EGE UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

(MASTER OF SCIENCE THESIS)

BIODIESEL PRODUCTION

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Department Code : 405.04.01
Presentation Date : 31.01.2011

Bornova-İZMİR

2011

Özge Kılınçlı tarafından yüksek lisans tezi olarak sunulan “Biodiesel Production” başlıklı bu çalışma E.Ü. Lisansüstü Eğitim ve Öğretim Yönetmeliği ile E.Ü. Fen Bilimleri Enstitüsü Eğitim ve Öğretim Yönergesi’nin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve 31.01.2011 tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

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ABSTRACT

BIODIESEL PRODUCTION

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January 2011, 45 pages

In this study, possibility of Lipozyme TL IM catalyzed biodiesel production from sunflower oil and methanol in a solvent free medium was investigated. Effects of different reaction conditions on enzymatic methanolysis of sunflower oil have been studied as well. Different enzyme loadings including 4.5wt%, 9wt%, 13wt%, 18wt%, 27wt% based on oil weight were utilized for 2:1, 4:1, 6:1, 8:1, and 10:1 molar ratios of methanol to oil, respectively. For experiments which were resulted in a high FAME (Fatty acid methyl ester) yield, effect of temperature was also investigated. Temperatures were varied between 30, 40, 45, and 55°C. After the determination of these parameters, optimum reaction time was studied. Samples were withdrawn from the reaction medium at 2, 4, 8, and 24hour periods and analyzed for their FAME contents. After optimizing the reaction conditions such as enzyme loading, alcohol/oil molar ratio and temperature, stability of the immobilized lipase during the repeated batches was also investigated.

Keywords: Biodiesel, Lipozyme TL IM, lipase, sunflower oil.

ÖZET

BİYODİZEL ÜRETİMİ

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Yüksek Lisans Tezi, Kimya Bölümü
Tez Danışmanı: Prof. Dr. Jale YANIK
Ocak 2011, 45 sayfa

Bu çalışmada, ayçiçek yağı ve metanol kullanılarak çözücüsüz ortamda Lipozyme TL IM katalizli biyodizel üretme olasılığı araştırılmıştır. Aynı zamanda farklı reaksiyon koşullarının ayçiçek yağının enzimatik metanolizine etkileri de çalışılmıştır. Yağ miktarına göre kütlece %4.5, %9, %,13, %18 ve %27 enzim ile sırasıyla 2:1, 4:1, 6:1, 8:1, ve 10:1 molar oranlarında metanol ve yağ kullanılmıştır. Yüksek YAME (yağ asidi metil esteri) verimi ile sonuçlanan denemeler için sıcaklığın etkisi de incelenmiştir. Kullanılan sıcaklıklar 30, 40, 45, ve 55°C dir. Bu parametrelerin belirlenmesinden sonra optimum reaksiyon süresi araştırılmıştır. Reaksiyondan 2, 4, 8 ve 24 saatlik periyotlarda örnekler alınarak YAME içerikleri açısından incelenmişlerdir. Ayrıca, optimum enzim miktarı, metanol:yağ molar oranı ve sıcaklık gibi reaksiyon koşullarının belirlenmesinden sonra tekrarlanan denemeler arasında enzim stabilitesi de araştırılmıştır.

Anahtar sözcükler: Biyodizel, Lipozyme TL IM, lipaz, ayçiçek yağı.

ACKNOWLEDGEMENT

I gratefully acknowledge Prof. Dr. Jale YANIK for her considerable contributions, supports, helpful suggestions and supervision during my study and for her interest in its progress.

I am also grateful to TÜBİTAK-BİDEB and Technical University of Eindhoven for their supports and technical help in interpretation of research.

My special thanks are to my family for their care and patience during the preparation of this thesis.

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SYMBOLS AND ABBREVATIONS

Symbol	Explanation
CO_2	Carbon dioxide.
$Wt\%$	Percent in weight.
Rpm	Revolutions per minute.
Wt/wt	Weight/ weight.
U/g	Unit per gram.
U/g_{oil}	Unit per gram oil.
μL	Micro liter.
$\mathring{A}$	Angstrom.

Abbrevation	
US	United States.
EU	European Union.
FAME	Fatty acid methyl ester.
GC	Gas chromatography.
ID	Inner diameter.
MSTFA	*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide.

1. INTRODUCTION

Current world energy demand is heavily based on fossil energy sources like oil, coal and natural gas. It is well known that, increasing demand in the consumption of fossil fuels in combination with their unsustainable character will eventually lead to the exhaustion of these fossil fuels. Furthermore, the greenhouse gas emissions associated with the usage of fossil fuels became an obvious threat in environmental terms. These greenhouse gas emissions are attributed to be the major part of global climate change threat and compatible mitigation strategies are required.

All concerns about fossil fuel exhaustion, climate change threat, economic issues and energy security have resulted in governmental actions and an increasing amount of attention is directed to the search for sustainable and environmentally friendly fuels. At that point, development of an efficient biodiesel fuel has a considerable importance to help replacement of fossil fuels with a renewable, sustainable and nonpetroleum fuel in long term. Besides, biodiesel is technically acceptable because it can be used in existing engines without the need of any modifications. Its energy content, physical and chemical properties are similar to conventional diesel fuel, hence it can be used either on its own or blended in any ratio with conventional diesel (Robles-Medina et al., 2009). Additionally, most of the researchers claim that engines operated on biodiesel have lower emissions of particles, sulphur, carbon monoxide and unburned hydrocarbons compared to conventional diesel (Van Gerpen, 2005; Meher et al., 2006; Demirbas, 2007).

Biodiesel is defined as the mono alkyl esters of long chain fatty acids derived from vegetable oils or animal fats, for use in diesel engines (Adamczak et al., 2009).In conventional biodiesel, these mono alkyl esters are usually methyl esters. Several types of vegetable oils like rapeseed, sunflower, soybean or tallow, lard, waste cooking oil and greases can be used for biodiesel production. Direct usage of these oils is also possible but has several drawbacks such as high fuel viscosity, low power output, low volatility, acid contamination, carbon deposition and free fatty acid formation resulting in gum formation by oxidation and polymerization (Akoh et al., 2007; Ranganathan et al., 2008). Thus, oils are processed first to provide the necessary properties for the diesel engine.

Transesterification is the most common processing technique to convert oils to their corresponding mono alkyl esters. By this process, triglycerides, the main component of oil, are successfully converted to their corresponding mono alkyl esters. Transesterification of triglycerides involves using a short chain alcohol in the presence of a suitable catalyst obtaining biodiesel and by product glycerol. The most important parameters that influence the transesterification reaction are temperature, ratio of alcohol to oil, type and amount of catalyst, mixing intensity and purity of starting materials. In any case, a surplus of alcohol is required in order to shift the equilibrium to the production of biodiesel and by product glycerol (Verdugo et al., 2010).

In transesterification process, methanol is the most preferred alcohol, mainly because of its lower cost and higher reactivity compared to other alcohols. Nevertheless, other alcohols such as ethanol, propanol, butanol, isopropanol and tert-butanol can be used for the process. Also the catalysts used for the transesterification reaction can be classified as: alkaline catalyst, acid catalyst, enzymatic catalyst, inorganic heterogeneous catalyst (Robles-Medina et al., 2009). Of all these catalysts, alkaline catalysts such as sodium hydroxide and potassium hydroxide are widely used for industrial applications. However, the alkaline catalyzed process has several drawbacks: it is energy intensive, recovery of glycerol has difficulties, the reaction is very sensitive to both free fatty acids and water, alkaline catalyst has to be removed after the reaction and the alkaline wastewater needs treatment (Al-Zuhair, 2007).

Enzymes have been proposed to overcome the drawbacks associated with the conventional alkaline catalyzed process, and have shown promising results. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are the most studied group of enzymes for enzyme catalyzed biodiesel production. They are part of the family of hydrolases that operate on carboxylic esters and widely spread in animals, plants, moulds and bacteria. Lipases are broadly classified as intracellular and extracellular. Under natural conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase but they can also catalyze esterifications, alcoholysis, and transesterifications in non-aqueous media (Caballero et al., 2009; Freitas et al., 2009).

Utilization of an enzyme catalyst offers several advantages over the conventional method. In general, enzymes work in milder conditions in contrast with alkaline catalysts which imply reduced energy consumption. Optimum reaction temperatures for enzymatic biodiesel synthesis vary between 20 and 60°C. On completion of transesterification process, glycerol phase can be easily separated from the biodiesel phase thus; the final product does not need deodorization and neutralization. Besides, enzymes are compatible with variations in the quality of the raw material because enzymes can catalyze the transesterification of both triglycerides and free fatty acids to yield biodiesel. And low water concentrations in reaction medium can have a positive impact on the biodiesel yield. If the lipase is immobilized, it can be easily separated from the reaction mixture by filtration and can be used several times. As a result of this, downstream separation and purification of the product is easier than with alkaline catalyzed process, in which the alkaline component needs to be eliminated, using a great quantity of water. In addition, enzymes have a high specificity and can be genetically engineered to improve their efficiency and/or obtain the desired product characteristics (Antczak et al., 2009; Robles-Medina et al., 2009).

Although enzymatic biodiesel production has been investigated intensively, it is presently employed industrially only in a 20,000 tons/year pilot plant in China (Fjerbaek et al., 2009) due to certain constraints. High cost of enzyme is the main obstacle for industrial scale applications. Enzyme must show high stability and must be reused many times to reduce the cost of the catalyst to compete with alkaline process. In addition, enzymatic process has a slower reaction rate compared to the alkaline process and there is a risk of enzyme inhibition due to methanol and by product glycerol (Robles-Medina et al., 2009). These are the key issues to be addressed for industrial use of enzymes in biodiesel production to be viable.

Stepwise addition of methanol or using a hydrophobic solvent in the reaction medium has been proposed to protect the enzyme and reduce the inhibition effect of insoluble methanol on enzyme activity. And in order to eliminate the negative effect of adsorbed glycerol, addition of silica gel into the reaction system or washing the enzyme with some organic solvents periodically have been applied by many researchers (Du et al., 2005; Xu et al., 2004; Selmi and Thomas, 1998). Moreover, in enzymatic process, immobilization of the enzyme is the key step that improves enzyme activity, recovery and reuse.

