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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต

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PROPYLOLEATE PRODUCTION USING IMMOBILIZED LIPASE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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น้ำมันหล่อลื่นชีวภาพที่ผลิตจากน้ำมันพืชและกรดไขมันเป็นผลิตภัณฑ์ที่เป็นมิตรต่อสิ่งแวดล้อม เนื่องจากมีความเป็นพิษต่ำ และย่อยสลายได้ง่าย ในงานวิจัยนี้ได้ศึกษาการผลิตสารหล่อลื่นชีวภาพด้วยปฏิกิริยาเอสเตอริฟิเคชันโดยใช้กรดโอเลอิกและโพรพานอลเป็นสารตั้งต้นในการผลิต ตัวเร่งปฏิกิริยาที่ใช้ในการบวนการผลิตนี้คือ โนโวไซม์ 435 ซึ่งเป็นเร่งปฏิกิริยาชีวภาพตรึงรูป ในการทดลองนี้ได้ทำการทดลองในเครื่องปฏิกรณ์แบบกะ ทดลองในเครื่องเขย่าแบบควบคุมอุณหภูมิ จากการทดลองนี้ได้ทำการศึกษาผลของเวลา โครงสร้างแอลกอฮอล์ (โพรพานอล กับไอโซโพรพานอล) ปริมาณของตัวเร่งปฏิกิริยา ความเร็วรอบ อัตราส่วนโดยโมลของโพรพานอลกับกรดโอเลอิก และอุณหภูมิ จากการศึกษาพบว่าสภาวะที่เหมาะสมในการทดลองนี้คือ อุณหภูมิ 45 องศาเซลเซียส อัตราส่วนโดยโมลของโพรพานอลกับกรดโอเลอิกคือ 2 ต่อ 1 ความเร็วรอบเท่ากับ 250 รอบต่อที และ 5% โดยน้ำหนักของตัวเร่งปฏิกิริยาเทียบกับกรดโอเลอิก ในสภาวะนี้ทำให้ได้ค่าการผลได้การเปลี่ยนเป็นผลิตภัณฑ์ของกรดไขมันที่ 88.9% นอกจากนี้เมื่อทำการกำจัดน้ำออกจากระบบโดยใช้โมเลกุลาซิฟ (Molecular sieve) จะได้ผลได้การเปลี่ยนเป็นผลิตภัณฑ์เป็น94.7% หรือเพิ่มขึ้นประมาณ 6.5% จากการศึกษาทางจลนพลศาสตร์พลังกระตุ้นของปฏิกิริยาเอสเตอริฟิเคชั่นคือ 34.05 กิโลจูลต่อโมล ท้ายสุดผลการทดลองแสดงให้เห็นว่าสามารถใช้เอนไซม์โนโวไซม์ 435 ได้อย่างน้อย 5 รอบโดยที่ ผลได้การผลิตโพรพิลโอลีเอทไม่เปลี่ยนแปลงมาก.

ภาควิชา วิศวกรรมเคมี ลายมือชื่อนิสิต……..……

สาขาวิชา วิศวกรรมเคมี ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก……...…

ปีการศึกษา 2554

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KEYWORDS: PROPYLOLEATE/ OLEIC ACID/ BIOLUBRICANTS/ NOVOZYM 435

KARNJANA SENA: PROPYLOLEATE PRODUCTION USING IMMOBILIZED LIPASE. ADVISOR: ASSOC. PROF. MUENDUEN PHIASALAPHONG, Ph.D., 65 pp.

Biolubricants derived from vegetable oils and fatty acids are environmentally compatible products due to their low toxicity and good biodegradability. In this work, biolubricant production by an esterification reaction of oleic acid and propanol was studied, where immobilized Novozym 435 lipase enzyme was used as a biocatalyst. The experiments were carried out in a shaking incubator to investigate effect of reaction time, alcohol structure (propanol vs. isopropanol), enzyme loading, rotation speed, molar ratio of propanol to oleic acid, and reaction temperature. The optimal condition were 45^{o}C, molar ratio of PrOH to Oleic acid of 2:1, Novozym 435 loading at 5% based on oleic acid weight and 250 rpm, in which the maximal FFA conversion at 88.9% was obtained. It was shown that the FFA conversion could be increased to 94.7% (or 6.5% increases) by removal of water during the reaction using molecular sieve. From kinetic study, the activation energy of the esterification reaction was 34.05 kJ/mol. Finally, it demonstrated that Novozym 435 could be used at least 5 cycles without considerable change in the conversion for propyloleate production.

Department: Chemical Engineering Student's Signature…….
Field of Study: Chemical Engineering Advisor's Signature……
Academic Year: 2011

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CHAPTER I
INTRODUCTION

1.1 Motivation

Lubricants are produced from based oil, which is one of the by-products of petroleum refinery. Lubricants are substances introduced to reduce friction between moving surfaces and to prevent wear of engine. Most modern lubricants are complex formulated products consisting of 70-80% based oils mixed with functional additives to modify the natural properties such as cold stability, hydrolytic stability, oxidation stability, viscosity and viscosity index to suit a specific application.

In recent years, demand for lubricants being used in engines (Akerman et al., 2011) and heating systems has increased, resulting in problems of lubricant contamination in soil, groundwater, and air with difficulty to degrade. Together with the continuous rising of the petroleum oil price and diminishing crude oil resources, the use of biolubricants derived from renewable source, like vegetable oils or biodegradable oleochemical esters has continuously increased (Brahmkhatri et al., 2011).

Biodegradable oleochemical esters derived from transesterification or esterification of oils on fatty acid and long chain alcohols were developed to provide an alternative for environmental friendly biolubricant fluids. Oils and fatty acids, products from agricultural and food industrial manufactures can be used as feedstocks. The most used fatty acids for production of biolubricants are palmitic acid and oleic acid (Filley et al., 2005).

Oleic acid, one of the most important fatty acids in nature is a product from palm oil. Esterification of biolubricants with oleic acid using sulphuric acid catalyst was studied to form natural lubricants (Filley et al., 2005). Furthermore, its ester can be produced by enzyme catalysis. Enzymatic conversion of fatty acid has been suggested as a realistic alternative to the conventional physio- chemical method, because the enzymatic methods require low energy and produce low wastewater. However, the main problem of the usage of lipase catalyst is the high cost of nature enzyme. Immobilized enzyme is therefore, becoming increasingly popular. The

advantages include mild operating conditions of the immobilized enzyme processing, ease of product isolation and reusability (Fukuda et al., 2001).

1.2 Objectives

To study the production of biolubricant from oleic acid and propanol using immobilized enzyme (Novozym435).

1.3 Working scopes

In this study, the biocatalyst was Novozyme 435, a commercial *Candida antractica* lipase (EC 3.1.1.3. *Triacylglycerol acylhydrolase*) immobilized on a macroporous acrylic resin. Oleic acid was used as fatty acid. Propanol and isopropanol were used as alcohol. The optimal conditions for biolubricant production from oleic acid in solvent free system were investigated. The effects of temperature, rotation speed (rpm), the molar ratio of alcohol to oleic acid and enzyme loading on FFA conversion were observed. Besides, the influence of alcohol structure, the reusability of Novozyme 435 and effect of water removal were examined. Furthermore, the kinetics of the esterification reaction was studied**.**

1.4 Expect benefits

The expected benefit of this research work is to obtain the optimal conditions for biolubricant production from oleic acid and propanol using immobilized enzyme (Novozym435) in solvent free system. The information gained from the study would be useful for the further development of biodiesel and biolubricant production processes.

CHAPTER II
BACKGROUND AND LITERATURE REVIEW

2.1 Lubricant

A lubricant is used to protect thin film between moving surface wear and device, to reduce friction between of mechanical device, to cool temperature down and to prevent corrosion and rust (Ting et al., 2011). Lubricants can also be used as working fluid in hydrostatic power transmission

2.1.1 Types of lubricant

2.1.1.1 Mineral oils

Mineral oils derived from crude oil (petroleum oils) are products of refining crude oil. There are three types of mineral oils: paraffinic, naphthenic and aromatic oils. (Hewstone et al., 1994)

2.1.1.2 Vegetable lubricants

Vegetable oils are environmentally friendly alternative to mineral oils since they are biodegradable. Vegetable oils have triglyceride structure with high viscosity property. The main disadvantages of vegetable lubricants are low oxidation and low temperature stability (Hewstone et al., 1994**).**

2.1.1.3 Synthetic lubricants

Synthetic lubricants are a blending of 70-80% based oils mixed with functional additives to modify the natural properties such as cold stability, hydrolytic stability, oxidation, stability, viscosity and viscosity index to suit a specific application.

2.1.2 Important properties of biolubricant (Saliha et al., 2011)

2.1.2.1 Viscosity

Viscosity of a fluid is its resistance to shear. The friction in a lubricated bearing is directly related to the fluid viscosity. The viscosity must be high enough to prevent wear to wear contract.

2.1.2.2 Viscosity index (VI)

Viscosity index is a measure for the change of viscosity with temperature. It is one of concerned characteristics of lubricant oil in the automotive industry.

2.1.2.3 Pour point

Pour point is the lowest temperature at which liquid will pour or flow before liquid will turn semi solid and will not flow (freezing point).

2.1.2.4 Flash point

Flash point is the lowest temperature at which liquid becomes vapor.

2.2 Biolubricant (Lazzeri et al, 2006)

Biolubricant can be produced from the transesterification and esterification reaction of vegetable oils/ fats or fatty acid feed stocks and long- chain alcohols. The impacts of usage of petroleum-based products on the environment have created an opportunity to produce environmentally acceptable lubricants from renewable source, like vegetable oils or biodegradable oleochemical. One of the major advantages of bio-based synthetic esters is better performances at low production cost compared to synthetic esters (Sanchez et al., 2006). Because of recent advances in the biotechnology of vegetable oils/ fat and the chemical modifications, it is possible to change biodegradable oleochemical esters into high performance biolubricant.

2.2.1 Transesterification reaction (Ting et al., 2011)

Transesterification of alcohol and triglyceride, vegetable oil or animal oil produces alkyl ester and glycerol. The general reaction of transesterification is shown in Fig 2.1. R', R'' and R''' of triglyceride are long chain fatty acid which may be same or different depending on type of oils. Catalysts are used to enhance the reaction rate. In the reaction, from stoichiometric, there are one mole of triglyceride to three moles of alcohol to produce three moles of alkyl ester and one mole of glycerol. It is a reversible reaction. Therefore, in the process, excess of alcohol is required to drive the reaction to the right side in order to increase the equilibrium conversion.

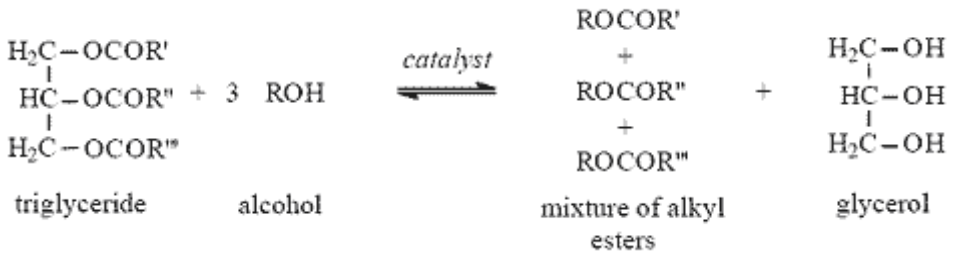

Figure 2.1 Transesterification reaction (Brahmkhatri et al., 2011)

2.2.2 Esterification reaction (Ting et al., 2011)

Esterification of one mole fatty acid with one mole alcohol produces fatty acid acid alkyl esters and water (Fig 2.2). Esterification is a reversible reaction. Therefore, excess of alcohol or removal of water might be used to increase the conversion yields of fatty acid alkyl esters.

