemical bond or an enzyme contained within restricted space as shown in Fig. 6 (Malcata *et al*., 1990; Illanes *et al*., 2008; Jegannathan *et al*., 2008).

The basic chemical techniques include adsorption and cross linking while the basic physical techniques include entrapment and encapsulation (Vaidya *et al*., 2008; Nasratun *et al*., 2009). Each of these techniques involves different levels of complexity, enzyme activity and conversion efficiency but essentially any immobilization improves the technological properties of the enzyme (Klibanov, 1983). The selection of which technique to employ is dependent on process specifications for the catalyst including: desired enzyme activity, cost limitations and desired final properties of the immobilized lipase (Nasratun *et al*., 2009; Malcata *et al*., 1990). The biocatalyst properties are ultimately defined by the choice of immobilization strategy (Dizge *et al*., 2009b). It is important to note that all of these techniques can be employed for both extracellular and intracellular lipases (Klibanov, 1983).

Adsorption: Adsorption is the simplest and cheap method of immobilization. It is defined as the attachment of the enzyme to the surface of the more or less hydrophobic supports by combination of hydrophobic, Van der Waals, or electrostatic forces (Yong and Al-Duri, 1996; Fernandez-Lafuente *et al*., 1998). The most common carriers used in adsorption via weak forces include: Toyonite, celite, cellulose polyprolene, acrylic, silica gel, textile membranes, spherosil, sepharose, sephadex and siliconized glass (Malcata *et al*., 1990; Jegannathan *et al*., 2008). The most common carriers used when covalent bonds are employed are: Porous glass and ceramics, sand, cellulose, synthetic polymers and metallic oxides (Klibanov, 1983). This technique may have a higher commercial potential because it is: (a) simpler, (b) less expensive, (c) no chemical additives are required; (d) there is large mass transfer rate of substrate and (e) can retain high catalytic activity (Fukuda *et al*., 2001: Gao *et al.*, 2006).

The adsorption of lipase onto porous support may be one of the most widely employed ways used in continuously operated packed beds and stirred tank reactors, especially in large-scale operations (Gao *et al.*, 2006). The major limitations of adsorption is the risk of the enzyme being stripped off the support and enzyme loss has been reported near the end of the transesterification reaction when the amount of glycerol becomes high (Malcata *et al*., 1990; Jegannathan *et al*., 2008). The stability of the enzyme when adsorbed is very low, which makes the reuse of the enzyme difficult when compared to other immobilization methods (Jegannathan *et al*., 2008)

Cross linking: Cross linking is the act of chemically linking lipase molecules with one another through the use of reagents to form a more robust structure and it becomes attractive because the final preparation is basically pure protein with a high concentration of enzyme per unit volume (Malcata *et al*., 1990; Lopez-Serrano *et al*., 2002). The reagents used include gluteraldehyde, bisdiazobenxidine and hexamethylene diisocyanate, with the most commonly used being gluteraldehyde (Jegannathan *et al*., 2008). Immobilization in this fashion does not involve any matrices, cross linking occurs both intermolecularly and intramolecularly (Klibanov, 1983). The use of cross linked enzyme aggregates accelerates the rate of transesterficiation. Overall conversions have been found to be rather high (90%) but sometimes it is difficult to separate them from the reaction mixture because of their small size (Jegannathan *et al*., 2008).

Entrapment: Entrapment entails the capture of lipase within the inner cavities of a matrix of polymer, often a gel such as alginate (Cheetham *et al*., 1979; Malcata *et al*., 1990; Shtelzer *et al*., 1992; Illanes *et al*., 2008). Lipases that are immobilized by entrapment are more stable and display better activities than those immobilized by adsorption (Malcata *et al*., 1990). Gels and other polymers employed for entrapment can either be covalent or noncovalent. The most common gels used are: methylenebisacylamide, calcium alginate and kappacarrageenan (Klibanov, 1983). The procedure used to entrap the lipase is relatively simple, quite robust and easy to recover during continuous operation but the cost factor is not as low as adsorption (Meter *et al*., 2007). The biggest disadvantage to entrapment is the mass transfer

limitation (Malcata *et al*., 1990). Because of the issues with mass transfer, the overall conversion is only approximately 65% which is lower than both adsorption and cross linking (Jegannathan *et al*., 2008).

Encapsulation: Encapsulation is relatively similar to entrapment, but encapsulation involves the confinement of the enzyme within a porous membrane such as small beads or capsules (Malcata *et al*., 1990) and successfully used for enzyme microencapsulating (Serralheiro *et al*., 1990; Vicente *et al*., 1994). The utilization of encapsulation allows for a separation of enzyme from the reaction mixture; it provides a cage which prevents the enzyme from leaking out, mixing with the reaction mixture and improves mass transfer (Khan and Vulfson, 2001). The conversion is to be low because of the limited permeability of the membrane which limits the lipases activity on large molecules such as triglycerides (Malcata *et al*., 1990). This also implies that the membrane may become clogged or a film layer may form, either of which would severely inhibit the reaction and decrease enzyme activity (Antczak *et al*., 2009; Fjerbaek *et al*., 2009).