In this study, some efforts have been made to explore the possibility of biodiesel production catalyzed by Lipozyme TL IM in a solvent free medium. Experiments were carried out in two steps. First of all, Lipozyme TL IM has been performed for the proposed reaction using sunflower oil and methanol. In the second step, effects of different reaction conditions on enzymatic methanolysis of sunflower oil have been investigated. After optimizing the reaction conditions such as enzyme loading, alcohol/oil molar ratio and temperature, stability of the immobilized lipase during the repeated batches was also investigated.

2. GENERAL INFORMATION

2.1 Biodiesel

Biodiesel is defined as the mono alkyl esters of long chain fatty acids derived from vegetable oils or animal fats, for use in diesel engines (Adamczak et al., 2009). Biodiesel can be used as a substitute or extender for conventional petroleum diesel because it can be used either in its pure form or can be blended with diesel fuel in any portion. The most important advantages of biodiesel are (Robles-Medina et al., 2009):

- Reduction of CO_2 emissions: The CO_2 released by the combustion of biodiesel balances CO_2 that previously fixed by photosynthesis
- Reduction of fossil fuel source depletion
- Reduction of dependence on fossil fuels
- Diversification of energy sources
- No modifications or extra equipment required for existing engines
- A new market for agricultural production surpluses
- Lower emissions of particles, sulphur, carbon monoxide and unburned hydrocarbons

Produced biodiesel needs to comply with ASTM Biodiesel Standard D6751 in US and Standard EN 14214 in EU according to existing legislation. Necessary biodiesel properties for both US and EU standards have shown in Table 2.1. The most important features are viscosity, cetane number, flash point, pour point, and cloud point. As stated to Rottig et al. (2010), high viscosities impair the transport pipelines and injection nozzles and can lead to an insufficient combustion in the cylinder. The viscosity rises with increasing chain length and degree of saturation. Cetane number is an indicator for the ignition behaviour of a chemical compound and a high cetane number stands for a short ignition delay. The flashpoint describes the tendency of the fuel to build a flammable mixture with air in order to estimate the ignition danger of the fuel. The pour and cloud points of biodiesel depend on degree of saturation and crucial for its cold flow properties. Below the temperature of the cloud point formed wax crystals may plug fuel lines and filters. Pour point is the lowest temperature at which fuel is still liquid and can be pumped through fuel lines. Additionally, output of carbon monoxide, particulate matter and nitrogen oxides are subjected to restrictions and are not allowed to exceed legally set values (Rottig et al., 2010; Canakci and Sanli, 2008).

Table 2.1 Biodiesel specificities for vehicle use according to American Standard (ASTM D-6751) and European Standard (EN 14214) (Robles-Medina et al., 2009).

Property	ASTM D-6751			EN 14214		
	Limits	Test methods	Units	Limits	Test methods	Units
Kinematic viscosity (40°C)	1,9-6,0	D 445	mm^2/s	3,5-5,0	EN ISO 3104	mm^2/s
Density (15°C)				860-900	EN ISO 3675/ EN ISO 12185	kg/m^3
Ester content				96,5 min.	prEN 14103	mass%
Cetane number	47 min.	D 613	_	51 min.	EN ISO 5165	_
Flash point	130,0 min.	D 93	°C	120 min.	ISO/CD 3679	°C
Cloud point	Report	D 2500	°C			
Water	0,050[a] max.	D 2709	volume%	500 max.	EN ISO 12937	mg/kg
Sulphated ash	0,020 max.	D 874	mass%	0,02 max.	ISO 3987	mass%
Sulphur	0,05 max.	D 5453	mass%	10,0 max.	DIN 51680	mg/kg
Copper strip corrosion	No.3 max.	D 130	_	No.1 (3h at 50°C)	EN ISO 2160	_
Carbon residue	0,050[b] max.	D 4530	mass%	0,3[c] max.	EN ISO 10370	mass%
Acid number	0,80 max.	D 664	mg KOH/g	0,5 max.	prEN 14104	mg KOH/g
Free glycerol	0,020 max.	D 6584	mass%	0,02 max.	prEN 14105	mass%
Total glycerol	0,240 max.	D 6584	mass%	0,25 max.	prEN 14105	mass%
Phosphorus content	0,001 max.	D 4951	mass%	10 max.	prEN 14107	mg/kg
Iodine number				120 max.	prEN 14111	_
Oxidative stability (110°C)				6 min.	prEN 14112	h
Monoacylglycerols				0,8 max.	prEN 14105	mass%
Diacylglycerols				0,2 max.	prEN 14105	mass%
Triacylglycerols				0,2 max.	prEN 14105	mass%
Distillation temp.	360[d] max.	D 1160	°C			

[a] Including sediment
[b] 100% of the sample
[c] 10% of the sample
[d] 90% recovered

2.2 Biodiesel Market

Biodiesel has emerged as the fastest growing industries worldwide in the last decade. In the United States, estimated biodiesel production increased from 2 million gallons (=7.56 million liters, approximately 6.700 metric tons) to 700 million gallons (=2.646 billion liters, approximately 2.33 million metric tons) between 2000 and 2008 with a great production increase. In Europe, estimated biodiesel production increased from 1.065 million metric tons to 7.715 million metric tons between 2002 and 2008 (Knothe, 2010). World biodiesel production and capacity is represented in

Figure 2.1. Biodiesel production in the world has increased from 1.8 million tons to 11.1 million tons between 2002 and 2008. At the same time, world biodiesel capacity has shown an increase from 2.2 million tons to 32.6 million tons (Thurmond, 2008).

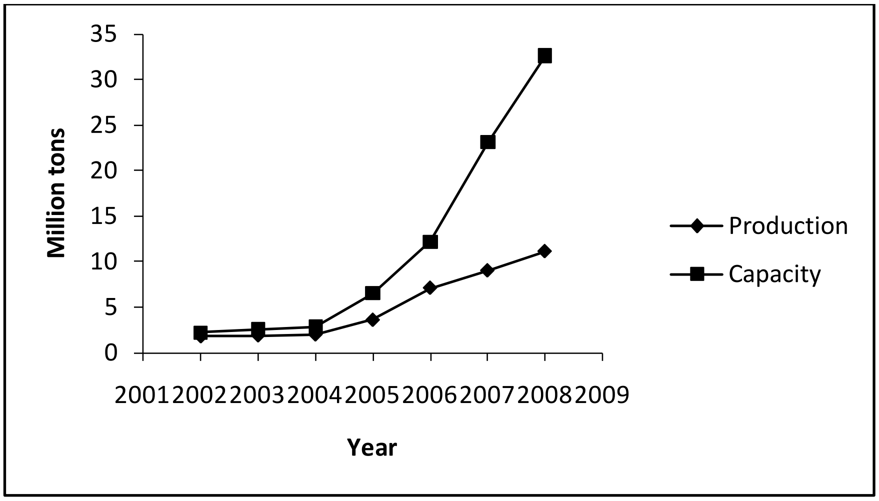


Figure 2.1 World biodiesel production and capacity (Thurmond, 2008).

Most of the total biodiesel production comes from European countries. Substantial support from the European government such as fuel tax reduction, tax incentives and loan guaranties for the production are claimed to be responsible for this situation (Lam et al., 2010). Figure 2.2 represents an overall picture of the Europe biodiesel capacity for 2010. Biodiesel production capacity in the EU for 2010 is estimated at 21.904 million metric tons, 4.933 million thereof in Germany, 4.100 million in Spain, 2.505 million in France, 2.375 million in Italy, and 1.036 million in The Netherlands. In 2009, total biodiesel production for EU was 9.046 million metric tons with 16.6% increase compared to 2008. The larger biodiesel producer in the EU was Germany with 2.539 million metric tons biodiesel production in 2009. France as the second largest biodiesel producer in the EU increased biodiesel production from 1.815 million metric tons to 1.959 million metric tons between 2008 and 2009. The third and the fourth largest producers in the EU were Spain and Italy, respectively European Biodiesel Board, 2011).

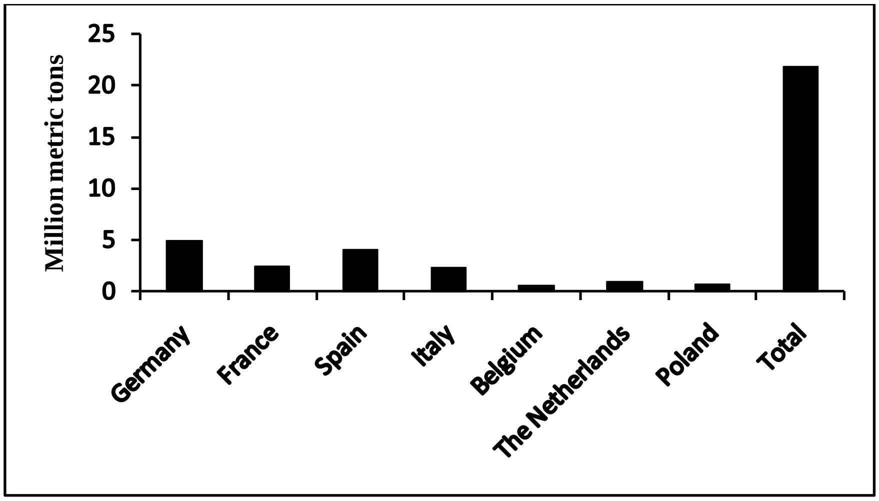


Figure 2.2 EU biodiesel production capacity 2010 (European Biodiesel Board, 2011).

2.3 Transesterification

Direct usage of vegetable oils with the existing diesel engine is possible by blending them with conventional diesel fuels in a suitable ratio. However, direct usage of these oils is unsatisfactory and impractical for long term usages due to the problems such as high viscosity, acid contamination, free fatty acid formation resulting in gum formation by oxidation and polymerization and carbon deposition (Ranganathan et al., 2008). Thus oils are processed first in order to get the necessary properties similar to those fossil fuels.

Transesterification is the most popular method for producing biodiesel from different oil types. Although vegetable oils are widely used for the transesterification process; animal fats, waste cooking oil, greases or algal oil can be used for biodiesel production as well. All vegetable oils and animal fats consist primarily of triglyceride molecules (Bisen et al., 2010). By transesterification process, those triglycerides, the main component of oil, are successfully converted to their corresponding mono alkyl esters. Transesterification of triglycerides involves using a short chain alcohol in the presence of a suitable catalyst obtaining biodiesel and by product glycerol. General transesterification reaction is shown below:

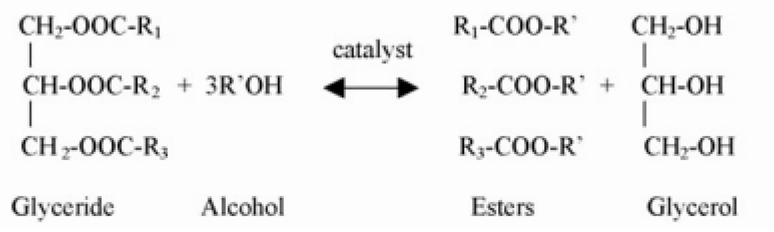


Figure 2.3Transesterificationreaction of triglycerides (Enweremadu and Mbarawa, 2009).