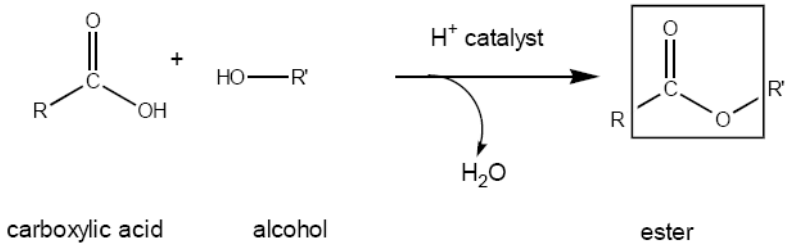

Figure 2.2 Esterification reaction (Schildhauer et al, 2009)

2.3 Propanol and iso propanol

2.3.1 Propanol

Propanol is a straight chain alcohol with the molecular formula of $CH_3CH_2CH_2OH$ (MW = 60.1, density = 0.8034 g cm^{-3} and viscosity =1.938 cP). It is also known as propan-1-ol, 1-propyl alcohol, *n*-propyl alcohol or *n*-propanol. It can be used as a solvent in pharmaceutical industries, and for production of resins and cellulose esters. It could be formed naturally in small amounts during many fermentation processes. Propanol can be produced from untreated plant biomass in aerobic growth conditions using an engineered strain of the actinobacterium, *Thermobifida fusca*. (Deng and Fong 2011).

2.3.2 Isopropanol

Isopropanol or Isopropyl alcohol (IPA) is a branch chain alcohol (Hanth et al., 2009) with the molecular formula C_3H_8O (MW= 60.1, density = 0.786 g cm^{-3}, viscosity = 1.96 cP). It is a colorless, flammable chemical compound with a strong odor. It is the simplest example of a secondary alcohol, where the alcohol carbon is attached to two other carbons sometimes showed as $(CH_3)_2CHOH$ (Santasalo et al., 2009).

2.4 Oleic acid

Oils and fatty acids, products from agricultural and food industrial manufactures can be used as feedstock for biolubricants. The most used fatty acids for production of biolubricants are palmitic acid and oleic acid. Oleic acid (cis–9-octadecenoic acid), $C_{18}H_{34}O_2$ is monounsaturated fatty acid found in many natural products. Oleic acid can be produced from plant oils. (Dormot et al., 2004)

2.5 Catalyzed process

2.5.1 Based catalyst

The common based catalysts in transesterification of oils and alcohols are potassium hydroxide (KOH) and sodium hydroxide (NaOH). Normally, the based catalysts proceed faster and less corrosive than acid catalysts; therefore, based catalysts have been generally used for ester production from oils in industrial processes (Guo et al., 2010)

2.5.2 Acid catalyst

Acid catalysts catalyze by Bronsted acids. These catalysts give very high yields in alkyl esters, but the rate of reaction is relatively slow, requiring typical temperatures above 100^{o}C for more than 3 h to reach completed conversion (Lou et al., 2008).

2.5.3 Enzyme catalyst

Although transesterification or esterification reaction using base or acid catalysts gives high reaction rate, it requires quite high energy and high reaction temperature. Besides, the separation of glycerol or water from esters is rather difficult and produces plenty of waste water. The process to remove the base or acid catalyst from the ester product also generates a lot of wastewater. Enzymatic conversion of fatty acid, therefore, has been suggested as a realistic alternative to the conventional physio-chemical method. The enzymatic methods require less energy and produce less wastewater. However, the main problem of the usage of biocatalyst is the high cost of enzymes. Hence, their reuse is required to overcome economical costs. Immobilized enzyme on solid is therefore, becoming increasingly popular. The advantages of the immobilized enzyme processing include mild operating conditions, ease of product isolation and reusability (Fukuda et al., 2001).

2.5.4 Immobilized enzyme (Novozym435)

The biocatalyst used for this research work is lipase acrylic resin from *Candida antarctica* (Novozym435, Sigma-Aldrich Co. LLC, USA) with its activity ≥10,000 U/g.

2.5.4.1 Properties

-Recombinant: expressed in *Aspergillus oryzae*

-Matrix: macroporous acrylic resin

2.5.4.2 Description

-Application

Immobilized preparation of a thermo stable lipase, particularly useful in the synthesis of esters and amides, and has broad substrate specificity.

-General description

Lipase from (B lipase) *Candida antarctica* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism and adsorbed on a macroporous resin.

-Legal Information

A product of Novozyme Corp. Novozym is a registered trademark of Novozymes A/S

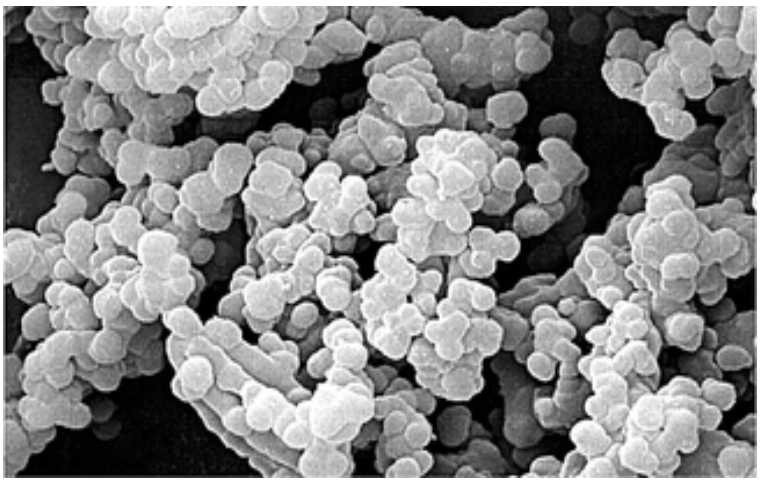

Figure 2.3 *Candida antarctica* lipase B (Juhla et al., 2010)

2.6 Literature review

Several development methods for biodiesel and biolubricant production have been previously reported. There are a number of factors affecting product qualities and production processes of biodiesel/ biolubricant. These include types of oil/fatty acid and alcohol, catalysts, and reaction conditions.

Steinke et al., (2000) studied lipase catalytic alcoholysis of crambe oil and camelina oil for preparation of long-chain esters by Novozym 435, Lipozyme IM and papaya latex lipase. The more yield was obtained by Novozym 435, Lipozyme IM and papaya latex lipase, respectively. The conversion of long-chain alcohols was higher than that of medium chain alcohols. The conversions of crambe oil and n-octyl esters using Novozym 435 were barely affected by the ratio of the alcohol, but with Lipozyme IM the conversions to esters distinctly reduced with excess of alcohol.

Dormoa et al., (2004) studied effect of water content, the molar ratio, chain length on production of biolubricant from oleic acid by enzymatic esterification in solvent-free system. It was shown that high water content could decrease reaction rates. The conversion of the oleates increased with increasing temperatures. The enzyme could be inhibited by high alcohol concentration. After pervaporation and membrane separation technique were applied to remove water from the product, the conversion yield at 99.8% was obtained under the optimal condition.

Salis et al., (2005) developed biodiesel production from triolien and short-chain alcohol though immobilized biocatalysis, using different commercial immobilized lipase such as *Cadida antartica B, Rizhomucor miehei*, and *Pseudomonas cepacia*. *Pseudomonas cepacia* lipase was found to be the most active enzyme. Temperature, water activity and molar ratio of substrate were studied. The optimal condition in this process is 40^{o}C, 6:1 molar ratio 1-butanol: triolein. It was found that methanol and 2-butanol were less efficient due to the immiscibility of the former of alcohol.

Bokade and Yadav (2007) investigated the influence of different alcohols (methanol, ethanol, n-propanol and n-octanol on transesterification of vegetable oil using Heteropoly-acids .loading on different supports, such as clay(K-10), activated carbon, ZSM-5, H-beta and TS-1 and reported the slightly decrease in ester

conversion with increasing number of alcohol. The catalyst loading at 10% on clay (K-10) was observed to give the best conversion.

Hernández-Martín and Cristina (2008) investigated alcoholysis of different oils in the presence of the commercially available immobilized lipases in solvent-free media. Optimum reaction parameters were determined: molar ratio of alcohol, reaction time, and enzyme loading. It was found that the loss of lipase activity was higher with methanol than with ethanol and Novozym 435 was stable than Lipozyme TL IM. The optimal volume of alcohol depended on loading of lipase. Alcoholysis of the vegetable oil with Lipozyme TL IM was faster than Novozym 435. By using a low enzyme loading of Lipozyme TL IM (10% w/w) with a large molar excess of alcohol, the initial rate was similar to that of Novozym 435 at a higher dosage (50% w/w). After 9 times in a batch reactor Novozym 435 maintained 85% of its initial activity.

Joelianigsih et al., (2008) studied the effects of reaction temperature and the kinetics of non-catalytic transesterification reaction of palm oil under atmospheric pressure from transesterification of vegetable oils with short-chain alcohol without using any catalysts. It was reported that the optimum reaction temperature with the highest conversion was 523 K; the kinetic constant increased with increasing temperature.

Hanth et al., (2009) investigated biodiesel production by esterification of oleic acid with short-chain alcohols by H_2SO_4. The results showed that the ethyl ester conversion depended on ethanol/oleic acid molar ratio. The conversion increased quickly with increasing ethanol/ oleic acid molar ratio to 3:1. However, the conversion decreased gradually after further increasing of alcohol ratio than 3:1. The ethyl ester conversion increased with rapidly increased catalyst concentration and slowly increased at a catalyst concentration above 5 %wt. The conversion of primary alcohols was higher than secondary alcohols. The optimal condition for this process was the molar ratio of alcohol to oleic acid at 3:1 with 5% H_2SO_4 at 60^{o}C.

Li and Yan (2010) developed the new technique of biodiesel product from Sapium sebiferum oil catalyzed by immobilized lipase from *Pseudomonas cepacia* G63. This work studied effects of enzyme dosage, effect of water concentration, effect of stirring rate and effect of temperature. The optimal reaction conditions were: 7% (w/w) water concentration, 40^{o}C of reaction temperature, 200 rpm for 12 h. It was

reported that high levels of molar ratio of methyl alcohol to oil, high temperature and high stirring rate could destroy the activity enzyme.

Brahmkhatri et al., (2011) studied recycling of the catalyst and showed that SBA-15 catalyst could be reused more than 5 times for the esterification of oleic acid and methanol. This research reported that the activation energy for diffusion limited reactions was 44.6 kJ/mol.

Yucel et al., (2011) investigated two different crude lipases immobilized onto micro porous poly matrix by both physical adsorption and covalent linking for the production of biodiesel by enzymatic transesterification at industrial scale. Biodiesel production was carried out with semi-continuous operation. Methanol was added into the reactor by three successive additions of 1:4 M equivalent of methanol to avoid enzyme inhibition. It was found that the optimal transesterification reaction conditions were: oil/methanol molar ratio of 1:4, temperature of 40°C and reaction time of 6 h. Lipozyme TL-100L lipase provided the highest yield of fatty acid methyl esters at 92%. Operational stability was determined with immobilized lipase and it indicated that a small enzyme deactivation occurred after used repeatedly for 10 batches with each of 24 h. Since the process was effective and enzyme did not leak out from the polymer, the method was proposed for industrial scale.