Properties of lipases: Lipase Specificity: The specificity of a lipase refers to its regioselectivity for specific positions on the triglyceride molecule. Lipases can be classified according to their selectivity for the acyl position (regioselectivity) on the glycerol backbone (Chandler, 2001). Each lipase has been deemed one of three types: 1,3 specific, 2 specific, or non specific (Koskinen and Klibanov, 1996; Rahman *et al*., 2005). 1,3 specific lipases act primarily on the ester bonds on the extreme positions of the triglyceride molecule and rarely attack the middle ester bond. 2 specific lipases primarily attack the middle ester bond on the triglyceride molecule. Non specific lipases show no preference to the ester bonds they attack (Macrae, 1983). The most common 1,3 specific lipases are *Rhizopus oryzae*, *Thermomyces lanuginosus*, *Aspergillus niger*, *Rhizopus delemar* and *Rhizomucor miehei* (Shimada *et al*., 1997; Fukuda *et al*., 2001; Lanser *et al*., 2002; Robles *et al*., 2009). The only 2 specific lipase that has been mentioned in the literature is *Geotrichum candidum* which is not commonly used for transesterification (Macrae, 1983). The most commonly used non specific lipases are *Candida antarctica*, *Candida cylindracea*, *Candida rugosa*, *Pseudomonas cepacia and Pseudomonas fluorescens* (Fukuda *et al*., 2001).

Regioselective lipases were not believed to be applicable to biodiesel production since they do not act on all ester bonds of the triglyceride molecules. It was however, later discovered that they efficiently catalyze transesterification with yields often greater than 90%, exceeding the estimated 66% yield (Antczak *et al*., 2009). It was suggested that the reason for the unexpected high yield is spontaneous acyl migration (Fukuda *et al*., 2001). It was later verified by thin layer chromotography, that acyl moities migrate from the 2 position to either the 1 or 3 positions on the partial triglyceride in aqueous environments (Fukuda *et al*., 2009). In order to promote acyl migration and, therefore, reaction productivity, it has been suggested to use polar immobilization supports and to add silica gel to the reaction mixture (Akoh *et al*., 2007; Robles *et al*., 2009).

Lipase Stability: The stability of the lipase without loosing its catalytic activity is the most important enzymatic characteristics when used in biodiesel synthesis (Moreira *et al*., 2007; Zheng *et al*., 2009). The environment in a reactor is often more harsh for the enzyme than when *in vivo* since enzymes are known to be more stable in their natural cell environment. Therefore, many enzymes do not remain stable when used industrially. The higher temperature, inactivating impurities and aggressive surfaces of the reactors assist in enzyme deactivation and inhibition (Klibanov, 1983). In addition to mechanical forces, lower chain alcohols, the by-product glycerol, water content and high alcohol to oil ratios can also cause destabilization and deactivation of the enzyme (Malcata *et al*., 1990; Marchetti *et al*., 2007; Robles *et al*., 2009). The loss of enzyme activity over time is often a result of thermal degradation and alcohol inhibitions (Torres *et al*., 2008). Methods that have been suggested to improve lipase stability include: Genetic engineering, molecular biology, chemical modification, physical treatments, immobilization techniques and reaction and reactor engineering (Malcata *et al*., 1990; Reetz, 2002; Mateo *et al*., 2007; Illanes *et al*., 2008).

Recovery and reuse: Competency of reuse and recycling of lipase is crucial factor in enzymatic biodiesel production as the high price of lipase enzymes is one of the constrains. In order to decrease the cost, enzymes must be reused while maintaining a high level of activity. Enzyme immobilization is an important approach that could be used as a tool to improve and optimize operation stability, activity and selectivity which allows the enzyme to study under harsher environmental condition and also provides their separation from the reaction mixture without filtration in case of packed bed reactor (Fernandez-Lafuente *et al*., 1998; Bhushan *et al*., 2009; Gao *et al*., 2006).) and,

hence, could lead to more favorable economical benefits. It is the cultivation method and strength of immobilization matrix which ultimately decides the longevity and durability of the enzyme (Fukuda *et al*., 2009; Robles *et al*., 2009). Stepwise addition of the alcohol (if inhibiting) has been shown to decrease the deactivation of the enzyme and, therefore, increase longevity. Stepwise addition of methanol in the transesterification of olive oil allowed for the repeated use of enzyme and the conversion rate was maintained over 85% after eight cycles (Lee *et al*., 2002). The use of solvents has been suggested to increase stabilization of the enzyme and, therefore, allows it to be used more times. It was demonstrated that a pretreatment of gluteraldehyde increased the longevity of enzymes (which normally decreased to a yield of 50% after 6 cycles of use) to yield over 70% over several cycles (Fukuda *et al*., 2008). Several cases have shown that the washing of the lipase between uses helps to increase its longevity. Li *et al*. (2007) washed immobilized lipase with tert-butanol between uses and found no obvious lose in FAAE yield even after 200 cycles of use. Huang *et al*. (2010) reported positive results using tert-butanol as a wash between cycles. The use of isopropanol allowed the reuse of the enzyme for 5 cycles with conversion over 80% (Lee *et al*., 2008). The use of hexane as a wash between cycles proved inefficient, only keeping the lipase sufficiently active for three cycles (Salah *et al*., 2007).