Transesterification reaction consists of a sequence of three consecutive reversible reactions. The first step is the conversion of triglycerides to diglycerides, followed by the conversion of diglycerides to monoglycerides, and finally monoglycerides to glycerol, yielding one fatty acid alkyl ester for each glyceride at each step (Enweremadu and Mbarawa, 2009). Since the reactions are reversible, a surplus of alcohol is required to shift the equilibrium to the production of biodiesel and by product glycerol. The most important parameters that influence the transesterification reaction are temperature, ratio of alcohol to oil, type and amount of catalyst, mixing intensity and purity of starting materials (Verdugo et al., 2010). Basic mechanism of the three consecutive steps is:

Triglyceride + Alcohol ↔Diglyceride + Fatty acid alkyl ester

Diglyceride + Alcohol ↔ Monoglyceride + Fatty acid alkyl ester

Monoglyceride + Alcohol ↔ Glycerol + Fatty acid alkyl ester

Among the alcohols that can be used in the transesterification process methanol is the most preferred alcohol for all the commercial development because of its low cost and its physical and chemical advantages such as being polar and shortest chain alcohol. Nevertheless, other alcohols like ethanol, propanol, butanol, isopropanol and tert-butanol can be used in the process (Bisen et al., 2010).

Catalysts used for the transesterification process may be classified as (Robles-Medina et al., 2009): alkaline catalysts (sodium hydroxide, potassium hydroxide, sodium metoxide); acid catalysts (sulphuric acid, phosphoric acid, hydrochloric acid, sulfonic acid); enzymatic catalysts (lipases) and inorganic heterogeneous catalyst (solid phase catalyst).

Today, most often used industrially process for biodiesel production is alkaline catalyzed transesterification process. In the alkaline catalyzed process alkaline catalysts are used along with methanol or ethanol. Initially, alcoxy is formed by reaction of the catalyst with alcohol and the alcoxy is then reacted with oil to form biodiesel and glycerol. This process is the most efficient and least corrosive of all the processes and the reaction rate is reasonably high even at a temperature of 60°C. However, the alkaline catalyzed process has several drawbacks: it is energy intensive, recovery of glycerol has difficulties, the reaction is very sensitive to both free fatty acids and water, soap formation is likely to take place, alkaline catalyst has to be removed after the reaction and the alkaline wastewater needs treatment (Al-Zuhair, 2007). If the starting oil contains small amount of free fatty acids soaps are formed that leads to a reduction in yield and increase the downstream processing of the biodiesel produced. The by-product glycerol is usually contaminated with alkaline catalysts that make the process even harder. Moreover, the need to treat the alkaline effluents generated, and the high water consumption during washing in purification steps shows that the alkaline catalyzed process is not so environmental friendly (Robles-Medina et al., 2009) .

The second way of producing biodiesel is using an acid catalyst instead of an alkaline catalyst. Acid catalysts are more suitable for oils with high free fatty acids and water contents because no soaps are formed when acid catalysts used for the transesterification reaction. However, the reaction rate is lower than the alkaline process and higher temperatures and substrate molar ratios are required. In addition, acid catalysts are rarely used on the industrial scale because they are more corrosive and may cause damage to the equipment (Bisen et al., 2010).

In order to eliminate the drawbacks of both alkaline and acid catalyzed processes, a two stage process, acid pretreatment on the oil following by the addition of an alkaline catalyst have been applied for oils that are low grade or have a high concentration of free fatty acid. The acid catalysts are used in the primary stage to convert free fatty acid to mono alkyl esters, followed by an alkaline catalyzed process to convert the remaining triglycerides to mono alkyl esters. An increased yield of reaction and faster reaction times obtained with this two stage method (Robles-Medina et al., 2009; Bisen et al., 2010).

The third way of producing biodiesel is using heterogeneous catalysts such as amorphous zirconia, titanium, aluminium, and potassium-doped zirconias. Research is still in progress in order to solve the problems observed in this process such as exhaustion of catalyst to yield higher conversions. Additionally, a new method has been improved for biodiesel production using alcohols in their supercritical state to yield mono alkyl esters. Researchers have experimented this process with methanol in its supercritical state (Ranganathan et al., 2008).

As a novel method, it has been found that enzymes such as lipase can be used to catalyze transesterification process to yield biodiesel. Enzymes have been proposed to overcome the drawbacks associated with the conventional alkaline catalyzed process, and have shown promising results. Utilization of an enzyme catalyst offers several advantages over the conventional method such as milder reaction conditions, reduced energy consumption, easy glycerol separation, insensitiveness to free fatty acids and water content in the oil, easy downstream separation and purification, reduced wastewater amount, and possibility to reuse and regenerate the catalyst. There are also some drawbacks with the usage of this enzyme catalyzed process. Main obstacles can be summarized as high costs of enzymes, slower reaction rate and the risk of enzyme inhibition due to methanol and by product glycerol (Lam et al., 2010).

2.4 Conventional Biodiesel Production

The conventional biodiesel production method involves the use of homogeneous alkaline catalysts under the temperature 50-60°C (Verdugo et al., 2010). In this process, alcohol and catalyst are mixed initially before entering a tank reactor together with oil. As mentioned before, a surplus of alcohol is added in order to shift the equilibrium to the products. After completion of the reaction, the product mixture is sent to a distillation tower for removal and recycle of excess methanol. The bottom product is then washed with water. Polar substances as glycerol, salts and residual substances are then neutralized with acid in the water phase and separated by distillation. The non-polar biodiesel phase is purified by distillation to meet the necessary standards (Sotoft et al., 2010).Furthermore, for low qualified oils having high content of free fatty acids and water, additional acid pretreatment step is usually being applied before starting the alkaline catalysis.

Downstream processing of biodiesel product can be summarized as (Enveremadu and Mbarawa, 2009): 1) separation of biodiesel, 2) purification of biodiesel a) alcohol recovery b) washing of biodiesel c) drying of biodiesel d) distillation of biodiesel.

Advantages and drawbacks of the conventional process have been discussed in the section 2.3. A schematic diagram of the conventional alkaline catalyzed biodiesel production process is represented in Figure 2.4.

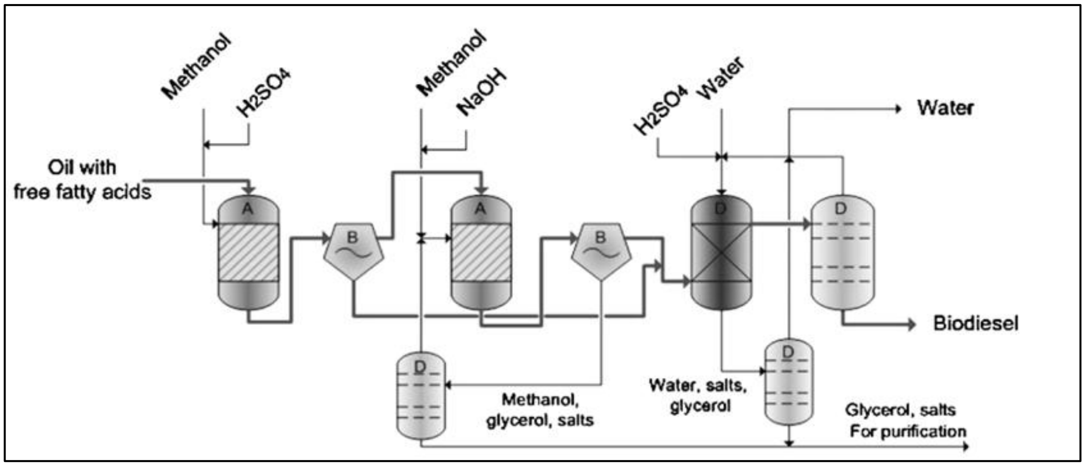


Figure 2.4 Traditional biodiesel process with an acidic pretreatment step followed by alkaline catalysis. (A) Reactor; (B) Separation (centrifuge or decanter); (D) Product purification and alcohol recovery (Fjerbaeket al., 2009).

2.5 Enzymatic Biodiesel Production

Biocatalysts have drawn more attention nowadays and have the potential to substitute chemical catalysts for biodiesel production in the future. Utilizing an enzyme catalyst seems to be a very promising alternative to those alkaline catalysts due to the downstream processing problems, huge amount of wastewater generation and difficulty in glycerol recovery associated with the use of alkaline catalysts (Lam et al., 2010).

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are the most studied group of enzymes for enzyme catalyzed biodiesel production. They are part of the family of hydrolases that operate on carboxylic esters and widely spread in animals, plants, moulds and bacteria. Under natural conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase but they can also catalyze esterifications, alcoholysis, and transesterifications in non-aqueous media (Caballero et al., 2009; Freitas et al., 2009).

As stated by Robles-Medina et al. (2009), there are two major categories of enzymatic biocatalyst. First group of enzymes are extracellular lipases those the enzyme has previously been recovered from the live-producing microorganism broth and then purified. The second group of enzymes are intracellular lipases which still remain either inside or in the cell-producing walls. Some of the commercially available lipases used for biodiesel production are: *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Candida antartica*, *Rhizomucor miehei*, *Thermomyces lanuginosa*, *Rhizopus oryzae*, *Candida rugosa*, *Candida cylindracea* and *Cryptococcus spp* (Bisen et al., 2010).