Birla et al., (2012) investigated a heterogeneous catalyst (CaO) prepared from snail shells and used for transesterification of waste frying oil. Reaction conditions were studied by varying the methanol/oil molar ratio, catalyst amount, reaction time and temperature. A kinetic study was also conducted to obtain the reaction rate constant (k) at various temperatures and activation energy (Ea). The results showed that the conversion of the ester was optimized at 6.03:1 methanol to oil molar ratio, catalyst amount 2.0 wt% relative to oil, 60^{o}C for 7 h. A highest conversion of esters as 99.58% was obtained. The reaction followed the1st order kinetics and the activation energy (Ea) was 79 kJ/mol.

Table 2.1 Review studies production of biodiesel/ biolubricant by different conditions

Reference	Objective	Optimal condition					
		Substrate	Catalyst	Temp (^{o}C)	Rx time (h)	Molar ratio Alc: Oil/acid	Conversion
Steinke et al., (2000)	To study 3 types of lipases (Novozym 435, Lipozyme IM and papaya latex lipase) for transesterification of crambe, camelina oil and various alcohols such as n-octanol, isopropanol, oleyl alcohol, crambe alcohols and camelina alcohol.	Camelina oil Oleyl alcohol	Novozym 435	-	24	3:1	>95%
Dormoa et al., (2004)	To find an application of fusel oil for bio-lubricant production. The alcohol compounds of fusel oil were esterified with oleic acid using enzyme catalysis in solvent-free media.	Oleic acid I-amyl-alcohol	Novozym 435	40	6	2.054:1	99.8%

Reference	Objective	Optimal condition					
		Substrate	Catalyst	Temp (^{o}C)	Rx time (h)	Molar ratio Alc: Oil/acid	Conversion
Salis et al (2005)	To study 4 immobilized lipases for esterification of oleic acid and short-chain (linear and branched) alcohols (C1-C5).	Triolien Butanol	Immobilized lipase *Pseudomonas cepacia*	60	6	6:1	>90%
Bokade and Yadav (2007)	To investigate effect of alcohols (methanol, ethanol, n-propanol and n-octanol), on transesterification of vegetable oil using different supports such as, clay(K-10), activated carbon, ZSM-5, H-beta and TS-1.	Vegetable oil Methanol	Heteropoly-acids Supported by Clay (K-10)	170	8	15:1	84%

Reference	Objective	Optimal condition					
		Substrate	Catalyst	Temp (^{o}C)	Rx time (h)	Molar ratio Alc: Oil/acid	Conversion
Hernández-Martín and Cristina (2008)	To investigate lipase-catalyzed alcoholysis of different oils in the presence of the commercially available immobilized lipases in solvent-free media.	Sunflower oil Methanol	Novozym 435	25	24	6.5:1	85%
Joelianigsih et al., (2008)	To study the effects of reaction temperatures and the kinetics of non-catalytic transesterification of palm oil under atmospheric pressure.	Vegetable oil Methanol	non-catalytic	250	5	-	95.17% k = 0.0034 min^{-1}

Reference	Objective	Optimal condition					
		Substrate	Catalyst	Temp (^{o}C)	Rx time (h)	Molar ratio Alc: Oil/acid	Conversion
Hanth et al., (2009)	To study the esterification of oleic acid with alcohol under irradiation condition.	Oleic acid Ethanol	H_2SO_4	60	10	3:1	>90%
Li and Yan (2010)	To study the biodiesel production in micro-aqueous phase catalyzed by the immobilized lipase and examine the operational stability and reusability of the immobilized lipase. To compare the fuel properties between the biodiesel and traditional diesel.	Sapium sebiferum oil Methanol	Pseudomonas cepacia G 63.	40	12	4:1	90%

Reference	Objective	Optimal condition					
		Substrate	Catalyst	Temp (^{o}C)	Rx time (h)	Molar ratio Alc: Oil/acid	Conversion
Brahmkhatri et al (2011)	To study the use synthesized catalyst for biodiesel production by esterification of oleic acid with methanol.	Oleic acid Methanol	SBA-15	40	4	4:1	1^{st} =90% 2^{nd} >85 % 3^{rd} =85% 4^{th} <85% 5^{th} <85% Ea = 44.6 kJ/mol
Yucel et al., (2011)	To investigate the production of biodiesel by enzymatic transesterification at industrial scale and the repeated use of lipases immobilized onto micro porous poly matrix by both physical adsorption and covalent linking.	Conalo oil Methanol	Lipozyme TL-100L	40	6	4:1	92%

Reference	Objective	Optimal condition					
		Substrate	Catalyst	Temp (^{o}C)	Rx time (h)	Molar ratio Alc: Oil/acid	Conversion
Birla et al., (2012)	To study the transesterification of waste frying oil by a heterogeneous catalyst (CaO) prepared from snail shells. A kinetic study was conducted; the reaction rate constant (k) at various temperatures and activation energy (Ea) was examined.	Waste frying oil Methanol	CaO	60	8	6.03:1	99.58% Ea= 79 kJ/mol

CHAPTER III
EXPERIMENTAL PROCEDURE

3.1 Materials and equipments

3.1.1 Chemicals

1. Propanol / Isopropanol
2. Phenolphthalein
3. KOH
4. Oleic acid
5. Novozyme 435 (Sigma-Aldrich Co. LLC, Canada)
6. Molecular sieve (Ajax Finechem Pty Ltd, Sydney, Australia)

3.1.2 Equipments

1. Hotplate stirrer with magnetic stirrer set
2. Vessel vial, flasks, Beaker
3. Burette
4. Centrifuge (5100, Kubota, Fujioka, Japan)
5. Incubator shaker (Innova 4000, ALT, Connecticut, USA)

3.2 Reaction procedure

The procedure used for ester production was as follows. The esterification reactions were carried out in 250 ml flask containing the mixtures of 40 g of oleic acid, propanol or isopropanol and Novozym 435 placed in a shaking incubator under controlled stirring and controlled reaction temperature for 24 h. For the analysis of FFA conversion, the samples of 3.5 ml were taken out from the reaction mixture at 15 min, 30 min, 1, 3, 6, 9, 12 and 24 h of the reaction. The remaining alcohol and water were removed from the samples by evaporation before the analysis. For the examination of the repeated uses of Novozym 435, after 24 h of the reaction, the products and the remaining substrates were removed from the flask and the fresh substrates were added to the flask. The reaction was then performed at the optimal conditions to examine the enzymatic activity of the reused Novozym 435.

3.3 Enzymatic esterification reaction

The procedure used for ester production was as follows:

3.3.1 Esterifications were carried out in shaking flasks containing 40 g oleic acid

3.3.2 The investigated variables on propyloleate production were:

- Reaction time (period 24 h)
- Alcohol structure (propanol vs. isopropanol)
- Enzyme loading (2.5, 5, 10 % based on oleic acid weight)
- Rotation speed (200, 250, 300 and 350 rpm)
- Molar ratio of propanol to oleic acid (1:1, 2:1, 3:1 and 4:1)
- Temperature (35, 40, 45, 50 and 60 ^{o}C)
- Repeated use of immobilized enzyme (Novozym 435)
- Removal of water by adsorption using molecular sieves (42.5 % by weight of oleic acid)

3.4 Analytical methods

3.4.1 Oleic acid conversion

Propanol and water were removed from the sample by evaporating and then centrifuging. Percentage of oleic acid conversion was determined by the titration method with 0.05 M KOH solution using phenolphthalein as the indicator. Concentration of oleic acid could be calculated from the titration volume of KOH solution.

Weight of FFA = KOH (ml) x [KOH] x 0.2824

$$\text{Weight of FFA per one gram sample} = \frac{\text{KOH(ml)x[KOH]x28.24614}}{\text{weight of sample}}$$

$$\%\ \text{FFA conversion} = \frac{(\text{initial weight} - \text{weight at time})}{\text{initial weight}} \text{ x } 100$$

3.4.2 Analysis of propyloleate by Nuclear Magnetic Resonance Spectrometer

Analysis of propyloleate by Nuclear Magnetic Resonance Spectrometer (NMR 500 MHz) with CP/MAS solid probe and Nano probe (Varian version INOVA, Lexington, USA) was carried out at Scietific and Technological Research Equipment Center, Chulalongkorn University, in order to confirm the results of the titration method. NMR analysis was performed by dissolving the propyloleate in $CDCl_3$. The dissolved sample was transferred to an NMR tube. All solid material must be removed from the solution before it was placed in the NMR tube. Then, the NMR tube was inserted into a sample turbine. Spectra were recorded on a Varian Mercury-500 spectrometer operating at 500 MHz at room temperature.

CHAPTER IV
RESULTS AND DISCUSSION

In this work, biolubricant was synthesized from the esterification of oleic acid with propanol using immobilized enzyme (Novozym 435) in batch process.

Optimal conditions for the biolubricant production were determined. Results and discussion are presented in 9 topics:

4.1 Effect of reaction time

4.2 Effect of alcohol structure (propanol vs. isopropanol)

4.3 Effect of enzyme loading

4.4 Effect of rotation speed

4.5 Effect of molar ratio of propanol to oleic acid

4.6 Effect of temperature

4.7 Reuseability of immobilized enzyme (Novozym 435)

4.8 Kinetics of esterification

- 4.8.1 Rate constant
- 4.8.2 Activation energy

4.9 Effect of water removal by adsorption using molecular sieve

4.1 Effect of reaction time

In the esterification reaction, the biolubricant was produced by using 40 g oleic acid, 45°C, at an enzyme (Novozym 435) loading of 5% (w/w) (based on oleic acid weight), 250 rpm and propanol to oleic acid molar ratio at 2:1 in order to investigate the effect of reaction time from 0- 24 h.

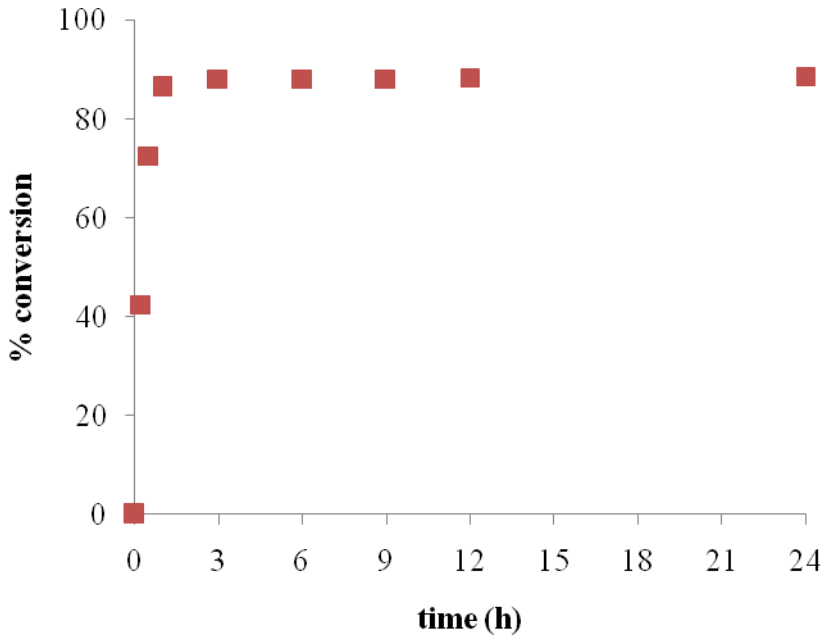

Figure 4.1 Effect of reaction time on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45°C, [PrOH]:[Oleic acid] = 2:1, 5% (w/w) Novozym 435 and 250 rpm.

Effect of reaction time on the conversion of FFA to propyloleate was studied. The results are showed in Figure 4.1. It was found that the conversion of FFA rapidly increased in the initial 1 h of the reaction and then slowly increased until the conversion approached the equilibrium point within 3 h.