Factors affecting enzymatic transestrification: There are several factors which affect the rate at which transesterification proceeds and the ultimate yield of biodiesel. These include: (a) selection of alcohol, (b) use of solvents, (c) lipase pretreatments, (d) alcohol to oil molar ratio, (e) water activity/content of the system and (f) reaction temperature.

Selection of alcohol: There are a number of different compounds that have been deemed acceptable acyl acceptors for transesterification. Methyl acetate and ethyl acetate have both been seen as appropriate acyl acceptors (Xu and Wu, 2003; Modi *et al*., 2007), but have also been found to be much more expensive than the more commonly used alcohols (Vasudevan and Briggs, 2008; Robles *et al*., 2009). The use of these two acyl acceptors also results in the production of a byproduct other then glycerol (Xu and Wu, 2003). Primary, secondary, straight chained and branched alcohols can all be employed in the transesterification reaction (Fukuda *et al*., 2001). Longer chain alcohols have also shown their effectiveness; however they give lower yields than methanol (Coggon *et al*., 2007). The most commonly used alcohols are: methanol, ethanol, propanol, iso-propanol, 2-propanol, n-butanol and iso-butanol (Iso *et al*., 2001; Antczak *et al*., 2009; Varma and Madras, 2010). Alcoholysis of triolein using *Pseudomonas cepacia* was carried out in a solvent free medium with a multitude of alcohols. Methanol showed a 40% conversion, ethanol showed a 93% conversion, propanol showed a 99% conversion, 1-butanol showed a 99% conversion, 2-butanol showed a 83% conversion, 2-methyl-1-propanol showed a 99% conversion and a mixture of pentanol isomers resulted in 99% conversion (Salis *et al*., 2005).

Even though the lower linear alcohols (methanol and ethanol) are seen as the only realistic and economically feasible options, they found to be liable for deactivation and inhibition of immobilized lipase (Chen and Wu, 2003; Samukawa *et al*., 2000). It was reported that lipase was deactivated by the insoluble methanol that existed as drops in the oil or fat (Salis *et al*., 2005; Al-Zuhair *et al*., 2007). Inaddition, the hydrophilic by-product glycerol get adsorbed easily onto the surface of the immobilized lipase as it is insoluble in the oil which also surplus the inactivation of lipase activity and its operational stability (Kumari *et al*., 2009).

The degree of deactivation is estimated to be inversely proportional to the number of carbon atoms in the alcohol which means that methanol is the most deactivating alcohol (Chen and Wu, 2003; Ranganathan *et al*., 2008). It is also thought that the rate of the transesterification reaction using lipase increases with the length of carbon chain of the alcohol, implying that the use of ethanol over the use of methanol increases the rate of the transesterification reaction (Antczak *et al*., 2009). The majority of the methanol today originates from fossil fuels sources whereas the majority of ethanol is derived from renewable sources (Fjerbaek *et al*., 2009). With the increase in world ethanol production, the price of ethanol is expected to decrease which suggests that ethanol is the best choice of acyl acceptor (Ranganathan *et al*., 2008) and potentially methanol with time is the realistic choice for enzymatic transesterification for biodiesel production on commercial scale (Fjerbaek *et al*., 2009).

Two solutions have been suggested to overcome the inhibiting effects of lower chained alcohols: (a) stepwise addition of the alcohol or the sequential addition of alcohol aliquots (Shimada *et al*., 1997; 2002; Watanabe *et al*., 2002; Soumanou and Bornscheuer, 2003; Matassoli *et al*., 2009) and (b) the use of solvents (Nelson *et al*., 1996; Mittelbach, 1990; Modi *et al*., 2007). Stepwise addition of alcohol is most

commonly used for methanol since ethanol inhibition has a much smaller effect than methanol inhibition. Little to no deactivation has been noticed when a methanol to oil molar ratio below 3 is used or an ethanol to oil ratio below 11 is used (Robles *et al*., 2009). Lee *et al*. (2008) reported a 98.92% conversion of stepwise addition of methanol and a 65% conversion when methanol was added in batch in methanolysis of olive oil. Bernardes *et al*. (2007) found a similar trend in the transesterification of soybean oil and ethanol using Lipozyme RM IM. Inhibition can also be masked by using extremely high amounts of enzyme. However, this solution is impractical since it would drastically increase the cost of production (Fjerbaek *et al*., 2009). The choice of lipase also has an effect on inhibition. Lipases sourced from *Pseudomonas* have shown more resistance to alcohol inhibition than lipases from *Thermomyces lanuginosus* and *Rhizomucor miehei* (Fjerbaek *et al*., 2009).