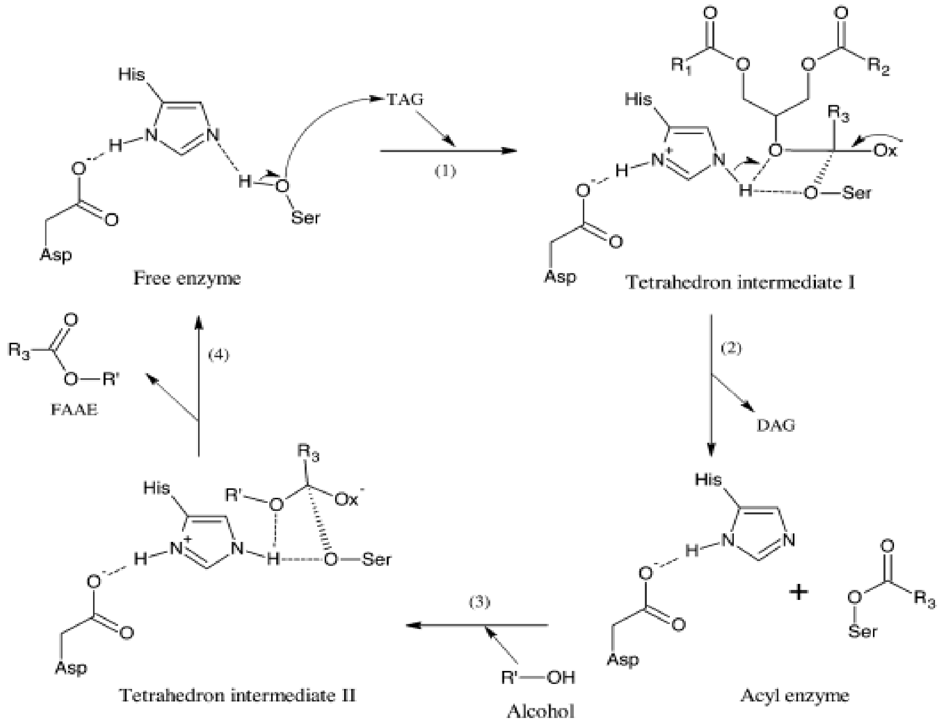


Figure 2.5 Mechanism of lipase-catalyzed transesterification (Rottig et al., 2010).

Advantages and drawbacks of the enzyme catalyzed biodiesel production have been discussed previously in the section 2.3. In Figure 2.5, a detailed mechanism of lipase catalyzed transesterification reaction can be seen. According to Rottig et al. (2010), first the histidine residue attracts the hydrogen atom of the hydroxyl group of serine building an oxygen anion. Afterwards, it attacks a carbonyl atom of triglyceride resulting in tetrahedron intermediate I. Then the proton of histidine is transferred to the separating diglyceride yielding an acyl enzyme intermediate. In the third step, the serine ester interacts with the alcohol molecule. First, the nitrogen atom of the histidine attracts the hydrogen atom of the alcohol building an alkyloxid anion. This alkyl oxid anion attacks the carbonyl carbon atom of the serine ester yielding the tetrahedron intermediate II. Finally, the fatty acid alkyl ester molecule and the free enzyme are released.

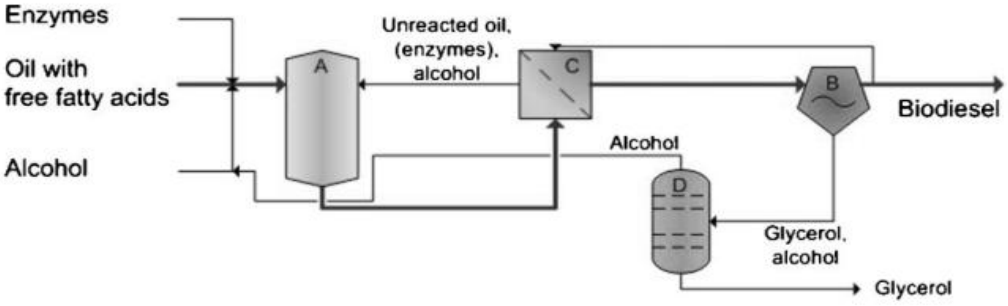


Figure 2.6 Ideal process design for enzymatic biodiesel production. (A) Reactor; (B) Separation (centrifuge or decanter); (C) Filter (Fjerbaek at al., 2009).

An ideal process design for enzymatic biodiesel production is represented in Figure 2.6. This estimated process design for enzymatic biodiesel production is much simpler in contrast with the conventional alkaline process. Since the enzymes are compatible with large variations in quality of oil, most of the alkaline process steps eliminated in this process. Unlike the alkaline catalysts enzymes can catalyze both triglycerides and free fatty acids together thus an extra acidic pretreatment step is not necessary in this enzymatic process. Furthermore, catalyst and glycerol recovery is easier with this enzymatic method that complicated separation and purification steps in alkaline process are eliminated as well.

Main factors affecting the reaction rate and biodiesel yield in enzymatic biodiesel production are: temperature, alcohol to oil molar ratio, water content of the system, type of alcohol, the use of solvents, mixing intensity, feedstock, and the properties of the used lipase.

2.5.1Lipase

The most desired characteristics of the lipases used for enzymatic biodiesel production are: its ability to utilize all mono, di, and triglycerides as well as the free fatty acids in transesterification, low product inhibition, high activity and yield in non-aqueous media, short reaction time, reusability, temperature and alcohol resistance (Bajaj et al., 2010).

As mentioned before, lipases are found in all living organisms and broadly classified as intracellular and extracellular. Both extracellular and intracellular lipases were tested for biodiesel synthesis but most of the research has been conducted by using extracellular lipases (Antczak et al., 2009). The most widely used commercial lipases are Novozym 435 from *Candida Antarctica* immobilized on a macroporous acrylic resin, Lipozyme RM IM from *Rhizomucor miehei* immobilized on an anionic resin, and Lipozyme TL IM from *Thermomyces lanuginosus* immobilized on a gel of granulated silica (Robles-Medina et al., 2009). In general, enzymes which are effective for biodiesel synthesis are able to reach conversions above 90% and reaction temperatures vary between 30 and 50°C (Fjerbaek et al., 2009).

There is an important key point for the lipase selection for enzymatic biodiesel synthesis that some of the lipases have a peculiar regioselectivity. In terms of regioselectivity on triglycerides, lipases have been divided into three groups: sn-1,3-specific those hydrolyze triglyceride ester bonds in R_1 or R_3 positions, sn-2-specific those hydrolyze ester bond in position R_2 , and nonspecific lipases those do not distinguish between position of the ester bonds (Antczak et al., 2009). In this regard, regiospecific character of the used lipase has to be taken into account for enzymatic biodiesel synthesis. In this sense, lipases having a narrow regiospecificity are considered to be inappropriate for biodiesel production. However, some sn-1,3-specific lipases efficiently catalyze transesterification reactions exceeding their maximum theoretical yield 66%. Acyl migration from position sn-2 to positions sn-1 and sn-3 is claimed to be responsible for this situation. Under appropriate conditions the yield of transesterification catalysed by sn-1,3-specific lipases can exceed 90% as a result of acyl migration. According to Robles-Medina et al. (2009), acyl migration is favoured under certain operational conditions including use of polar immobilization supports such as the ionic exchange resin or using silica gel in the reaction mixture. The regioselectivity of lipases can be affected by several factors and most of them have not been recognized yet.

Another key point for the lipase selection for enzymatic biodiesel synthesis is substrate specificity of the lipase. Regarding to Antczak et al. (2009), this substrate specificity consists in capability of distinguishing structural features of acyl chains such as the length, the number, position, or configuration of double bonds, or the presence of branched groups, as well as the nature of the acyl source. And in triglyceride and alcohol reactions, lipases distinguish the length and type of fatty acid contained in triglyceride and the length of alcohol.

Genetic engineering can be used to improve regiospecificity, substrate specificity and other properties of the lipases such as thermostability, alcohol tolerance, pH stability, and the efficiency for biodiesel synthesis. In that case, large quantities of recombinant lipases can be produced with desired properties eliminating the economic obstacles for enzymatic biodiesel production.

As discussed earlier, the main obstacle for enzymatic biodiesel production is the high costs of lipases. In order to make the process cost efficient, the lipase has to be recovered and reuse. At that point, immobilization of the lipase has a great importance. Immobilization of the lipase on a carrier increases the enzyme stability towards denaturation and offers ease handling and recovery of the lipase. Additionally, immobilization is a good way of increasing the surface area of the lipase. However, immobilization may pose internal and external mass transport limitations because large molecules such as triglycerides and fatty acid alkyl esters have to diffuse through small pores to reach the enzymes while only sparingly soluble reactants like methanol have to travel through oil filled channels (Fjerbaek et al., 2009).

2.5.2 Immobilization methods

Common immobilization methods include physical adsorption onto a solid support, covalent bonding to a solid support, ionic bonding to a solid support and physical entrapment within a polymer matrix support (Noureddini et al., 2005). A schematic representation of the immobilization methods can be seen in Figure 2.7.

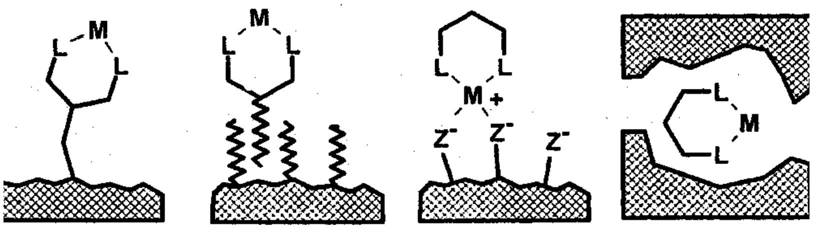


Figure 2.7 Immobilization methods (Martin, V.C., 2007).

According to Al-Zuhair (2007), physical adsorption has been selected by most researchers due to its ease, absence of expensive and toxic chemicals, ability to retain the activity, and feasibility of regeneration. On the other hand, poor adsorption of lipase results in its leaching off the support surface, which favours other lipase immobilization methods.

Entrapment of lipase entails capture of the lipase within a polymer matrix and the immobilization with this method is much more stable than physically adsorbed lipase. Furthermore, this method uses a simple procedure maintaining the lipase activity and stability in contrast with the covalent bonding method (Noureddini et al., 2005).

2.5.3 Feedstock

The use of a triglyceride feedstock for biodiesel production depends on regional availability and economics of the feedstock. The cost of the feedstock represents the major part of the total production costs thus the feedstock used for biodiesel production must be cheap, rich in oil percentage and have a low cost of production. Oils used for biodiesel production can be classified as: edible vegetable oils like sunflower and soybean oil, non-edible vegetable oils like jatropha and castor oil, animal fats including tallow, lard and greases, and waste vegetable oils.

According to Rodrigues et al. (2008), the main difference between oils are their fatty acid composition and strongly affects some important properties of the biodiesel such as cetane number, heat of combustion, melting point, and viscosity. As an example, the iodine index is related to the grade of oil unsaturation and biodiesel produced from high unsaturated fatty acid containing oils are less viscous, show greater cloud point and pour points which make it suitable for cold weather conditions. However, it also promotes the oxidation, has a lower cetane index and lower combustion heats (Robles-Medina et al., 2009).