The percentage of conversion at 24 hours was 88.7%. This result was in agreement with other studies on synthetic esters. Li et al. (2010) investigated transesterification of Sapium sebiferrum oil and methanol by immobilized lipase and reported that the reaction was very fast in the first hour; after that the reaction slowed down. However, the equilibrium time depended on conditions in the process.

4.2 Effect of alcohol structure (Propanol vs. Isopropanol)

The alcohols used in this study were propanol (linear chain) and isopropanol (branch chain). The conditions of esterification reaction were: 45°C, 2:1 propanol to oleic acid molar ratio, 5% (w/w) Novozym 435 (based on oleic acid weight) and 250 rpm.

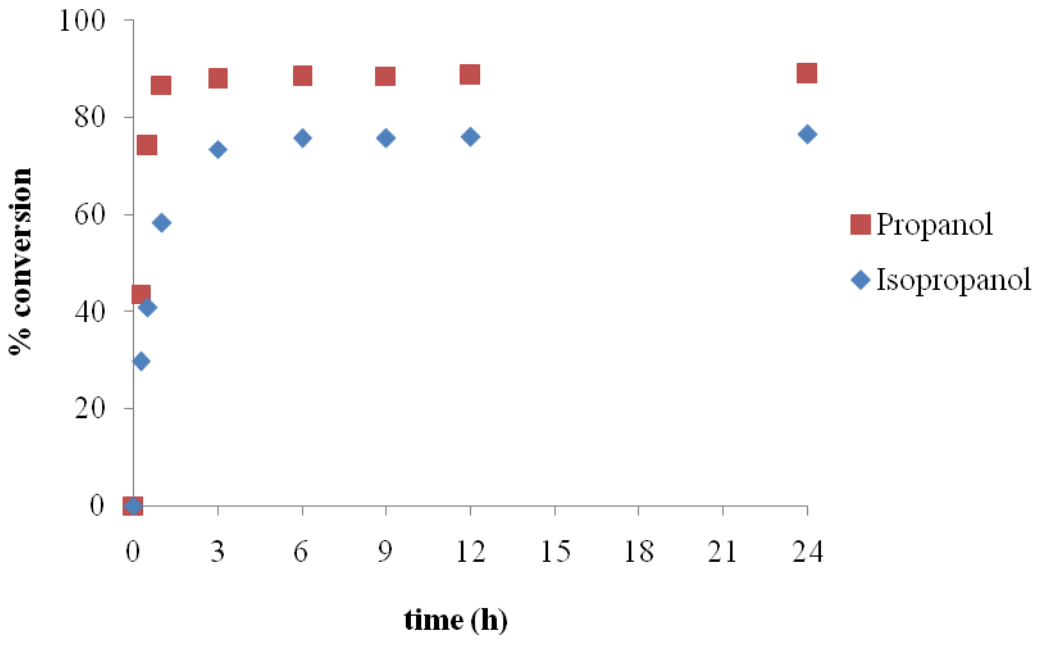

Figure 4.2 Effect of alcohol structure (Propanol vs. Isopropanol) on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45°C, [PrOH]:[Oleic acid] = 2:1, 5% (w/w) Novozym 435 and 250 rpm.

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The conversions of FFA are shown in Figure 4.2. The percentages of the conversion by using n-propanol reached 89.0% which was higher than that using isopropanol (76.4%).

The percentage conversion of isopropyloleate was lower than the conversion of propyloleate. Since isopropanol is a branched chain alcohol, according to its structure, it could be more difficult to reach the active site of the enzyme than a linear chain alcohol. According to Salis et al. (2005) who studied the biodiesel production from trilein and alkyl alcohols through biocatalysis, it was found that the secondary alcohol (isobutanol) reached the active sites slower than the primary alcohols (Butanol). Later in 2009, Hanh et al. investigated the esters production of various alcohols and reported the higher reaction rate of primary alcohols than the secondary alcohols, such as propanol/2-propanol and butanol/isobutanol.

4.3 Effect of enzyme loading

In order to investigate the effect of enzyme loading at 2.5, 5, and 10% (w/w) Novozym 435 based on oleic acid weight. The esterification reaction of the biolubricant was performed at 45 ^{o}C, with 2:1 propanol to oleic acid molar ratio, at 250 rpm for 24 h.

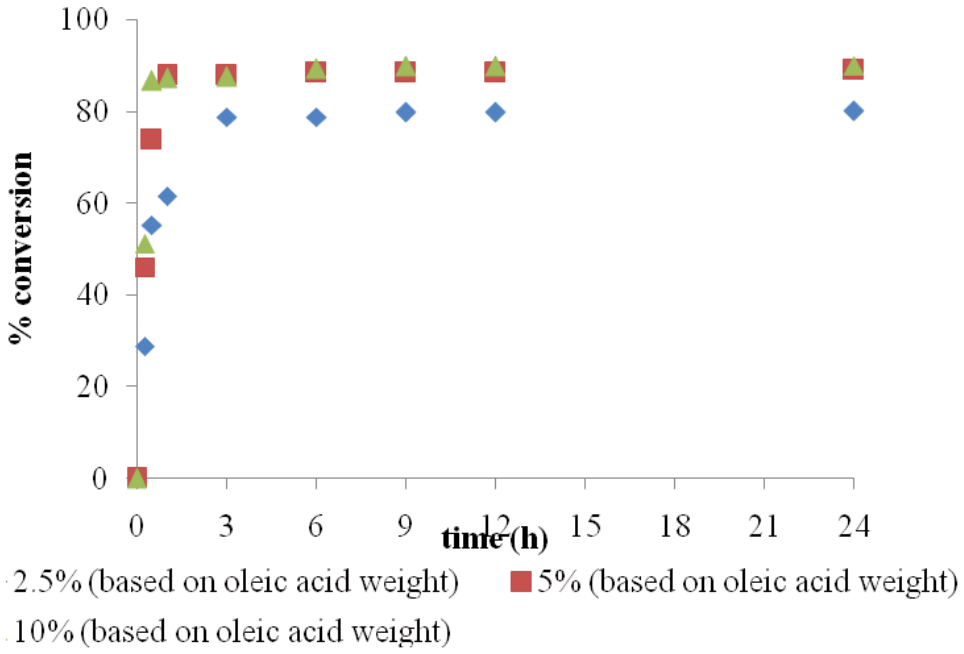

Figure 4.3 Effect of enzyme loading (based on oleic acid weight) on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid and 250 rpm.

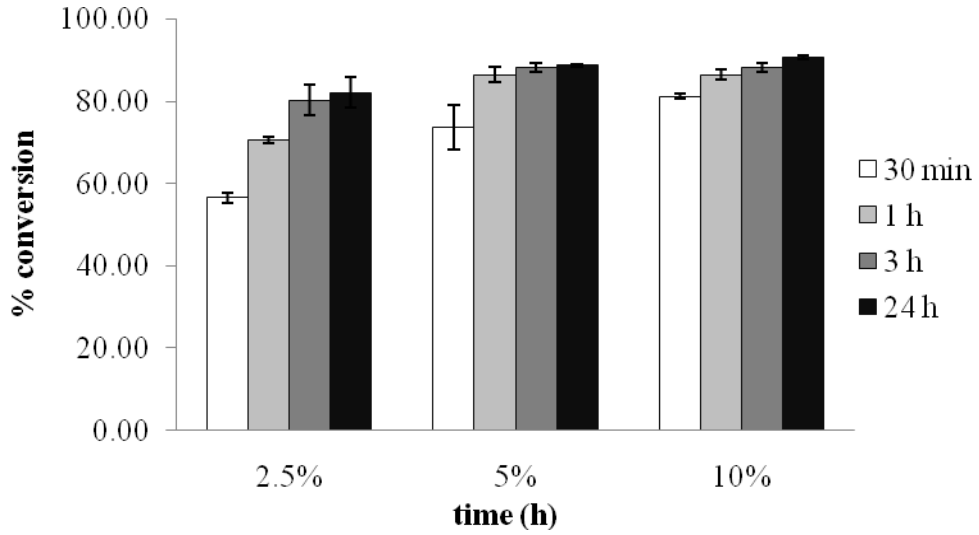

Figure 4.4 Effect of enzyme loading on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45°C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 250 rpm at 30 min, 1 h, 3 h and 24 h.

The effects of enzyme loading varied from 2.5% to 10% (w/w of oleic acid) are shown in the Figure 4.3 and Figure 4.4. The FFA conversion increased when increasing enzyme loading. The percentages of propyloleate conversion at 24 h for 2.5 to 5.0% of enzyme loading were 80.1% and 88.7% respectively. The higher conversion rate at enzyme loading of 5% than 2.5% was observed, especially in the initial period (0-1 h). The maximum FFA conversion at 90.6% after 24 h was obtained by using enzyme loading at 10%, which was slightly higher compared with that at 5.0%.

At low enzyme loading, the conversion rate is slow. The increase of enzyme concentration will lead to an increase in the rate of reaction. The conversion continuously increases until it reaches the equilibrium point. However, at high enzyme loading, the substrate becomes saturated with enzymes. Further increasing of enzyme loading, therefore, will not affect the rate as shown in Figure 4.4. It can be observed that the times taken for the reaction to approach the equilibrium were about 3 h for the system using 2.5% of Novozym 435 and about 1 h for the systems using 5 and 10% Novozym 435.

This result is in agreement with Li et al., (2010), who investigated biodiesel production and reported that the conversion yield of biodiesel was increased quickly with increased enzyme dosage until 2.5%. However, the yield of biodiesel was slightly increased after 2.5% enzyme dosage. Yucel et al., (2011) studied the enzymatic (Novozym 435) biodiesel production of canola oil and methanol by using enzyme loading at 5, 10 and 20% (w/w of oil). They found that the conversion increased with increasing in amount of the biocatalyst up to 5%. While at higher amount of catalyst of 10% (w/w of oil), the conversion was not distinguished different. They all reported that the conversion of esters slightly increased when excessive enzyme loading was used.

From the economic point of view, the chosen condition is the one with relatively high FFA conversion at low enzyme loading. Due to high cost of the enzyme and no considerable difference in the conversion yields between the systems with 5% and 10% enzyme loadings, therefore, the further experiments were carried out by using enzyme loading at 5% (w/w, based on oleic acid weight).

4.4 Effect of rotation speed

Effect of rotation speed in the batch esterification process was investigated at 200, 250, 300, and 350 rpm. The other controlled parameters were as follows: reaction temperature at 45^{o}C, propanol to oleic acid molar ratio at 2:1 and enzyme Novozym 435 loading at 5% (w/w) (based on oleic acid weight).

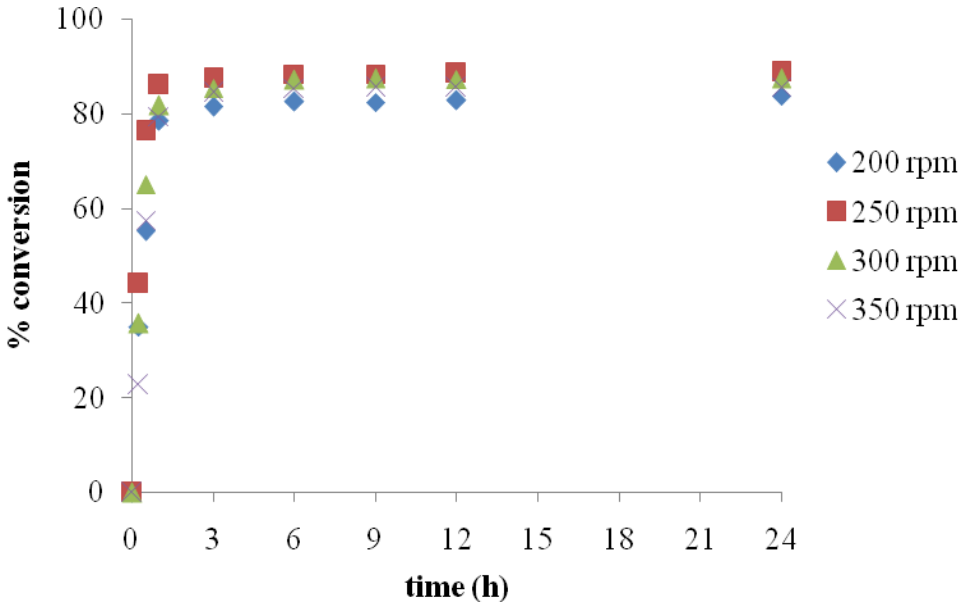

Figure 4.5 Effect of rotation speed on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45°C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid and 5% (w/w) Novozym 435.