Use of solvents: Inhibition by lower chained alcohols is often due to alcohol insolubility. Solvents are used to protect the enzyme from denaturation by alcohol by increasing alcohol solubility (Kumari *et al*., 2009). The solvent can also increase the solubility of glycerol which is beneficial since the byproduct can coat the enzyme and inhibit its performance (Royon *et al*., 2007). The use of a common solvent for the reactants and products not only reduces enzyme inhibition but also ensures a homogeneous reaction mixture, reduces the reaction mixture viscosity and stabilizes the immobilized enzyme (Ranganathan *et al*., 2008; Fjerbaek *et al*., 2009). This is beneficial because homogeneous reaction mixture decreases problems associated with a multiple phase reaction mixture and a reduced viscosity reduces mass transfer problems around the enzyme (Fjerbaek *et al*., 2009). The use of solvents significantly increases the reaction rate in comparison to solvent free systems (Vasudevan and Briggs, 2008).

The most common solvents used in transesterification are hydrophobic organic ones: hexane, isooctane, n-heptane, petroleum ether, cylohexane, 2-butanol and tert-butanol (Holmberg and Hult, 1990; Nelson *et al*., 1996; Soumanou and Bornscheuer, 2003; Ghamguia *et al*., 2004; Lara and Park., 2004; Coggon *et al*., 2007). Tert-butanol is the most popular among all these solvents (Li *et al*., 2006). It is only moderately polar, has stabilizing effects on the enzyme and is not easily influenced by the polarity of other solvents (like water) or by any of the reactants or products (Fjerbaek *et al*., 2009). Tert-butanol and 2-butanol have been suggested as treatments for the regeneration of deactivated lipase (Robles *et al*., 2009).

Tert-butanol has been tested as an effective solvent in several cases. Methanolysis conversion using *Candida antarctica* was increased when tert-butanol was added to the system (Royon *et al*., 2007). *Thermomyces lanuginosa* used for methanolysis produced a 10% conversion in a solvent free system which was increased to 75% when tert-butanol was added to the system (Li *et al*., 2006). Qin *et al*. (2008) tested various solvents and determined n-heptane to be the most efficient when *Rhizopus chinensis* was used for the methanolysis of soybean oil. The conversion was 73.4% when acetone was used, 65.8% when tert-butanol was used, 71.1% when cyclohexane was used, 73.5% when petroleum ether was used, 76.5% when n-hexane was used, 82.4% when isooctane was used and 84.2% when n-octane was used.

The use of solvents has become a recognized solution for reducing inhibitory effects of lower chained alcohols. However, several disadvantages of the use of solvents have been identified (Ranganathan *et al*., 2008). These include: (a) solvent must be separated from the final desired product (biodiesel) which requires addition processing (Vasudevan and Briggs, 2008), (b) the use of organic solvents can compromise safety since they are generally volatile and hazardous and (c) reactor volumes must also increase to compensate for the additional volume of solvent added to the reaction mixture. All of these disadvantages of using solvents could ultimately lead to increased capital and running costs of biodiesel production (Fjerbaek *et al*., 2009).

Enzymatic transesterification for biodiesel production has been also studied in absence of solvent by various researchers. Kose *et al*. (2002) investigated the alcoholysis of the refined cotton seed oil with primary and secondary alcohols in the presence of an immobilized enzyme from *Candida antarctica* in a solvent-free medium and found the yield of methyl ester to be 72 and 94%, respectively. Selmi and Thomas (1998) studied the ethanolysis of sunflower oil with immobilized 1,3 specific *Mucor miehei* lipase in a solvent-free medium with methyl ester and reported a yield of 83%.

Lipase pretreatment: Pretreatment of immobilized lipase often involves soaking the enzyme in a medium prior to use in the transesterification reaction. This pretreatment is believed to minimize the deactivation of the enzyme which is most commonly due to the use of lower chained alcohols (Ranganathan *et al*., 2008). Pretreatment in a polar organic solvent is thought to transform the enzymes hydrophobic closed active site to a hydrophobic open active site, thus enhancing its activation (Jegannathan *et al*., 2008).

Pretreatment mediums that have been employed on a small scale include: Isopropanol, methyl oleate, tert-butanol and the feedstock employed for the transesterification reaction (Fjerbaek *et al*., 2009). The pretreatment of immobilized *Candida antarctica* lipase in isopropanol showed an increased FAAE conversion over the no pretreatment (Jegannathan *et al*., 2008). Samukawa *et al*. (2000) reported on the pretreatment of immobilised *Candida Antarctica* lipase enzyme preincubated in methyl oleate for 0.5 h and subsequently in soybean oil for 12 h to reduce the deactivation of the lipase. They observed a methyl ester yield of 97% within 3.5 h of the stepwise addition of 0.33 mol equivalent of methanol at 0.25-0.40 h intervals which was maintained even after 20 cycles of methanolysis.