Vegetable oils mainly consists of triglycerides (90-98 wt%) and in smaller amounts of monoglycerides, diglycerides, free fatty acids, phospholipids, phosphatides, carotenes, tocopherols, as well as traces of sulphur components and water (Rottig et al., 2010). Typical compositions of oils and animal fats that can be used for biodiesel production are presented in Table 2.2.

Table 2.2 Composition (in-wt%) of biodiesel feed stocks (Nielsen et al., 2008).

	Rapeseed	Soybean	Palm	Tallow	Palm fatty acid distillate	Used cooking oil
Triglycerides	96	98,6	87	74	8	62
Diglycerides	2	0,8	6	12	5	16
Monoglycerides	0,5	0,1	2	4	2	7
FFA	1,5	0,5	5	10	85	15

Edible oils such as soybean, rapeseed, palm, sunflower, and olive oil can easily serve as feedstock for enzymatic biodiesel synthesis. Nevertheless, the largest benefits can be obtained with using low cost, low quality oils with high free fatty acid content such as waste oils, acid oils and animal fats. Because, as mentioned before, enzymes are capable with the use of low quality oils containing high amount of free fatty acids and can esterify both triglycerides and free fatty acids. In addition, production of biodiesel from waste oils is an important step in reducing and recycling waste oil.

2.5.4 Type of alcohol and alcohol:oil molar ratio

A wide selection of alcohols has been used for enzymatic biodiesel production. Some of the alcohols used for enzymatic biodiesel synthesis are methanol, ethanol, propanol, isopropanol, 2-propanol, n-butanol and iso-butanol. The choice of alcohol in biodiesel production has some influences such as cold flow properties and lubricity but it does not draw as much attention as the cost of alcohol (Fjerbaek et al., 2009). Alcohol used for industrial applications must be cheap and available in large quantities.

Among the alcohols used for biodiesel production, the most utilized alcohol is methanol mainly because of its lower cost and higher reactivity compared to other alcohols. However, methanol is insoluble in oil and the lipases are easily inactivated by contacting this insoluble methanol in the reaction medium. Because lipases are usually unstable in short-chain alcohols and the contact of the insoluble methanol existed as drop in the oil causes inactivation of the lipase. As stated in many papers, methanol to oil molar ratio over 1.5 equivalents causes a remarkable deactivation on the lipase. But the effect of methanol varies from lipase to lipase that some of the lipases display more resistance towards methanol inhibition and have a higher optimum molar ratio of methanol to oil (Fjerbaek et al., 2009).

In order to reduce the deactivating effect of methanol, two different solutions have been suggested. One of them is using solvents for both methanol and oil and the other alternative consists of stepwise addition of methanol to the reaction mixture. Stepwise addition of methanol can avoid high amounts of methanol in the reaction mixture and therefore can reduce the inhibition effect of methanol on the lipase. In this way, methanol amount can keep below its solubility. The use of an organic solvent to dissolve methanol and thus protecting the lipase towards deactivation will be discussed in another section.

Another important key point to eliminate the deactivation effect of methanol is using very high enzyme loadings for the synthesis reaction. Using a 50% immobilized enzyme based on oil weight can mask the methanol inhibition on the enzyme (Fjerbaek et al., 2009).

Some researchers used methyl acetate as an acyl acceptor instead of methanol in order to eliminate the inhibition effect of methanol. The major advantages of this method were that no glycerol was formed; the method is suitable for crude, refined, bleached, and deodorized oils; and the operational stability of the lipase was great and could be reused many times (Akoh et al., 2007).

2.5.5 Use of organic solvent

Biodiesel can be enzymatically produced in organic solvent or in no solvent. Nevertheless, there are several examples on the use of organic solvents in enzymatic biodiesel synthesis. Organic solvents can be used to ensure a homogeneous reaction mixture eliminating the problems with having reactants in two phases. Solvents reduce the viscosity of the reaction mixture increasing the diffusion rate reducing mass transfer problems around the enzyme. And non-polar solvents might force the residue water to stay around the lipase increasing the water activity (Fjarbaek et al., 2009). Organic solvents increase the transesterification yield through improving mutual solubility of oil and alcohol and protect enzymes from deactivation by high concentrations of alcohols such as methanol. Solvents used for enzymatic biodiesel synthesis can be classified as polar and non-polar solvents.

Enzymatic biodiesel synthesis was carried out in several organic solvents. Some of the used organic solvents are n-hexane, cyclohexane, n-heptane, petroleum ether, isooctane, acetone, chloroform, 1,4-dioxane, and tert-butanol (Antczak et al., 2009).

Among polar and non-polar organic solvents used for enzymatic synthesis of biodiesel, non-polar solvents such as hexane and petroleum ether were found to be suitable in contrast with polar solvents. Polar organic solvents could deprive the lipase molecules of their essential water and deactivate the lipase making it unable to reuse. However, tert-butanol is an exception to this knowledge keeping high conversions. Due to the steric hindrance, tert-butanol is not accepted by the lipases in the esterification reaction and as a solvent it has the ability to dissolve oil, alcohol and glycerol. Additionally, solubilisation of methanol and glycerol has a positive effect on the enzyme stability (Nielsen et al., 2008).

On the other hand organic solvents have several drawbacks for industrial applications. In general, organic solvents are volatile and potentially hazardous.

Besides, reactor volumes have to be larger to accommodate both solvents and reactants. These solvents have to be recovered after the production adding extra costs for distillation equipment (Fjarbaek et al., 2009). Hence, solvent-free production for enzymatic biodiesel is an important issue. A solvent-free system avoids problems of separation, toxicity, and flammability of organic solvents lowering the cost of the final product and permitting recovery of product without organic solvent evaporation step (Selmi and Thomas, 1998). All these positive and negative issues of organic solvents have to be taken into account for the use of these solvents in enzymatic biodiesel production.

2.5.6 Water content of the system

Protection of the water surrounding the lipases is important for optimal conformation of the enzyme and removal of the water can lead to changes in the protein structure (Fjarbaek et al., 2009). Lipases acts at the interface between an aqueous and an organic phase and activation of the enzyme requires the presence of oil-water interface. With the increased addition of water, the amount of water available for oil to form oil-water droplets increases thus increases the available interfacial area (Noureddini et al., 2005). Therefore, it is important to determine influence of this key factor and ascertain its optimal value.

Optimal water activity for the enzymatic transesterification is specific for different lipases and, reaction medium composition and the type of enzyme catalyzed reaction are also effective on optimal water activity. It also relates to the enzyme preparation (Antczak et al., 2009).

Since lipases usually catalyse hydrolysis in aqueous media, excess water may also promote the competing hydrolysis reaction. Namely, the optimum water content is a balance between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction (Noureddini et al., 2005).

According to Fjarbaek at al. (2009), in the latter case, too high a water activity can decrease the lifetime of the enzyme so the optimal water content must be evaluated for each given lipase with regard to optimal yield as well as stability. Therefore, when deciding on the water activity, it is an important factor if the enzyme is already stabilized in its active conformation due to immobilization or water is needed to stabilize the enzyme.

2.5.7 Temperature

Ideal process for enzymatic biodiesel synthesis is that occurs at the lowest temperature and reaches the highest conversion in the shortest time. Optimum temperature results from the interplay between the operational stability of the lipase and the rate of transesterification. Moreover, it also depends on alcohol to oil molar ratio, use of organic solvent and thermostability of enzymatic preparation (Antczak et al., 2009).

In general, optimum temperatures for enzyme catalyzed transesterification vary between 20-60°C. Usually reaction rate increases with the increase of the temperature. However, further increment in temperature does not result in a yield increment due to the thermal deactivation of the enzyme. Additionally, when the molar ratio of alcohol to oil is higher, an increase in temperature causes faster deactivation of the lipase (Antczak et al., 2009).

According to Fjarbaek et al. (2009), an enzyme might lead to the same yield at different temperatures or that different enzymes have the same yield at different temperatures. Besides, the optimal temperature is expected to increase when immobilizing an enzyme, because binding to the carrier material gives stability to the enzyme and decreases the effect of thermal deactivation compared to the free enzyme.

2.5.8 Mixing intensity

Mixing intensity is another key variable for enzymatic biodiesel production. Mixing of the reaction medium is important in order to reduce mass transfer limitations. However higher mixing intensities might cause separation of the immobilized enzyme from the support material and damage the enzyme thus, an optimum mixing intensity should be determined for enzyme catalyzed biodiesel synthesis reactions.

Sim et al. (2010) tested different mixing intensity values for enzymatic biodiesel synthesis in their study. Among the mixing intensities 150, 175, 200, and 225rpm; 150rpm covered both the minimum and maximum biodiesel yield thus, they decided that mixing intensity does not have a significant effect on their experiments. They hypothesized that amount of the enzyme is effective on biodiesel yield and higher mixing intensities did not improve the biodiesel yield which may be due to the substantial enzyme particles that were dispersed and stuck to the wall.

3. LITERATURE REVIEW

Sim et al. (2010) experimented transesterification of crude palm oil with methanol using Lipozyme TL IM as catalyst. The enzyme loading, agitation speed and reaction time were also studied for the temperatures 30 and 40°C. Other reaction parameters were 0.37, 0.10 and 0.53 proportions for crude palm oil, methanol and tert-butanol respectively as in their previous study. Among the mixing intensities 150, 175, 200, and 225rpm; 150rpm covered both the minimum and maximum biodiesel yield thus, they decided that mixing intensity does not have a significant effect on their experiments. The combination of optimum enzyme loading of 6.67 wt% and 150rpm agitation speed for the system at 30°C gave 81.73% FAME yield at 4h and a production rate of 85.86% FAME yield/h. Enzyme loading determined to be the most influential parameter for 30°C. Nevertheless, same reaction parameters resulted in 96.15% FAME yield for 40°C. Heavy viscosity of the reaction mixture at 30°C compared to 40°C was attributed to be responsible for this yield difference. Additionally, the enzyme denaturation constant at 40°C was 1.79-fold greater than at 30°C, but they claimed that the low yield due to enzyme instability was circumvented by the high production rate observed at 40°C. Thus, 40°C was defined as the optimum temperature for this study.