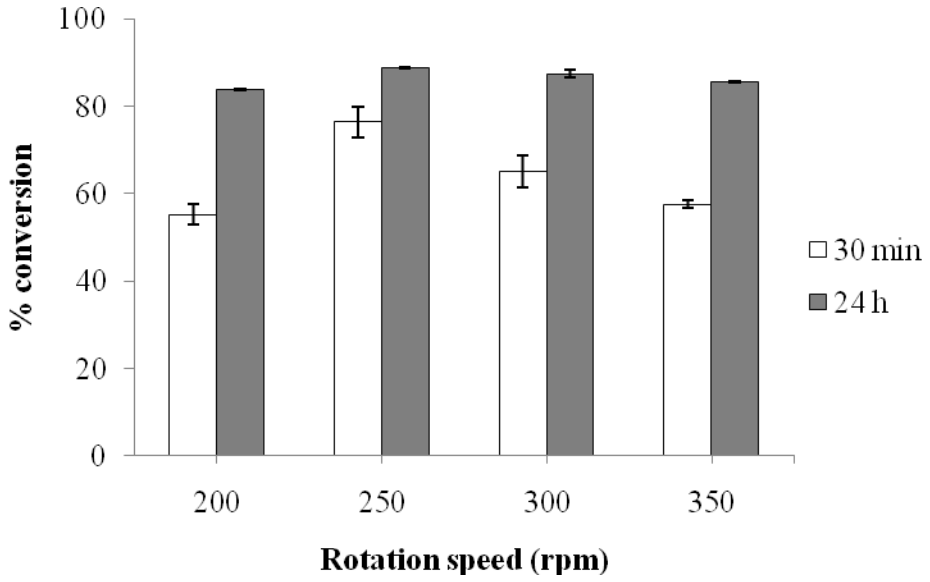

Figure 4.6 Effect of rotation speed on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45°C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 at 30 min and 24 h.

Oleic acid and propanol formed a two phase mixture when added together. Well Stirring was required for efficient contact of two liquid phases. The results for the effect of rotation speed in the range of 200-300 rpm are shown in Figure 4.5 and Figure 4.6. The percentages of FFA conversion were 83.8, 88.9, 87.5 and 85.7% at the rotation speeds of 200, 250, 300 and 350 rpm, respectively. The conversion of FFA increased to the maximum at 250 rpm, in which the maximal conversion was 88.9%. Beyond this (300 and 350 rpm), the conversion relatively decreased, which could be due to the negative effect of high shear rate on the enzymatic activity. Similarly, it was previously reported that the conversion yield of biodiesel in micro-aqueous phase catalyzed by the immobilized lipase increased with increasing of stirring rate from 100 to 200 rpm, but decreased at the stirring rate over 250 rpm (Li et al., 2010).

4.5 Effect of molar ratio of propanol to oleic acid

Effect of molar ratio of propanol to oleic acid was investigated in this study. The molar ratio were conducted at 1:1, 2:1, 3:1, and 4:1 molar ratio of propanol to oleic acid at 45 ^{o}C with 5%(w/w) Novozym 435 (based on oleic acid weight) at 250 rpm for 24 h of reaction period.

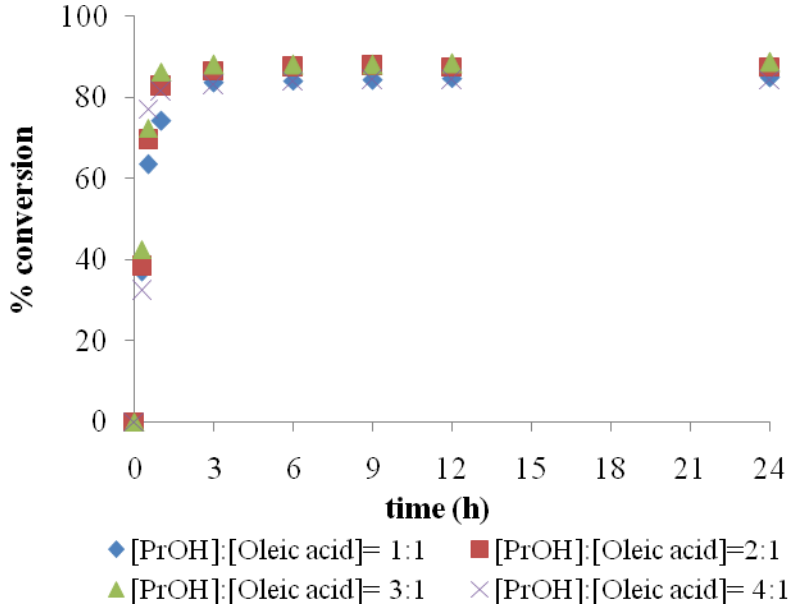

Figure 4.7 Effect of molar ratio [oleic acid]:[propanol] on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45^{o}C, 5% (w/w) enzyme loading and 250 rpm.

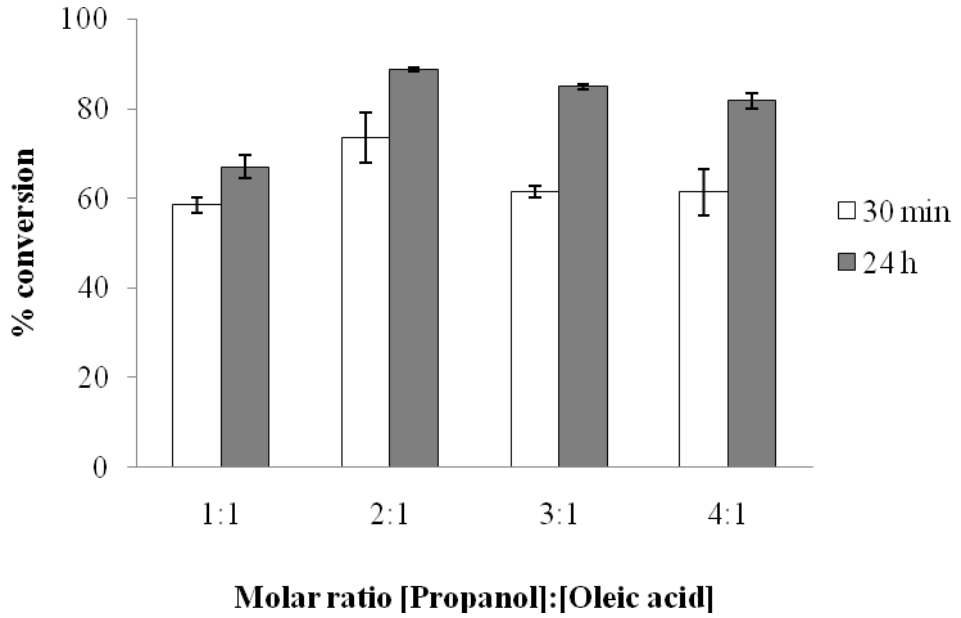

Figure 4.8 Effect of molar ratio of propanol to oleic acid on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45^{o}C, 40 g oleic acid, 5% w/w Novozym 435, 250 rpm at 30 min and 24 h.

From stoichiometric esterification, the reaction needs one mole of oleic acid and one mole of propanol to produce one mole of propyloleate and one mole of water. The reaction is reversible reaction so that the excess of propanol is required to drive the reaction forward until it reaches the equilibrium concentration. In order to gain the approached irreversible reaction, the process must use an excess of alcohol. The results from the study are shown in Figure 4.7 and Figure 4.8. It was found that the percentages of FFA conversion at 1:1 and 2:1 molar ratios of propanol and oleic acid were 67.2% and 88.7%, respectively. After that, the FFA conversions slightly reduced when the ratios were increased to 3:1 and 4:1. The possible reason was due to the reduction of enzyme and oleic acid contact at higher alcohol concentration and/or the decline of enzymatic activity from propanol inhibition effect. Moreover, surplus addition of alcohol would cause large energy consumption in product separation.

From a previous result by Rodrigues et al. (2011), the immobilized lipase was inactivated when the molar ratio of methanol to oil ratio was higher than 24:1.

Feifei et al. (2010) investigated the transesterification of soybean oil with various amounts of methanol using 1, 3-specific lipase. The maximal percentage of conversion was obtained from 4:1 molar ratio of alcohol to oil. At molar ratio more than 4:1, the lipase catalyst was found inactivated. The inactivation of lipase in esterification process of isoamyl alcohol and oleic acid at molar ratio of alcohol to oleic acid over 2:1 was also reported (Dormoa et al., 2004). From some previous reports (Gulati et al., 2003; Dormoa et al., 2004), the optimal ratio of alcohol to fatty acid for esterification by lipase was between 1:1 and 3:1.

4.6 Effect of temperature

Temperature is an important factor for enzymatic catalysis. Higher temperature causes quickly transformation of substrate, but at the same time, high temperature cause denaturation of enzyme. In this work, we investigated the effect of reaction temperatures at 35, 40, 45, 55, and 60^{o}C. The constant system parameters are as follows: [PrOH]:[Oleic acid] at 2:1, 5%(w/w) lipase loading (based on oleic acid weight), 250 rpm and reaction period of 24 h.

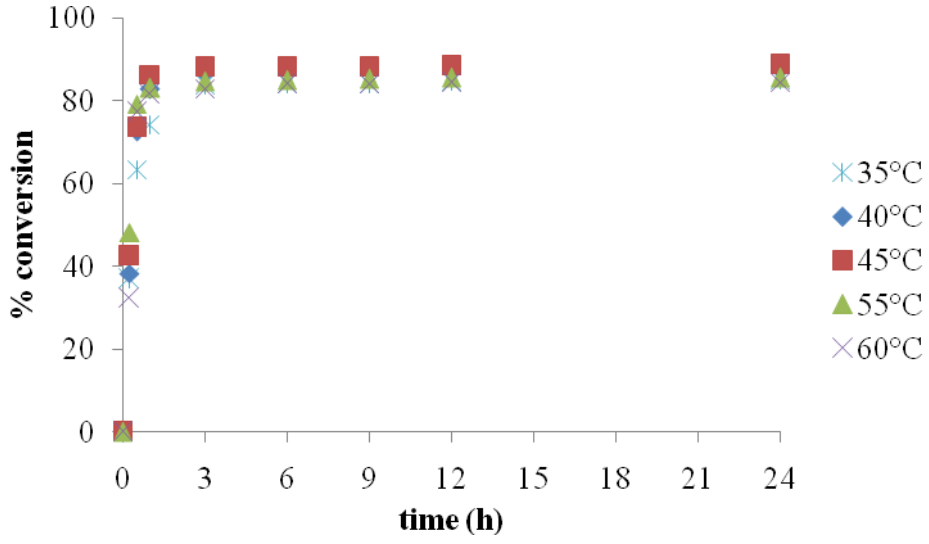

Figure 4.9 Effect of temperatures on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm.