The methanolysis of soybean oil progressed much more rapidly when the immobilized *Candida antarctica* lipase was preincubated in methyl oleate for 30 min and subsequently in the soybean oil for 12 h (Fukuda *et al*., 2001). In both cases, the inhibitory effects of lower chained alcohols were reduced and relatively high FAAE conversions were reached (Ranganathan *et al*., 2008). To further stabilize *Rhizopus oryzae* cells, a gluteraldehyde treatment was employed. Without the treatment of the cells in a 0.1% glutaraldehyde solution conversion levels dropped to 50% after its sixth reuse whereas with the treatment, the conversion level was maintained above 70% after six cycles were completed (Ranganathan *et al*., 2008).

The pretreatment of immobilized enzymes has shown to be beneficial in small scale transesterification reactions but has yet to be used in large scale processes. It is predicted that a pretreatment would have a significant impact when batch reactors are used, but have little to no impact when continuous reactors are used (Fjerbaek *et al*., 2009). It is important to note that the use of a medium for pretreatment could greatly impact the overall cost of biodiesel production.

Alcohol to substrate molar ratio: A molar excess of alcohol to oil is needed for the transesterification reaction to proceed at a reasonable rate. Generally, the greater the molar ratio of alcohol to oil the faster the reaction rate, as long as the alcohol is soluble in the reaction mixture (Antczak *et al*., 2009). When a portion of the alcohol remains insoluble (in excess) it forms droplets which coat the enzyme causing ite deactivation. Many authors stressed that the alcohol employed in transesterification must be completely dissolved (especially methanol) implying that there is an optimum alcohol to oil molar ratio which allows for the fastest reaction rate (Jeong and Park, 2008). However, in enzyme-catalyzed methanolysis, this alcohol solubility is the limiting factor since it greatly impacts the activity of the enzyme as methanol concentration increases in solvent free reactions (Iso *et al*., 2001; Kose *et al*., 2002; Chen *et al*., 2006).

As a guideline, if the alcohol has less than three carbons it is likely to inhibit the lipase enzyme since its solubility is less than the stoichiometric ratio. Methanol and ethanol typically are soluble at 1/2 and 2/3 of their stoichiometric amounts respectively. Alcohols with greater than three carbons typically do not cause any inhibition since they often dissolve in the feedstock in their stoichiometric ratios (Shimada *et al*., 2002; Robles *et al*., 2009). In an organic solvent reaction, an excess amount of alcohol is needed in order to achieve a satisfactory reaction rate and a FAAE yield. Typically, in a solvent system, methanol to oil molar ratios should be in the range of 3:1 - 6:1 (Matassoli *et al*., 2009).

It has been suggested by many researchers that alcohol need to be added in a stepwise manner in a solvent free system so that inhibition of the enzyme is minimized (Selmi and Thomas, 1998; Kose *et al*., 2002; Vasudevan and Briggs, 2008). When methanol is employed in a solvent free system, any molar ratio of methanol to oil above 3:1 will cause significant inhibition of the enzyme (Antczak *et al*., 2009). It has been noticed that higher ratios of alcohol to oil can be employed when ethanol is used since it results in lower enzyme inhibition. In a solvent free system, inhibitory effects only become significant when an ethanol to oil ratio over 11:1 was used in the ethanolysis of fish oil using a lipoprotein lipase (Robles *et al*., 2009; Munio *et al*., 2008).

Salis *et al*. (2005) experimented with ratios of 3:1, 6:1, 9:1 and 12:1 in the butanolysis of triolein with *Pseudomonas cepacia*. The best ratios were found to be 3:1 and 6:1, both reached 100% conversion after 4 h. Ratios of 9:1 and 12:1 resulted in 100% conversion after 5 and 6 h. Jeong and Park (2008) evaluated the effect of methanol to rapeseed oil between 1:1 and 6:1 using *Candida antarctica*. It was determined that any ratio between 2:1 and 5:1 resulted in a high conversion and any ratio above 6:1 reduced conversion. However, it is important to realize that the optimum alcohol to oil molar ratio is vastly dependent on the individual system employed and the alcohol, feedstock and enzyme used.

Water activity/content: The amount of water in the reaction mixture is an important factor in enzymatic transesterification since it has an impact on both the reaction rate and FAAE yield. Water is essential in order to maintain the specific three dimensional structure of the enzyme (Lu *et al*., 2009).

The water content of a reaction mixture is expressed as water activity, or more commonly percentage concentration (Antczak *et al*., 2009). Biocatalysts often require a minimum amount of water present to maintain their activity (Jegannathan *et al*., 2008). Unmasking and restructuring of the active site of lipase can be possible in the presence of oil-water inferface as lipase activity generally depends on the available interfacial area.