Xu et al. (2004) investigated conversion of soybean oil to biodiesel in the presence of Lipozyme TL IM in a solvent-free medium. The reaction was performed by a three-step addition of 1 molar equivalent of methanol at 40°C, 150rpm, 10% enzyme quantity based on oil weight and the maximum yield was 98% after 12 h reaction. They found that the lipase was inactivated when more than 1.5 molar equivalent of methanol was added to the reaction mixture. Operational stability of the lipase was also investigated. At the end of the reaction, they transferred the recovered lipase to a fresh reactant mixture but the FAME yield decreased dramatically for this second methanolysis reaction. During the repeated use of the lipase, they also observed that a glycerol layer formed on the surface of the enzymatic support which may cause lipase deactivation by limiting substrate and product diffusion. In order to eliminate the glycerol inhibition, they adopted iso-propanol to remove this glycerol by washing the immobilized lipase during the repeated use. More than 94% FAME yield could be achieved with this washing method even after 15 cycles.

Hernandez-Martin and Otero (2008) performed enzymatic synthesis of biodiesel via alcoholysis of sunflower, borage, olive and soybean oils. Lipases such as Lipozyme TL IM, Lipozyme RM IM and Novozym 435 were utilized for catalyzing the biodiesel synthesis. Since methanol caused stronger inhibition on the lipase, they selected ethanol for their experiments. And they observed that, there were no significant differences in the kinetics of the different oils investigated. Optimum volume of ethanol found to be depending on the loading of the enzyme and was higher for preparations of Novozym 435 than for Lipozyme TL IM. Maximum rates obtained with Lipozyme TL IM with a molar ratio of alcohol to oil 0.33 and by contrast, at least 2:1 molar ratio was required for Novozym 435. Another point was that the alcoholysis of vegetable oils was faster with Lipozyme TL IM than with Novozym 435. Use of a high loading of Novozym 435 (50% w/w) and a large molar excess of ethanol were required in order to obtain a similar initial rate that obtained with Lipozyme TL IM at a lower enzyme loading. Nevertheless, Lipozyme TL IM was not able to produce conversions greater than 84% even with three stage stepwise addition of ethanol. In contrast, Novozym 435 was able to produce higher conversions, although it required use of higher alcohol to oil molar ratios. Quantitative conversions could be obtained with Novozym 435 in 7h at 25°C with 4.5:1 ethanol to oil molar ratio and 50% enzyme loading in a single step process. And under the optimum conditions, Novozym 435 retained 85% of its initial activity after nine cycles of reaction.

Liu et al. (2010) studied biodiesel synthesis with immobilized lipases in solvent-free and tert-butanol media. Novozym 435, Lipozyme TL IM and Lipozyme RM IM were utilized with different alcohols such as methanol, ethanol, propanol, isopropanol, isobutanol and isoamyl alcohol. Reactions were performed 20h at 45°C and 5% lipase loading with 1:4 molar ratio of waste baked duck oil to alcohol. They observed that each lipase presented a different kinetic pattern depending on the monohydric alcohols in solvent-free and tert-butanol systems. They produced an alcohol mixture for biodiesel synthesis mixing suitable amounts of 15% isobutanol, 80% isoamyl alcohol and 5%methanol. In addition, combined use of Novozym 435 and Lipozyme TL IM lipases with this alcohol mixture was performed. The conversion rate observed to be increased with the proportion of Lipozyme TL IM for combined lipases, and the maximal conversion rate of waste baked duck oil to the corresponding alkyl esters reached over 90% in solvent-free medium and over 70% in tert-butanol medium catalyzed by combined lipases of Novozym 435 and Lipozyme TL IM with weight ratio of 2:4.

Deng et al. (2005) investigated enzymatic alcoholysis of sunflower oil with different lipases under solvent-free conditions. Six lipases were tested with seven alcohols, including straight and branched-chain primary and secondary alcohols. Reaction conditions were: 40°C, 10%wt lipase dosage, 24h, and 3:1 molar ratio of alcohol to sunflower oil added in four steps. For Lipozyme TL IM, the highest conversion was obtained with methanol (89.8%) and other conversions were 77.5%, 85.1%, 78.1%, 24.6%, 77.9%, and 72.4% for ethanol 99%, ethanol 96%, 1-propanol, 2-propanol, 1-butanol, isobutanol respectively. Besides, they observed that Novozym 435 produced the highest conversions for most of the alcohols, with yields over 90% for methanol, absolute ethanol, and 1-propanol. Highest conversions were obtained with ethanol 96% and increasing the water content in the system resulted in an increased degree of conversion for all lipases except Novozym 435. They pointed out that the secondary alcohol 2-propanol significantly reduced the alcoholysis reaction with all lipases; however, the branch-chain isobutanol was more advantageous than linear 1-butanol for Novozym 435, Lipozyme RM IM, and Lipase PS-C.

Rodrigues et al. (2008) studied biodiesel synthesis from soybean, sunflower and rice bran oils with methanol, ethanol, propanol and butanol by Novozym 435, Lipozyme TL IM and Lipozyme RM IM lipases. They pointed out that all three lipases displayed similar reaction kinetics with all three oils; however, each lipase displayed the highest alcoholysis activity with a different alcohol. Novozyme 435 presented higher activity in methanolysis at 5:1 methanol to oil molar ratio, Lipozyme TL IM presented higher activity in ethanolysis at a 7:1 ethanol to oil molar ratio, and Lipozyme RM IM presented higher activity in butanolysis at a 9:1 butanol to oil molar ratio. Additionally, the optimal temperatures were varied between 30-35°C for all lipases. Under optimum conditions, lipase stability over repeated batches was also investigated by washing the immobilized lipase with n-hexane, water, ethanol or propanol after each batch. Lipases found to retain 90% of their activity when washing with n-hexane even after seven cycles.

Soumanou and Bornscheuer (2003) produced methyl esters from sunflower oil by lipase catalyzed reactions in solvent-free and solvent systems. Among the organic solvents investigated, n-hexane and petroleum ether were presented the highest conversions (80%). And among the lipases tested in a solvent-free system, the best conversion over 90% was obtained with *Pseudomonas fluorescens* lipase at 1:4.5 molar ratio of oil to methanol. In order to reduce the deactivation effect of methanol for solvent-free systems, a three-step methanolysis was employed by adding 1M equivalent of methanol initially, after 5 and 10h. A high conversion (>80%) was obtained for Lipozyme RM IM lipase with this method. Besides, immobilized lipase RM could be used at least 5 times with this stepwise addition method without significant loss of activity. Reaction conditions were 10% (w/w) lipase, 3:1 methanol to oil molar ratio, 24h, and 40°C. Additionally, conversions for the Lipozyme TL IM lipase were 72.9% in n-hexane system and 40% in solvent-free system with stepwise method.

Wang et al. (2008) optimized the process of biodiesel production from corn oil catalyzed by Lipozyme TL IM. The optimum conditions for the reaction were 25.9 U/g_{oil} of enzyme, 0.58 volume ratio of tert-butanol to oil and 0.5, 0.5, 2.8 molar equivalent of methanol to oil added at the reaction time of 0, 2, and 4h. 85.6% methyl ester yield could be obtained in 12h with the conditions described. Additionally, they found waste oil to be more suitable for Lipozyme TL IM catalyzed methanolysis due to high free fatty acid content of this oil. The conversion was 93.7% when waste oil used. Different kinds of additives were also investigated for their potential to improve acyl migration in this study. Among the additives tested for corn oil, 92% methyl ester yield was achieved with addition of 20% triethylamine based on oil weight, which was much higher than the yield of 85.6% obtained previously.

Wang et al. (2006) studied lipase catalyzed methanolysis of soybean oil deodorizer distillate for biodiesel production. Optimum reaction conditions were considered as 3.9:1 molar ratio of methanol to oil, 3% Lipozyme TL IM and 2% Novozym 435 based on the oil weight, 80% tert-butanol based on oil weight, and 40°C. Conversion of 94% could be obtained with these conditions. There was no obvious loss in lipase activity even after being repeatedly used for 120 cycles. Additionally, effect of adding adsorbents on the methanolysis was investigated and fine-pored silica gel and 3Å molecular were found to be effective to control by product water concentration. The highest yield of 97% was achieved with 3Å molecular sieve as the adsorbent with the reaction conditions of 3.9:1 molar ratio of methanol to oil, 2% Novozym 435 and 3% Lipozyme TL IM based on the oil weight, 20% tert-butanol based on the oil weight, 10-fold adsorbent based on the maximal water weight, 40°C, and 12h.

Li et al. (2006) experimented combined use of Lipozyme TL IM and Novozym 435 for methanolysis of rapeseed oil for biodiesel production. The highest biodiesel yield of 95% could be achieved under the conditions of 1:1 tert-butanol to oil volume ratio, 4:1 methanol to oil molar ratio, 3% Lipozyme TL IM and 1% Novozym 435 based on the oil weight, 35°C, and 12h. They did not observe obvious loss in lipase activity after being repeatedly use of the catalyst for 200 cycles. Furthermore, waste oil was also tested for the reaction. Although the biodiesel yield was below 80% without molecular sieve present in the reaction system, yields over 90% could be obtained with molecular sieve existing systems. It has demonstrated that water amount present in the system has a considerable importance for enzyme catalyzed biodiesel production.

Du et al. (2005) studied different factors affecting the acyl migration on Lipozyme TL IM catalyzed methanolysis of soybean oil. They have investigated parameters such as enzyme loading and water amount present in the reaction medium. Surprisingly, they have found that the silica gel acting as the immobilized material contributed significantly to the promotion of acyl migration in the process. The final biodiesel yield was only 66% when 4% free Lipozyme TL used, while about 90% biodiesel yield could be obtained when combining 6% silica gel with 4% free Lipozyme TL, almost as high as that of 10% immobilized lipase used for the reaction.

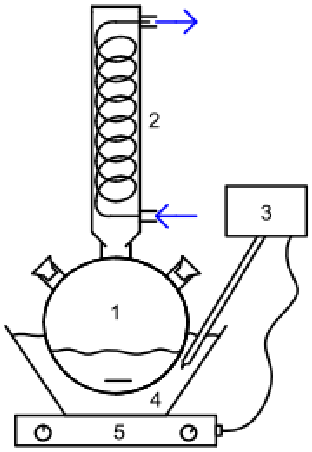
4. MATERIALS AND METHODS

4.1 Materials

Lipozyme TL IM (Lipase immobilized on immobead 150 from *Thermomyces Lanuginous*) 3423U/g was purchased from Sigma-Aldrich. Commercial food-grade sunflower oil was supplied from a local market. All other chemicals used in this study were analytical grade.