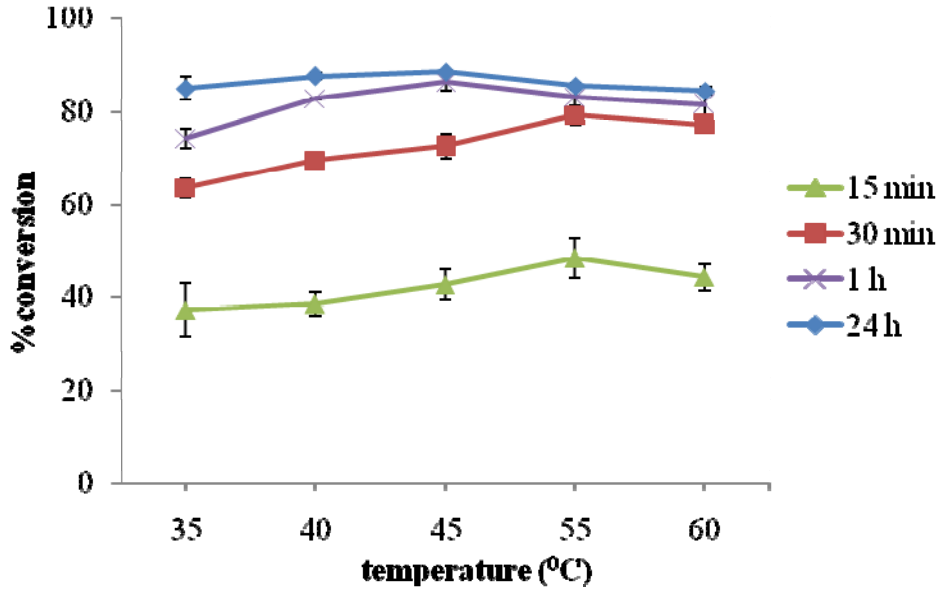

Figure 4.10 Effect of temperatures on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435, 250 rpm at 15 min, 30 min, 1 h and 24 h

Esterification of oleic acid and propanol was studied at various controlled temperatures in range 35-60^{o}C. The results were shown in Figure 4.7. It was found that the maximal FFA conversion at 88.7% was obtained at 45^{o}C. In the first 30 min, the conversion increased with increasing of temperature. The increase of reaction rate by temperature is due to increasing of kinetic energy in the system, which can be described in form of Arrhenius equation ($k = Ak^{-E/RT}$). However, it was found that increasing temperature over 45^{o}C resulted in a relatively lower final FFA conversion, which could be due to the thermal denaturation of enzyme at high temperature.

The conversion in the initial time period (observed at 15 and 30 min) tended to increase with the increase of temperature (Figure 4.8). However, when the temperature reached 55^{o}C, further increasing temperature resulted in a slightly lower conversion. Significant effect of thermal denaturation was observed when the reaction continued from 1-24 h. On the whole, the optimal temperature for maximum conversion was at 45^{o}C. This result quite agrees with other previous reports as shown in Table 4.1.

Table 4.1 Comparison of the optimal temperatures in various reaction conditions

Reference	Optimal temperature	Reaction conditions
This work	45^{o}C	Esterification of oleic acid and propanol using Novozym 435 catalyst
(Rodrigues et al., 2011)	40^{o}C	Transesterifacation of waste cooking oil and methanol using lipase catalyst
(Li et al., 2010)	40^{o}C	Transesterifacation of Sapium sebiferum oil and methanol using immobilized lipase
(Salis et al., 2005)	40^{o}C	Esterifacation of waste oleic acid and methanol using immobilized lipase
(Dormoa et al.,2004)	60^{o}C	Esterification of oleic acid and i-amyl-alcohol using Novozym 435 catalyst

All reports observed the increased conversions with increasing temperature up to the optimal temperature; further increasing temperature then resulted in lower conversions. From the comparison, it should be noted that the optimal temperature of short-chain alcohols is relatively lower than that of long-chain alcohols. This result indicated that the optimal temperature depended on structure of alcohols.

4.7 Repeated use of the immobilized lipase Novozym 435

The experiments were performed in order to investigate activity of the immobilized enzyme (Novozym 435) after the repeated uses. The immobilized enzyme (Novozym 435) was used in the repeated batch experiments at 40^{o}C and 45 ^{o}C under constant shaking at 250 rpm.

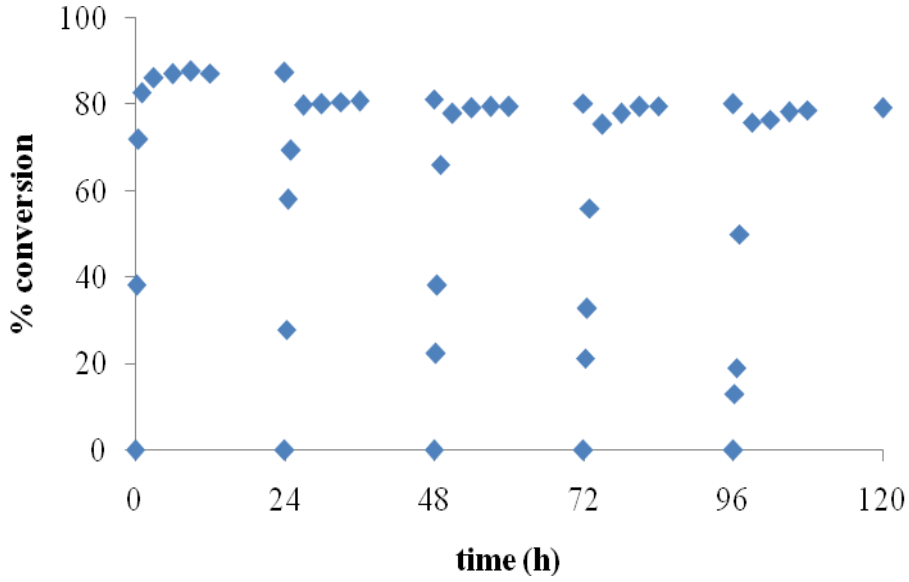

Figure 4.11 Repeated use of the immobilized lipase Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 40^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5 % (w/w) Novozym 435 and 250 rpm.

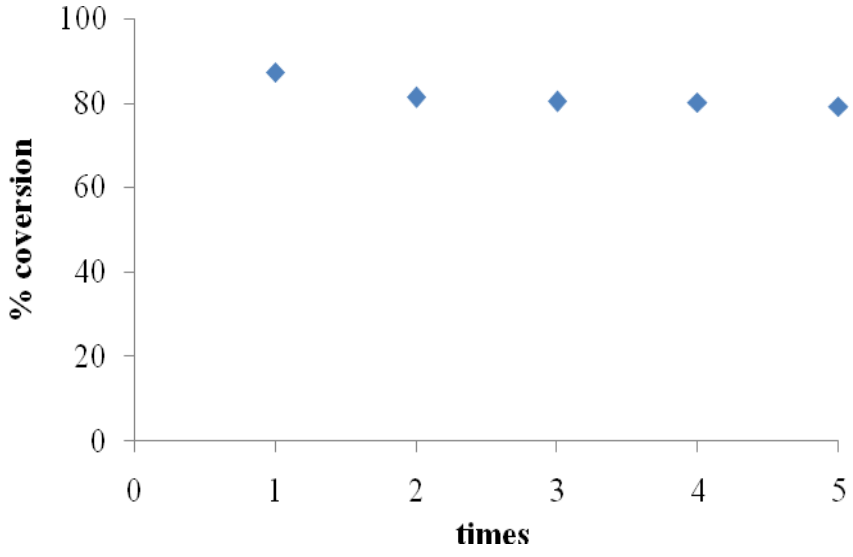

Figure 4.12 Repeated use of the immobilized lipase Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 40^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435, 250 rpm at 24 h.

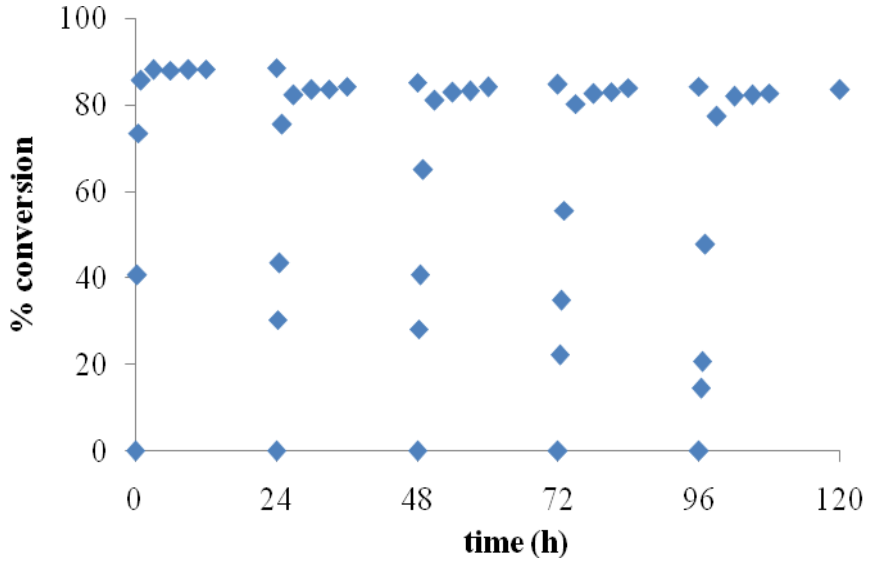

Figure 4.13 Repeated use of the immobilized lipase Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45°C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm.

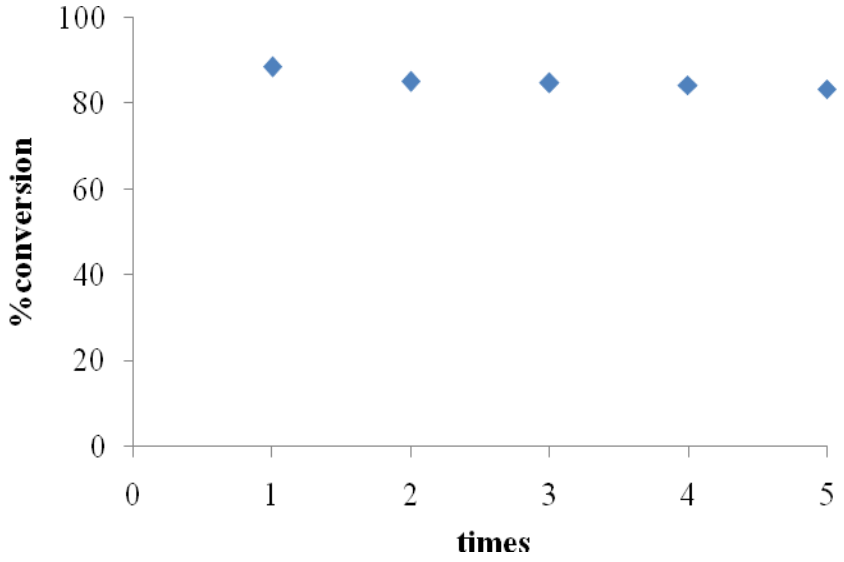

Figure 4.14 Repeated use of the immobilized lipase Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45°C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435, 250 rpm at 24 h.

The results at 40^{o}C are shown in Figure 4.12. Under the reuse of the enzyme, the final FFA conversion for the 1st, 2nd, 3rd, 4th and 5th run were 87.4, 81.2, 81.4, 80.2 and 79.3%, respectively. If the final FFA conversion by using freshly prepared immobilized lipase in the first run was defined as 100%, it was found that the final conversion for propyloleate production after Novozym 435 having been used for 5 times was at 100, 93.0, 91.8, 91.8 and 90.7% of that of the 1st run, respectively. Therefore, it could be concluded that after 5 cycles in the batch system at 40^{o}C, Novozym 435 was still effective in the esterification reaction with less than 10% loss in its conversion.