The optimum water content minimizes hydrolysis and maximize enzyme activity for the transesterification reaction (Noureddini *et al*., 2005; Jegannathan *et al*., 2008) even if it is employed with lower chained alcohols (Akoh *et al*., 2007). The optimal water content is ultimately dependent on the system used, feedstock used, source of lipase, immobilization technique, enzyme stability and type of alcohol used (Jegannathan *et al*., 2008; Antczak *et al*., 2009). If the system is water free, no reaction takes place when using *Candida rugosa*, *Pseudomona cepacia* and *Pseudomonas fluorescens*. These lipases displayed an increased rate of reaction with increase water content between 1 and 20% (Akoh *et al*., 2007; Fjerbaek *et al*., 2009). *Candida antarctica* shows the highest dislike for water (Deng *et al*., 2005; Fjerbaek *et al*., 2009). *Rhizopus oryzae* lipase was found to respond in the same manner when water content was between 4 and 30% (Fukuda *et al*., 2001). It has been suggested that the reaction rate of transesterification decreases when over 0.1 grams of water is present per gram of dry enzyme. This is believed to happen because the excess water floods the pores of the enzyme support which decreases the enzymes exposure to the reaction medium (Robles *et al*., 2009).

Qin *et al*. (2008) investigated the effect of water content on methanolysis using *Rhizopus chinensis* in the absence of a solvent. The methyl ester yield reached 93% at a water concentration of 2%. Any water concentration above or below 2% resulted in a lower conversion Li *et al*. (2006) found that a water content of 2% was best suited for transesterification and a water content above 2% for methanolysis using a combination of *Thermomyces lanuginosa* and *Candida antarctica* in a tert-butanol solvent was found to dramatically decrease the methyl ester yield. Lu *et al*. (2009) investigated the effect of water content (5-20%) on methanolysis in the presence of a n-hexane solvent using *Candida* sp. 99-125 and found the maximum yield to be 80.6% at a 20% water content. Dizge and Keskinler (2008) found that methyl esters content was decreased by increasing water quantity up to 0.5% (water/total reaction volume, wt/wt).

Reaction temperature: Lipase is known to have a fairly large thermal stability (Marchetti *et al*., 2008). The conversion of transesterification is rarely influenced by temperature fluctuations so long as the temperature remains between 20 and 70°C. However, most lipases have optimal temperatures between 30 and 60°C. It is important to note that the optimum temperature for a given lipase increases when the enzyme in immobilized (Fjerbaek *et al*., 2009). Overall, the optimum temperature is dependent on enzyme stability, alcohol to oil molar ratio and the type of organic solvent used (Antczak *et al*., 2009).

Table 3: Enzymatic production of biodiesel using lipase *Candida antarctica*

Authors	Lipase Form	Feed stock	Acyl -acceptor	Alcohol to Substrate ratio	Solvent	Temp (°C)	Other conditions	Yield (%)
Mittelbach (1990)	Imm.	Sunflower oil	Methanol	-	None	-	-	3
Mittelbach (1990)	Imm	Sunflower oil	Methanol	-	Petroleum ether	-	-	79
Belafi-Bako *et al*. (2002)	Imm	Sunflower oil	Methanol	4:1 molar ratio added continuously	-	50	12 h, 130 rpm	97
Deng *et al*. (2005)	Imm	Sunflower oil	Methanol	3:1 molar ratio added in 4 steps	Propanol		24 h	93.20
Xu *et al*. (2003)	Imm	Sunflower oil	Methyl acetate	12:01	None	40	10 h	92
Mittelbach (1990)	Imm	Ethanol	Ethanol	-	None	-	-	82
Samukawa *et al*. (2000)	Imm	Soybean oil	Methanol	- -	-	-	Pre incubated in ethyl oleate for 0.5 h	97
Watanabe *et al*. (2002)	Imm	Soybean oil	Methanol	-	None	-	Stepwise addition of methanol	93.80
Ha *et al*. (2007)	Imm	Soybean oil		4:01	Ionic liqid [Emim][TfO]	40	12 h	80
Du *et al*. (2004)	Imm	Soybean oil	Methyl acetate	12:01	None	40	14 h, 150 rpm	92
Lee *et al*. (2002)	Imm	Tallow	Methanol	3:1 molar ratio added in 3 steps	-	30°C	72 h, 200 rpm	74
Li *et al*. (2006)	Imm	Rapeseed oil	Methanol	-	tert-butanol	-	-	95
Royon *et al*. (2007)	Imm	Cottonseed oil	Methanol	-	tert-butanol	-	-	97
Modi *et al*. (2006)	Imm	Jatropha oil	2-propanol	4:01	Hexane	50°C	8 h, 150 rpm	92.8-93.4

Methanolysis using *Candida antarctica* was performed in a temperature range of 25-55°C (Jeong and Park, 2008). The optimum temperature was found to be 40°C, anything above this causes a decrease in overall conversion. This was again varified by Lu *et al*. (2009) using *Candida* sp. 99-125. Salis *et al*. (2005) evaluated butanolysis using *Pseudomonas cepacia* within a temperature range of 20-70°C over time sing and fond the optimal temperature to be 50°C after 1 h which dropped to 40°C after 2 h. Methanolysis using *Rhizopus chinensis* was found to have an optimal temperature of 30°C in the range of 20-60°C (Qin *et al*., 2008). Methanolysis using a combination of *Rhizopus oryzae* and *Candida rugosa* showed *a*n optimal temperature of 45°C (Lee *et al*., 2008).