4.2 Experimental Setup

The equipment used to carry out the transesterification consists of a 100ml flask with a reflux condenser, a controller for the measurement of the temperature, and a heated oil bath with a magnetic stirrer. The experimental setup is shown in Figure 4.1. First, the necessary amount of enzyme and sunflower oil were mixed together. After adding the calculated amount of methanol to the flask, the flask was set into the preheated oil bath. During the reaction, the mixture was stirred continuously at 280rpm. The reaction was continued for 24 hours. The initial reaction conditions were 27wt% immobilized enzyme loading based on oil weight together with 0.01mol sunflower oil and 0.04mol methanol at 40°C reaction temperature. In the optimization studies, only one reaction parameter was varied at a time. At the end of the reaction; lipase was separated from the reaction mixture by vacuum filtration. During the filtration, lipase was washed with a small amount of methanol in order to take the residual product. After filtering the lipase, the formed biphasic mixture transferred to a separation funnel and the yellow biodiesel phase was separated for further analysis.



1. 100ml flask
2. Reflux condenser
3. Temperature controller
4. Oil bath
5. Magnetic stirred heater

Figure 4.1 Schematic representation of the experimental set up.

4.3 Analysis

Analysis of biodiesel samples was done using a Shimadzu GC17A GC-FID device with a Varian biodiesel column designed to determine glycerides. The column was 15m long with a 0.32mm ID and a maximum operational temperature of 400°C. The column temperature was kept at 50°C for 1 min, raised to 180°C at 15°C/min, raised to 230°C at 7°C/min, then raised to 370°C at 10°C/min and maintained as this temperature for 5min. The detector temperature was 380°C. Treatment of biodiesel samples for GC analysis was done as follows: 15µl of biodiesel sample was taken and 100µl of internal standard was added as well as 100µl of MSTFA. This mixture was shaken and left for 15min. Afterwards, this mixture diluted with 8ml of n-heptane then got ready for the GC analysis.

5. RESULTS AND DISCUSSIONS

In this study, some efforts have been made to explore the possibility of biodiesel production catalyzed by Lipozyme TL IM lipase in a solvent-free medium and the effects of different reaction conditions on the enzymatic methanolysis of sunflower oil has been investigated. Conditions such as enzyme loading, methanol to oil molar ratio, and operation temperature were varied in order to determine optimum reaction conditions. Stability of the immobilized lipase during the repeated batches was also investigated.

5.1 Effect of Methanol Content on Enzymatic Methanolysis

The alcohols used for biodiesel production must be cheap and available in large quantities for optimization of production costs; therefore methanol is the most preferred alcohol for industrial applications. However, it is well known that too much methanol would lead to serious inactivation of lipase. Thus, methanol to oil molar ratio has a significant influence on lipase catalysed methanolysis of sunflower oil. According to stoichiometry of the reaction, three molar equivalents of methanol are needed for the complete methanolysis of sunflower oil. Besides, the use of an excess of alcohol is necessary to ensure high reaction rates, to minimize diffusion limitations, and to keep the glycerol formed during the reaction in solution (Rodrigues et al., 2008).

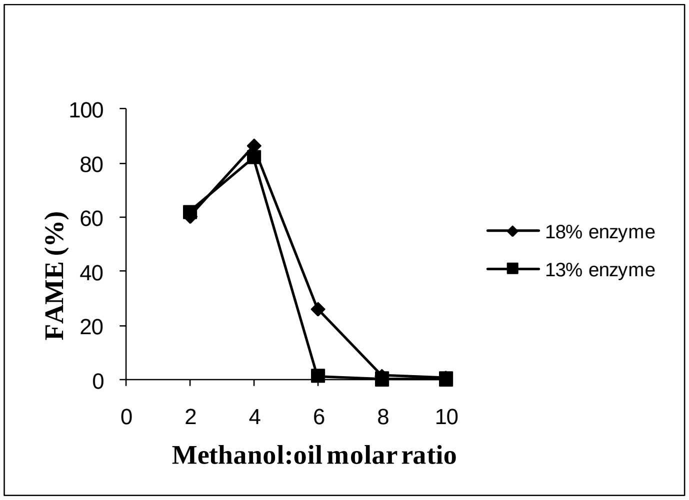


Figure 5.1 Effect of oil:methanol molar ratio. All reactions were performed at 45°C and 24h.

Different methanol:oil molar ratios (in a solvent-free media employing a one-step addition of the methanol to the reaction system) were investigated in order to study the influence of methanol. Both a higher and lower methanol concentration lead to a decrease in FAME yield as can be seen from Figure 5.1. When more than 4 molar equivalent methanol was present initially in the reaction mixture, FAME yield decreased dramatically by the inactivation of lipase through contact with excess of methanol. In the same way, at a 2:1 molar ratio of methanol to oil, a decrease in the FAME content was observed. The results indicated that an increase in the number of moles of methanol resulted in an increase in the FAME production. However, the formation of FAME reached a maximum level and further increases in the methanol concentrations resulted in a yield decrease due to lipase inactivation. Maximum FAME yield was obtained with 4:1 molar ratio of methanol to oil for both enzyme loadings where about 86% and 82% FAME were formed for 18wt% and 13wt% enzyme loadings respectively at 45°C.

Although 4 molar equivalents of methanol was added to the reaction medium in one-step, lipase inactivation was not observed. This amount of methanol was claimed to cause significant deactivation of the lipase by many researchers, nevertheless we did not observed this phenomenon in our study. Therefore, stepwise addition of methanol or use of an organic solvent was not necessary to protect lipase against methanol. However, for molar ratios higher than 4:1 methanol to oil, there was a considerable inhibition caused by methanol as can be seen from Figure 5.1. According to Al-Zuhair (2006), the initial rate of reaction increases with substrate concentration and/or alcohol concentration. Hence, much higher alcohol concentrations were required for inhibition to take place at high substrate concentrations. Increasing the substrate concentration reduced the probability of attachment of enzyme to the alcohol since alcohol inhibition was competitive. At high substrate concentrations, effectively all the enzyme was attached to the substrate available in abundance. The phenomenon observed in our study was in an agreement with this explanation.

5.2 Effect of Enzyme Loading on Enzymatic Methanolysis

As the cost of the lipase contributes significantly to the total cost of biodiesel production, the enzyme loading should be minimized as much as possible. In order to investigate the effect of enzyme loading on methanolysis of sunflower oil, the amount of Lipozyme TL IM was varied from 4.5wt% to 27wt%based on oil weight while keeping the molar ratio of methanol to oil constant at 45°C.

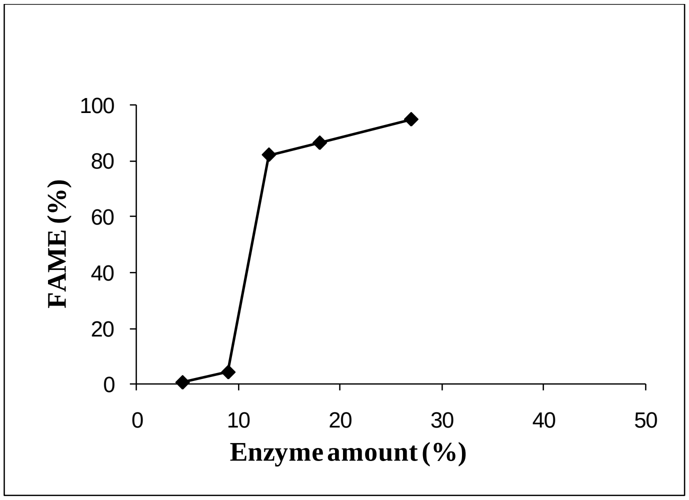


Figure 5.2 Effect of enzyme amount. All reactions were performed at 45°C, using 1:4 oil:methanol molar ratio, and 24h.

The effect of enzyme loading on the methanolysis of sunflower oil is shown in Figure 5.2. A decrease in the FAME yield is seen for enzyme loadings below 13wt% and a significant increase observed for 13wt% and higher loadings. The yield of FAME increased with increasing enzyme loading. However, the FAME yield at the highest dosage of 27wt% was almost similar to that of 18wt% and 13wt%. To minimize the cost of process, 18wt% and 13wt% enzyme loadings were chosen for further study.

Since Lipozyme TL IM is known to exhibit 1,3-regiospecifity, theoretically the maximum FAME yield should be 66%. However, higher FAME yields were obtained in this research that might be explained by acyl migration from the sn-2 position to the sn-1 or sn-3 position. This situation has also been observed by other researchers (Xu et al., 2004; Du et al., 2005; Soumanou and Bornscheuer, 2003). Parameters such as lipase quantity, water existing in the reaction mixture, presence of additional organic solvents or immobilization support materials thought to be responsible for acyl migration by different researchers. In this study, no additional organic solvent or water, except the water combined in the immobilized Lipozyme TL IM was added into the reaction system. In order to illuminate the detailed reaction mechanism, further study is needed.

5.3 Effect of Temperature on Enzymatic Methanolysis

The optimum temperature for enzymatic biodiesel synthesis results from the interplay between the operational stability of the lipase and the reaction rate. It also depends on alcohol to oil molar ratio, use of an organic solvent, and thermostability of enzymatic preparation.

Effect of temperature on enzymatic methanolysis of sunflower oil was also investigated. Several trials were conducted at temperatures of 30, 40, 45 and 55°C in order to determine the optimum temperature. Generally, it is known that increasing the temperature increases the FAME yield but surprisingly, the highest FAME yield was obtained at the lowest temperature in our study. Hernandez-Martin and Otero (2008) observed the same phenomenon in their study that the conversions decreased when they increased the temperature.

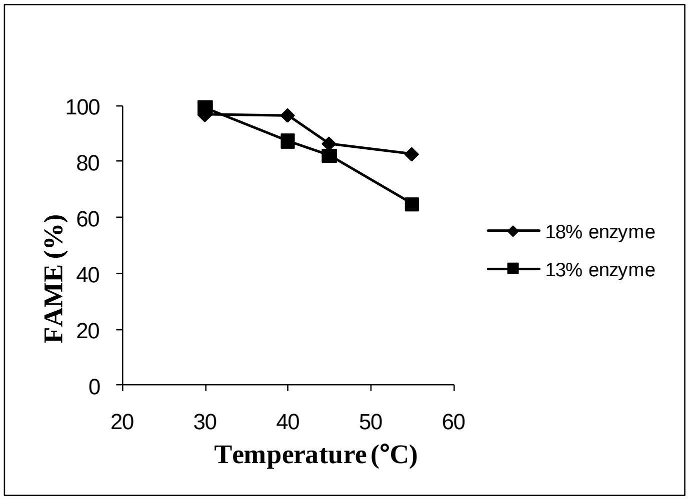


Figure 5.3 Effect of temperature. All reactions were performed using 1:4 oil:methanol molar ratio and 24h.