The experimental results at 45^{o}C are shown in Figure 4.13. The final FFA conversions were 88.54, 85.19, 84.75, 84.34 and 83.4% respectively. It was found that the final conversions for propyloleate production after Novozym 435 having been used for 5 times were at 100, 96.2, 95.7, 95.3, and 94.2% of that of the 1st run, respectively. After 5 cycles in a batch reactor at 45^{o}C, Novozym 435 was still effective in the esterification reaction with less than 6% loss in its conversion.

Previously, biodiesel by esterification of oleic acid and methanol using SBA-15 catalyst was studied. It was reported that under the repeated use for 5 times (of 4 h for each cycle), the conversion reduced less than 6% (Brahmkhatri et al., 2011). The FFA conversions in this work were higher compared to the previous work by Brahmkhatri et al., (2011). Therefore, the process conditions for production of biolubricant using immobilized lipase (Novozym 345) in this work could be considered more effective.

4.8 Kinetics of esterification

4.8.1 Rate constant

The common esterification of oleic acid and propanol follows the reaction:

$$\text{Oleic acid} + \text{PrOH} \rightleftharpoons \text{PrE} + H_2O$$

Where PrOH is propanol and PrE is propyl ester.

In this study, the model of kinetic reaction based on the esterification reaction which was assumed to proceed in the second order reaction as a function of the concentration of oleic acid and propanol as shown below.

$$R = \frac{-d[\text{Oleic a}]}{dt} = k\,[\text{Oleic a}]\,[\text{propanol}] \quad (1)$$

$$[\text{Oleic a}] = [\text{Oleic a}]_0\,(1\text{-}X_{\text{oleic a}}) \quad (2)$$

$$[\text{Propanol}] = [\text{Propanol}]_0\,(1\text{-}X_{\text{propanol}}) = [\text{Oleic a}]_0\,(M\text{-}X_{\text{oleic a}})$$

$$M=\frac{[\text{Propanol}]}{[\text{Oleic a}]} \quad (3)$$

Substitute (2) and (3) into (1)

$$\frac{-d[\text{Oleic a}]_0(1-X_{\text{Oleic a}})}{dt} = k\,[\text{Oleic a}]_0\,(1\text{-}X_{\text{oleic a}})\,(M\text{-}X_{\text{oleic a}}) \quad (4)$$

Integrate Equation. (4)

$$\ln\frac{(M-X_{\text{oleic a}})}{M(1-X_{\text{oleic a}})} = [\text{Oleic a}]_0\,(M\text{-}1)k\,t \quad (5)$$

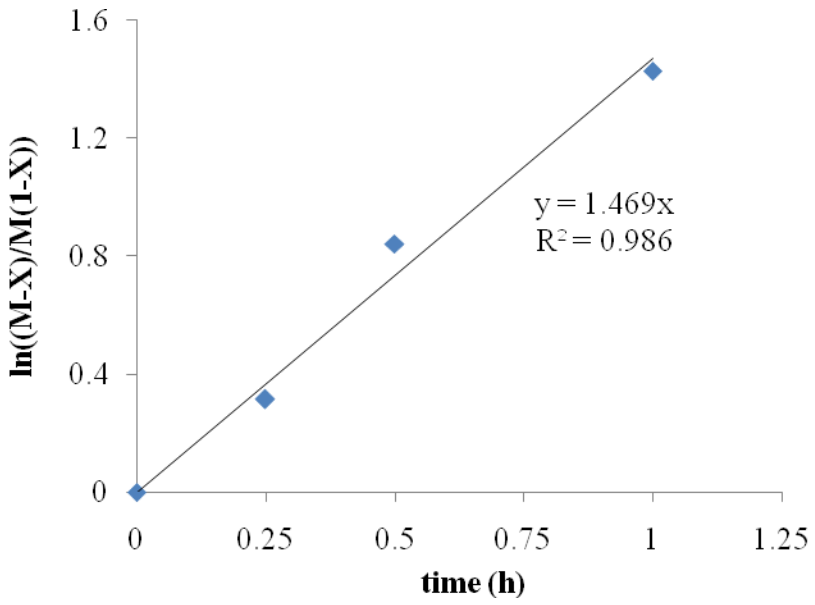

Figure 4.15 The plot for the determination of kinetic constants of Novozym 435 catalyzed alcoholysis in oleic acid. Conditions: 45°C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm

The conversions from various time were used to calculate the kinetic constants of the initial rate (0-1 h) from plot of $\ln((M-X_{oleic\ a})/M(1-X_{oleic\ a}))$ and time as shown in Figure 4.15, the slope of this graph is 1.481 and the kinetic constants (k) is found to be 1.469/(2.14(2-1)) = 0.686 $(h^{-1}(kmol/m^3)^{-1})$

Table 4.2 Determination of the kinetic constants and FFA conversion on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: various temperature, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435, 250 rpm

Temperature (°C)	Conversion at 24 h (%)	Rate constant, k $(h^{-1}(kmol/m^3)^{-1})$	R^2
35	85.0	0.306	0.945
40	87.6	0.413	0.950

Temperature (^{o}C)	Conversion at 24 h (%)	Rate constant, k ($h^{-1}(kmol/m^3)^{-1}$)	R^2
45	88.7	0.467	0.980
55	85.6	0.450	0.842
60	84.5	0.412	0.851

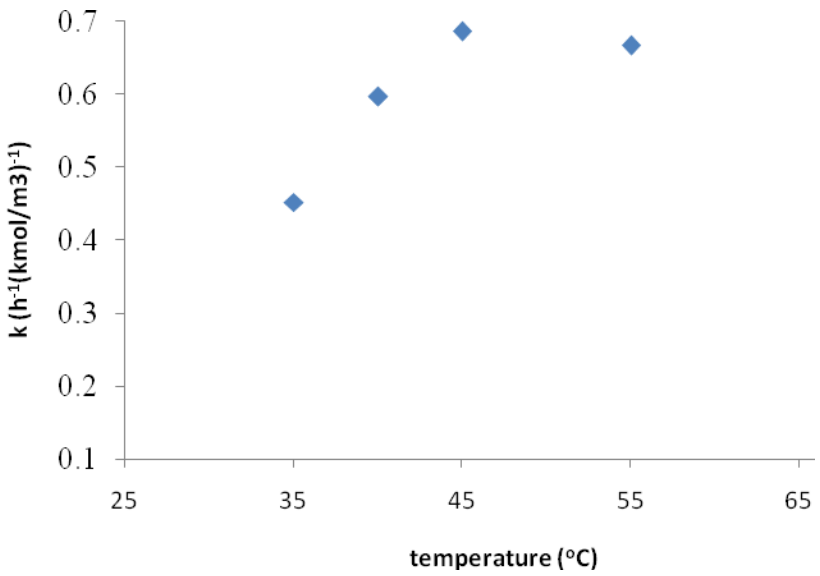

Figure 4.16 The kinetic constants on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: various temperature, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435, 250 rpm at 24 h.

From the experimental data at various temperatures, the results could be used for the analysis in term of the kinetic constants. The model of kinetic reaction based on the esterification reaction was assumed to proceed in the second order reaction as a function of the concentration of oleic acid and propanol as described by Equation (5). The plot shown in Figure 4.15 is for the determination of the rate constants or k values. The kinetic constant (k) could be calculated from the slope of the plot at the initial rate of reaction (0-1 h). The results are shown in Table 4.2 and Figure 4.16. It was found that the rate constant (k) increased with increasing temperature up to 45°C, at which the highest kinetic constant (k) was 0.467 $h^{-1}(kmol/m^3)^{-1}$. The lower k values

at temperature over 45^{o}C were due to the denaturation of immobilized lipase at high temperature.

This result agrees with the previous result (Joelianingsihaj et al., 2008) studied on biodiesel fuels from rapeseed oil and methanol using non-catalytic. The model of kinetic reaction based on the transesterification reaction was assumed to proceed in the first order reaction of the initial rate. The k value increased (0.0034 - 0.056 min^{-1}) with increasing temperature (532- 563 K), according to the Arrhenius equation.

The kinetic constants in the repeated uses of Novozym 435 at 40^{o}C and 45^{o}C are shown in Table 4.3 and Table 4.4, respectively. The kinetic constant and FFA conversion decreased with the number of the repeated use of Novozym 435. The decrease of FFA conversion could be due to the deactivation of Novozym 435 and/or the loss of the enzyme in the recycle process.

Table 4.3 Determination of the kinetic constants and FFA conversion on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 40 ^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm.

Run	% conversion at 24 h	k ($h^{-1}(kmol/m^3)^{-1}$)	R^2
1	87.4	0.607	0.940
2	81.2	0.384	0.947
3	80.4	0.294	0.981
4	80.2	0.226	0.994
5	79.3	0.171	0.926

Table 4.4 Determination of the kinetic constants and FFA conversion on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45 ^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm.

Run	% conversion at 24 h	k (h-1(kmol/m3)$^{-1}$)	R^2
1	88.55	0.673	0.991
2	85.20	0.406	0.966
3	84.75	0.302	0.994
4	84.34	0.227	0.998
5	83.43	0.120	0.956

4.8.2 Activation energy

From Arrhenius equation

$$k = Ae^{-Ea/RT} \quad (6)$$

$$\ln k = \ln A - E_a/RT \quad (7)$$

$\ln A = 12.52$

$A = 2.74\times10^{5}\ h^{-1}(kmol/m^3)^{-1}$

$-E_a/R = -4095.0\ h^{-1}(kmol/m^3)^{-1}$

$E_a = 4095.0 \times 8.314 = 34{,}045.8$ J/mol = 34.05 kJ/mol

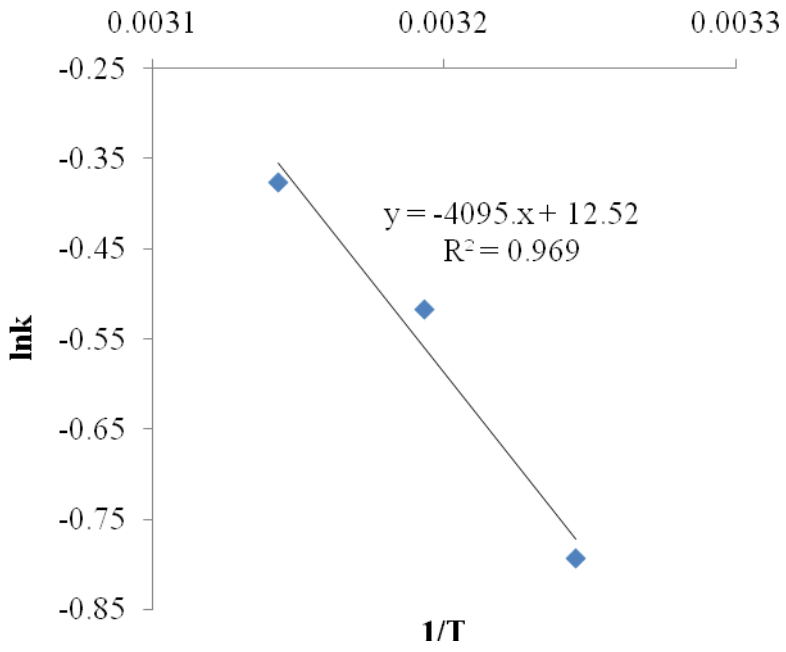

Figure 4.17 Second order reaction rate in Arrhenius plot during the esterification reaction of oleic acid and propanol at various temperatures.