RESULTS AND DISCUSSION

Examples of research reported on production of biodiesel by enzymatic transesterification using different lipases (*Candida antarctica*, *Pseudomonas cepacia*, *Pseudomonas fluoresces*, *Rhizomucor miehei*, *Rhizopus oryzae*, *Thermomyces lanuginose*, *Chromobacterium viscosum* and *Mucor miehei*) are presented in Table 3-11.

Table 4: Enzymatic production of biodiesel using lipase *Candida* sp. 99-125

Authors	Lipase form	Feed stock	Acyl -acceptor	Alcohol to Substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)
Deng *et al*. (2005); Nie *et al*. (2006); Tan *et al*. (2006)	Imm.	Rapeseed oil	Methanol	3:1 molar ratio added in 3 steps	Petroleum ether	40	36 h, 180 rpm, batch stirred reactor	83
Deng *et al*. (2005); Nie *et al*. (2006); Tan et al. (2006)	Imm.	Salad oil	Methanol	-	n-hexane	40	30 h, 180 rpm, batch stirred reactor	95
Deng *et al*. (2005); Nie *et al*. (2006); Tan *et al*. (2006)	Imm.	Waste oil	Methanol	-	Petroleum ether	40	22 h, 180 rpm, 3 packed bed reactors	92
Deng *et al*. (2005); Nie *et al*. (2006)	Imm.	Vegetable oil	Methanol	-	petroleum ether	40	30 h, 180 rpm, batch stirred reactor	96

Table 5: Enzymatic production of biodiesel using lipase *Pseudomonas cepacia*

Authors	Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temp (°C)	Other conditions	Yield (%)
Kaieda *et al*. (2001)	Free	soybean oil	methanol	3:1 molar ratio added in 3 steps	-	35	90 h, 150 rpm	>80
Deng *et al*. (2005)	Imm.	Sunflower oil	1 butanol	3:1 molar ratio added in 4 steps	-	40	24 h	88.4
Noureddini *et al*. (2005)	Imm.	Soybean oil	Methanol	-	None	-	-	67
Noureddini *et al*. (2005)	imm.		Ethanol	-	None	-	-	65
Abigor *et al*. (2000)	Imm.	Palm kernel oil	Ethanol	-	None	-	-	72
Abigor *et al*. (2000)	Imm.	Palm kernel	T-butanol	-	None	-	-	62
Abigor *et al*. (2000)	imm.	Oil	N-propanol	-	None	-	-	42
Kumari *et al*. (2007)	Imm.	Mahua oil	Ethanol	4:1 molar ratio	-	40	6 h, 200 rpm	96
Shah and Gupta (2007)	Imm.	Jatropha oil	Ethanol	4:1 molar ratio	-	50	8 h, 200 rpm	98
Kumari *et al*. (2007)	PCMC	Mahua oil	Ethanol	4:1 molar ratio	-	40	2.5 h, 200 rpm	99

Table 6: Enzymatic production of biodiesel using lipase *Pseudomonas fluoresces*

Authors	Lipase form	Feedstock	Acyl-acceptor	Alcohol to Substrate ratio	Solvent	Temp (°C)	Other conditions	Yield (%)
Kaieda *et al*. (2001)	Free	soybean oil	methanol	3:1 molar ratio added in 3 steps	none	35	90 h, 150 rpm	90
Lou *et al*. (2006)	imm.	Soybean oil	Methanol	-	N-heptane	-	Use of recombinant Lip B68	92
Soumanou and Bornscheuer (2003)	Imm.	Sunflower oil	Methanol	4.5:1 molar ratio added in 3 steps	None	40	24 h, 200 rpm	>95
Deng *et al*. (2005)	imm.	Sunflower oil	Iso butanol	3:1 molar ratio added in 4 steps	-	40	24 h	45.3

Table 7: Enzymatic production of biodiesel using lipase *Rhizomucor miehei*

Authors	Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temp (°C)	Other conditions	Yield (%)
Soumanou and Bornscheuer (2003)	Imm.	Sunflower oil	methanol	3:1 molar ratio added in 3 steps	n-hexane	40	30 h, 200 rpm	>80
Soumanou and Bornscheuer (2003)	Imm.	Sunflower oil	Ethanol	3:1 molar ratio added in 4 steps	n-hexane	40	24 h	79.1
Shieh *et al*. (2003)	imm.	soybean oil	methanol	-	n-hexane	-	-	92.2