As shown in Figure 5.3., for 18wt% enzyme loading, FAME yield was almost same for the temperatures 30 and 40°C. In contrast, 12% FAME yield decrease was observed for 13wt% of enzyme loading, when the temperature increased from 30°C to 40°C. It shows that temperature difference is more effective on smaller enzyme loadings. Considering that the highest yield obtained at the lowest temperature is more economical for industrial applications, 30°C is a very promising temperature for biodiesel production. Therefore, 30°C was selected as optimal temperature for further experiments.

5.4 Effect of Reaction Time on Enzymatic Methanolysis

In order to determine the optimum reaction time for enzymatic methanolysis of sunflower oil, samples were withdrawn from the reaction medium at 2, 4, 8, and 24hour periods and analyzed for their FAME contents. Figure 5.4 presents the results of the reaction conversion as a function of time. From this figure it can be observed that after the 2h period of reaction, almost 70% conversion was obtained for an enzyme loading of 13wt%. It was followed by a slower rate as the reaction progresses and ultimately, a state of equilibrium was approached. Results were demonstrated that the initial reaction rate was very high for Lipozyme TL IM lipase. This observation was in agreement with the results of Hernandez-Martin and Otero (2008). They have also obtained higher initial reaction rates with Lipozyme TL IM compared to other lipases. The initial reaction rate was almost twice that for other lipases when they used Lipozyme TL IM as catalyst. Since the complete conversion was reached after 24h in our study, 24h was selected as optimum reaction time for further experiments.

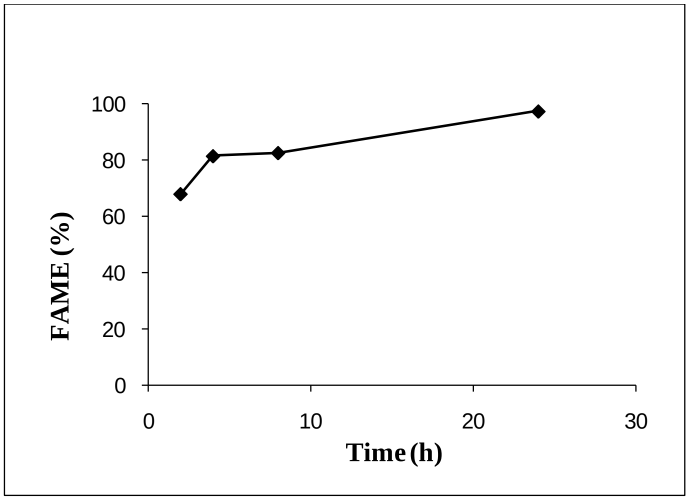


Figure 5.4 Effect of time. All reactions were performed at 30°C, using 1:4 oil:methanol molar ratio, and 13wt% enzyme amount.

5.5 Enzyme Reuse

One of the most important characteristics of an enzyme is the stability and reusability over an extended period of time due to the high cost of enzyme. Methanol and by-product glycerol inhibition on the lipase are the primary factors which effect stability and reusability of the lipase. Immobilization of enzymes has generally been used to obtain reusability of the enzyme. Reuse is easier with immobilization due to lipase stability towards temperature, methanol, and glycerol. It also enables easy handling and recovery of the enzyme.

Experiments were performed to examine the reusability of Lipozyme TL IM. At the end of the transesterification reaction, lipase was recovered by vacuum filtration and subsequently reused transferring the lipase to a fresh reaction mixture. Reaction conditions were 4:1 molar ratio of methanol to oil, 30°C, 24h, and 18wt% and 13wt% enzyme loadings respectively. Figure 5.5 shows the relative activity of the lipase in each cycle. As seen from the figure, activity of the lipase decreased dramatically after the first usage of lipase, for both enzyme loadings. For 18wt% enzyme loading, there was almost a 77% yield loss between the first and the second usage of the lipase. And the lipase was completely deactivated in its third cycle. As a comparison, the lipase was completely deactivated in its second cycle for 13wt% lipase loading. It was not possible to reuse the lipase for this enzyme loading. This difference between the behaviors of two enzyme loadings, demonstrated that higher lipase amounts are more stable towards deactivation factors. As mentioned earlier, the same phenomenon was also observed towards the temperature for two different enzyme loadings. Although 18wt% enzyme loading could maintain some of its activity for the next cycle, both enzyme loadings were not able to show a considerable stability between repeated batches. Therefore, we tried to protect the lipase against deactivation and improve its stability.

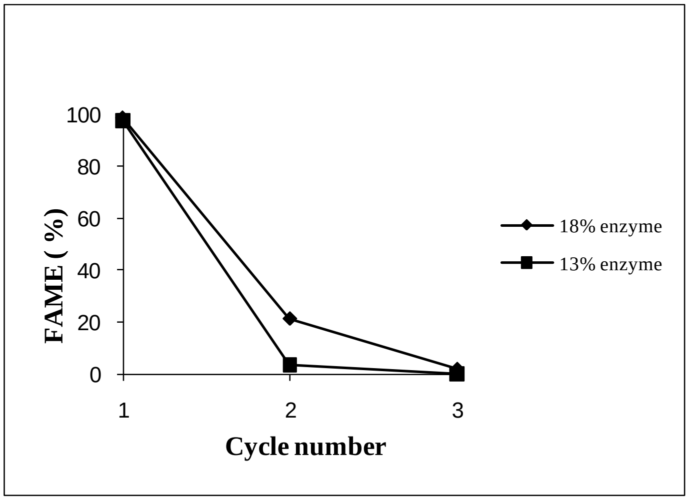


Figure 5.5 Enzyme reuse. All reactions were performed at 30°C, using 1:4 oil:methanol molar ratio, and 24h.

According to Ognjanovic et al. (2009), a way of improving the performance of lipase in the media with a high polarity as constituted by methanol was lipase regeneration by washing its solid particles with C3- C5 alcohols. Besides, Xu et al. (2004) performed washing the lipase with isopropanol between the repeated batches in order to recover the adsorbed glycerol and maintain lipase activity for the next cycles. They could achieve a yield more than 94% even after 15 reaction cycles with this method. Considering these observations, the lipase was recovered by filtration and washed with isopropanol before being reused. This procedure was repeated several times to examine the extent of the stability of the immobilized lipase. Experiments were performed with 4:1 molar ratio of methanol to oil, 13wt% immobilized lipase, 30°C, and 24h. Experimental results summarized in Figure 5.6 show a considerable activity of the lipase upon repeated uses. Another point was that a 16% FAME yield decrease appeared for the first use of the lipase in comparison with our previous experiments. In our previous experiments which were repeated three times, FAME yield was 98% under optimum conditions. Nevertheless, the maximum FAME yield was 82% under optimum conditions during reuse experiments. This situation was attributed to be a result of using a new purchase of Lipozyme TL IM. Lipases have a unique nature and immobilization process has a significant effect on the lipase activity. Therefore, this new purchase of lipase showed a slight different behavior on methanolysis of sunflower oil. In spite of this, washing lipase with isopropanol caused greater activity of lipase. About 87% of the activity of Lipozyme TL IM was maintained even after 5 batches when the lipase was washed with isopropanol during the repeated uses.

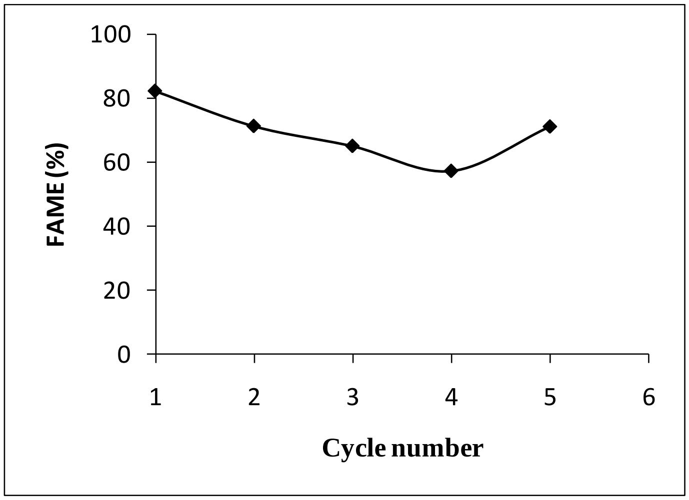


Figure 5.6 Enzyme reuse with isopropanol washing method. All reactions were performed at 30°C, using 1:4 oil:methanol molar ratio, 13wt% enzyme amount, and 24h.

6. CONCLUSION

In this study, some efforts have been made to explore the possibility of biodiesel production catalyzed by Lipozyme TL IM in a solvent free medium. Effects of different reaction conditions on enzymatic methanolysis of sunflower oil have been investigated. After optimizing the reaction conditions such as enzyme loading, alcohol/oil molar ratio and temperature, stability of the immobilized lipase during the repeated batches was also investigated.

Different enzyme loadings including 4.5wt%, 9wt%, 13wt%, 18wt%, 27wt% based on oil weight were utilized for 2:1, 4:1, 6:1, 8:1, and 10:1 molar ratios of methanol to oil, respectively. For experiments which were resulted in a high FAME yield, effect of temperature was also investigated. Temperatures were varied between 30, 40, 45, and 55°C. After the determination of these parameters, optimum reaction time was studied. Samples were withdrawn from the reaction medium at 2, 4, 8, and 24hour periods and analyzed for their FAME contents.

The optimum conditions determined for Lipozyme TL IM catalyzed methanolysis of sunflower were 4:1 molar ratio of methanol to sunflower oil, 13% enzyme loading, 30°C, and 24h reaction time. Under optimum conditions, almost 98% FAME yield could be achieved.

After determination of the optimum parameters, enzyme reusability during the repeated batches was also investigated. Lipozyme TL IM lipase was not able to show a considerable stability between repeated batches. Therefore, lipase protection against deactivation and stability improvement were aimed. Washing lipase with isopropanol was found to cause greater activity of lipase. About 87% of the activity of Lipozyme TL IM was maintained even after 5 batches when the lipase was washed with isopropanol during the repeated uses.

Consequently, the results of this study showed that Lipozyme TL IM lipase can be used for enzymatic biodiesel production from sunflower oil and methanol. Since the reuse of lipase was possible during the repeated batches, biodiesel production with this method could reduce cost of production and eliminate drawbacks of the conventional alkaline catalyzed process.

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