Table 8: Enzymatic production of biodiesel using lipase *Rhizopus oryzae*

Authors	Lipase form	Feedstock	Acyl-acceptor	Alcohol to Substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)
Tamalampudi *et al*. (2008)	Imm. whole cell	Jatropha oil	Methanol	3:01	-	30	60 h, glutaraldehyde treatment	80
Matsumoto *et al*. (2001)	Imm	Soybean oil	Methanol		-	37	165 h, 150 rpm	71
Kaieda *et al*. (2001)	Imm	Soybean oil	Methanol	-	None	-	-	80-90
Ban *et al*. (2001)	imm	soybean oil	methanol	-	-	-	Stepwise addition of methanol, glutaraldehyde treatment	90

Table 9: Enzymatic production of biodiesel using lipase *Thermomyces lanuginose*

Authors	Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)
Soumanou and Bornscheuer (2003)	Imm.	Sunflower oil	Methanol	3:1 molar ratio added in 3 steps	n-hexane	40	30 h, 200 rpm	>60
Deng *et al*. (2005)	Imm	Sunflower oil	1 propanol	3:1 molar ratio added in 4 steps	-	40	24 h	89.8
Deng *et al*. (2005)	Imm	Sunflower oil	2 propanol	3:1 molar ratio added in 4 steps	-	40	24 h	72.8
Li *et al*. (2006)	Imm	Rapeseed oil	Methanol	4:01	tert-butanol	35	12 h, 130 rpm	95

Table 10: Enzymatic production of biodiesel using lipase *Chromobacterium viscosum*

Authors	Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)
Shah *et al*. (2004)	Free	Jatropha oil	Ethanol	4:01	None	40	8 h, 200 rpm, addition of 1% (w v-1) water	73
Shah *et al*. (2004)	Imm	Jatropha oil	Ethanol	4:01	None	40	8 h, 200 rpm, addition of 0.5% (w v-1) water	92

Table 11: Enzymatic production of biodiesel using lipase *Mucor miehei*

Authors	Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)
Selmi and Thomas (1998)	Imm	Sunflower oil	Ethanol	3:01	None	30	5 h	83.0
Mittelbach (1990)	Free	Sunflower oil	Ethanol	3.6:1	Petroleum ether	45	5 h	82.0
Nelson *et al*. (1996)	Free	Tallow	Methanol	3:01	Hexane	45	8 h, 200 rpm	94.8
Nelson *et al*. (1996)	Free	Rapeseed oil	Methanol	3:01	Hexane	45	5 h, 200 rpm	77.3
Nelson *et al*. (1996)			Ethanol	3:01	Hexane	45	5 h, 200 rpm	98.2
Nelson *et al*. (1996)	Free	Soybean oil	Methanol	3:01	Hexane	45	5 h, 200 rpm	75.4
Nelson *et al*. (1996)			Ethanol	3:01	Hexane	45	5 h, 200 rpm	97.4

CONCLUSION

All kinds of plant oils, animal fats, greases and waste materials such as animal rendering, fish processing and cooking oil wastes have been espoused as the feedstocks for biodiesel production through transesterification. Chemical transesterification using alkalis and acids has traditionally been used. However, transesterification in presence of alkaline catalysts has been the method of choice for biodiesel production and is 100% commercialized. The multi-step purification of end products, separation of glycerol, wastewater treatment and the intensive energy use of the chemical transesterification have given chance to the less energy intensive, robust and highly active enzymatic transesterification. It has been shown that enzymatic transesterification can be carried out successfully with variety of lipases with higher yields using a large variety of oil, fats and acyl acceptors. Higher FFA and water content of substrate can be catalyzed with complete conversion to alkyl esters with significantly condensed amount of wastewater.

The process has been proved to be most limited by its high cost. Therefore, the optimal choice of lipase, alcohol and feedstock will minimize the cost of biodiesel production. There are several ways that the cost of enzymatic transesterification can be reduced. All lipases are thought to be generally expensive but some are more than others. A combination of enzymes could reduce the overall cost of the catalyst. The enzymatic approach becomes more practical through the use of different acyl acceptors, addition of solvents and enzyme modification. Solvents have been shown to decrease enzyme inhibition from low chain alcohols, increase the reaction rate and the overall conversion.

Although the use of solvents is beneficial, they are expensive and could cause increases in cost. The benefits of solvents should be investigated and weighed against increases in cost. If a solvent is used, a low cost alcohol must be used (ethanol or methanol). The benefit of using lipase as catalysts is the opportunity for catalyst regeneration and reuse which can be achieved using immobilized lipases. Pretreatments and washes between cycles should be investigated in order to increase longevity of the lipase and ultimately decrease cost. Optimization of parameters of reaction systems will reduce the cost of the production of biodiesel and will make enzymatic transesterification for biodiesel production more promising.

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