According to the experimental data, the k values increased with increasing temperature up to 45^{o}C; then the k values decreased from thermal denaturation at 55 and 60^{o}C (Figure 4.16). Therefore, only k values obtained from the experiments with controlled reaction temperatures in the range of 35-45^{o}C was used for Ea determination. According to the Arrhenius relation (Equation (6)); Ea is the activation energy, R is the molar gas constant (8.314 J/mol K) and A is the frequency factor. From the plot of ln k and 1/T as shown in Figure 4.17, the activation energy (Ea.) was found to be 34.05 kJ/mol and a frequency factor (A) was 2.74×10^{5} ($h^{-1}(kmol/m^{3})^{-1}$). The comparison of activation energies of esterification and transesterification using different types of catalysts and substrates are shown in Table 4.5. Activation energy is a minimum energy required to start a chemical reaction. The Ea value of the process for production of biolubricant using immobilized enzyme (Novozym 435) is lower than the others, which indicated that this process requires less activation energy.

Table 4.5 Comparison of activation energy with different catalysts and substrates.

Reference	Catalyst	Oil/fatty acid	Ea (kJ/mol)
(Rodrigues et al., 2011)	Heterogeneous catalyst	Waste frying oil	79.0
(Feifei et al.,2010)	Super critical CO_2	Jatropha oil	45.2
(Brahmkhatri et al., 2011)	SBA-15	Oleic acid	44.6
This work	Novozym 435	Oleic aci	34.05

4.9 Effect of water removal by adsorption using molecular sieve

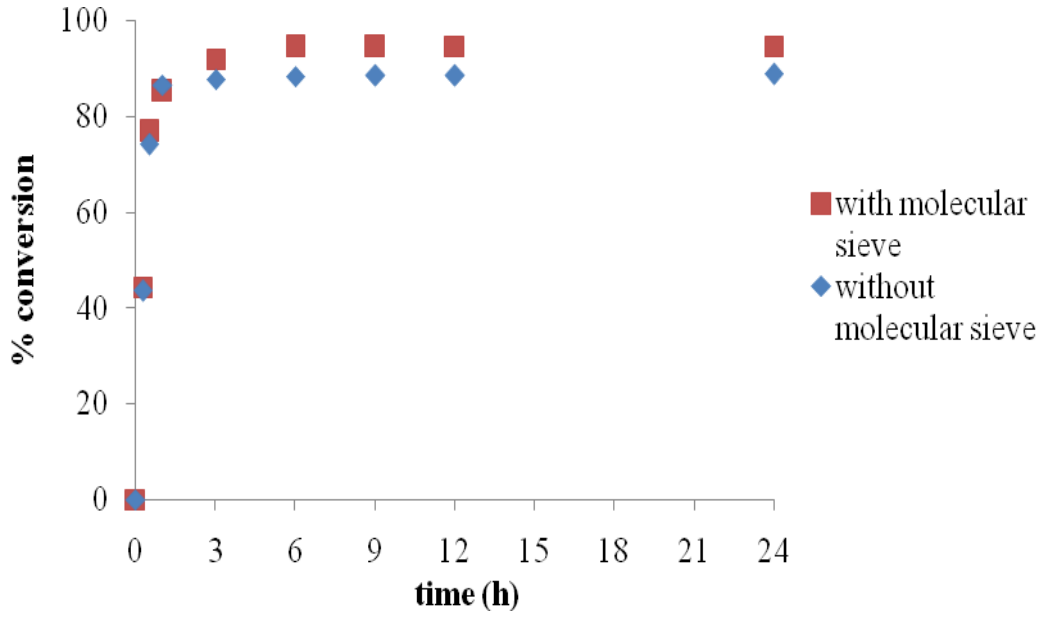

Figure 4.18 The removal of water produced of Novozym 435 catalyzed alcoholysis in oleic acid. Conditions: 45^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435, 250 rpm and molecular sieve 17 g.

The esterification reaction of oleic acid and propanol produces water as a by-product. Therefore, if water generated during the reaction can be removed, the reaction will shift to the right, towards products. Moreover, high water content can

inhibit the enzyme activity. In addition, high water content would dilute substrate concentration (Gayot et al, 2003), so that the limitation diffuseness of the substrate was occurred (Dormoa et al 2004). Al-Zuhair et al., (2006) studied effect of water content; they reported that the conversion after 1 h was decreased when the water content increased. Dormoa et al., (2004) investigated the removing of excess water production by pervaporation. They found that the conversion of oleic acid without water removal was 92.0% and the conversion was higher (99.8%) when excess water was removed. Excess water could be removed from product by evaporation but this method uses large energy. The alternative method was by using water adsorbent material such as micro gel and molecular sieve. In this experimental study, molecular sieve was used as an adsorbent to remove excess water from the esterification process under the reaction condition as follows: 45^{o}C, [PrOH]:[Oleic acid] = 2:1, 5%(w/w) Novozym 435, 250 rpm and reaction period of 24 h.

The obtained FFA conversions were compared with those of the non-water removal system. The results are shown in Figure 4.18. During the initial period (0-1 h), the addition of the molecular sieve only slightly affected the conversion due to low amount water content. However, the result at the final period indicated that excess water significantly affected the FFA conversion. Hence, when water was removed, the final conversion increased from 88.9% to 94.7%. This illustrates the attribution of water removal by adsorption with molecular sieve. The removal of water during the esterification could enhance the equilibrium conversion of ester synthesis by about 6%.

CHAPTER V
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The main purposes of this research were to develop biolubricant production of oleic acid and propanol using immobilized enzyme, where Novozym 435 was used as a biocatalyst.

The experiments were carried out in a shaking incubator to investigate effect of reaction time, alcohol structure, enzyme loading, rotation speed, molar ratio of propanol to oleic acid, and reaction temperature. In this process, the optimal condition were 45^{o}C, 2:1 molar ratio of propanol to oleic acid, Novozym 435 loading at 5% based on oleic acid weight, 250 rpm, in which the maximal FFA conversion at 88.9% was achieved. From the experimental results, it could be concluded that:

i. The conversion of FFA rapidly increased during the initial 1 h of the reaction and then slowly increased until the conversion approached the equilibrium point within about 3 h.
ii. The FFA conversion by using n-propanol was higher than that using isopropanol.
iii. FFA conversion increased appreciably when increasing enzyme loading up to about 5%.
iv. The rotation speed on or after 250 rpm made well mixing of two substrates. However, the rotation speed greater than 250 rpm showed negative effect on enzymatic activity.
v. Temperature affected to rates of reactions, which can be explained by Arrhenius equation. The reaction rate increased with temperature up to 45^{o}C. The significant effect of thermal inactivation of enzyme resulted in low FFA conversion was observed at the reaction temperature more than 45^{o}C.
vi. In this process, the activation energy of the esterification was 34.05 kJ/mol.
vii. The removal of water generated during the reaction by water absorption using molecular sieve could enhance the equilibrium conversion by 6%.
viii. It demonstrated that Novozym 435 could be used at least 5 cycles without considerable change in the conversion for propyloleate production.

5.2 Recommendations for further study

i. Development of technique to avoid or reduce deactivation of immobilized enzyme in the presence of high concentration of propanol, high temperature and high rotation of speed in order to be used for a long period practice.

ii. Development of reaction processes that ease product separation. We recommend some techniques such as using a packed bed reactor in continuous operation.

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APPENDICES

APPENDIX A
EXPERIMENTAL DATA AND FOR ANALYSIS

Experimental data of enzymatic esterification reaction of oleic acid and propanol using immobilized enzyme (Novozym 435) catalyst in batch system.

Table A-1 Effect of alcohol structure (Propanol vs. Isopropanol) on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45^{o}C, [Alcohol]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm.

Reaction time (h)	% conversion	
	Propanol	Isopropanol
0	0.00	0.00
0.25	43.6	29.7
0.5	74.2	40.9
1	86.6	58.2
3	87.8	73.5
6	88.4	75.7
9	88.4	75.7
12	88.6	76.0
24	88.9	76.4

Table A-2 Effect of enzyme loading (% based on oleic acid weight) on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid and 250 rpm.

Reaction time (h)	% conversion		
	2.5% (w/w) Novozym 435	5% (w/w) Novozym 435	10% (w/w) Novozym 435
0	0.00	0.00	0.00
0.25	31.6	42.5	49.6
0.5	56.5	73.6	81.2
1	70.5	86.4	86.4
3	80.2	88.1	88.2
6	81.1	88.1	88.4
9	81.6	88.2	89.3
12	81.6	88.4	89.4
24	82.1	88.7	90.6

Table A-3 Effect of rotation speed on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid and 5% (w/w) Novozym 435

Reaction time (h)	% conversion			
	200 rpm	250 rpm	300 rpm	350 rpm
0	0.00	0.00	0.00	0.00
0.25	35.0	44.0	35.7	22.6
0.5	55.3	76.4	65.1	57.5
1	78.5	86.0	81.8	79.5
3	81.5	87.5	85.4	84.4
6	82.5	88.4	87.1	85.4
9	82.5	88.4	87.4	85.5
12	82.9	88.5	87.3	85.6
24	83.8	88.9	87.5	85.7

Table A-4 Effect of molar ratio [oleic acid]:[propanol] on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45^{o}C, 40 g oleic acid, 5% (w/w) Novozym and 250rpm.

Reaction time (h)	% conversion			
	1:1 Propanol to oleic acid molar ratio	2:1 Propanol to oleic acid molar ratio	3:1 Propanol to oleic acid molar ratio	4:1 Propanol to oleic acid molar ratio
0	0.00	0.00	0.00	0.00
0.25	41.9	42.5	38.6	33.0
0.5	58.5	73.6	61.4	61.4
1	71.7	86.4	75.9	75.2
3	73.1	88.1	84.7	82.0
6	70.7	88.1	85.1	82.9
9	70.3	88.2	85.1	82.9
12	69.8	88.4	85.3	83.1
24	67.2	88.7	85.4	83.8

Table A-5 Effect of temperatures on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym and 250 rpm

Reaction time (h)	% conversion				
	35°C	40°C	45°C	50°C	60°C
0	0.00	0.00	0.00	0.00	0.00
0.25	37.	38.3	42.5	48.3	44.1
0.5	63.5	69.6	72.6	79.3	77.2
1	74.3	82.8	86.4	83.1	81.5
3	83.7	86.7	88.1	84.8	83.0
6	84.2	87.4	88.1	85.1	84.1
9	84.3	87.8	88.2	85.3	84.2
12	84.8	87.6	88.4	85.7	84.3
24	85.0	87.7	88.7	85.6	84.5

Table A-6 Repeated use of the immobilized lipase Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 40^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm.

Reaction time (h)	% conversion				
	First run	Second run	Third run	Fourth run	Fifth run
0	0.00	0.00	0.00	0.00	0.00
0.25	38.3	28.0	22.5	21.3	13.1
0.5	72.0	58.1	38.1	32.8	19.2
1	82.8	69.4	65.1	56.1	50.0
3	86.2	79.8	78.0	75.5	75.9
6	87.7	80.2	79.2	78.1	76.6
9	87.7	80.6	79.5	79.5	78.3
12	87.3	80.8	79.6	79.7	78.8
24	87.4	81.2	80.4	80.2	79.3

Table A-7 Repeated use of the immobilized lipase Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435, 250 rpm and reaction period of 24 h

Reaction time (h)	% conversion				
	First run	Second run	Third run	Fourth run	Fifth run
0	0.00	0.00	0.00	0.00	0.00
0.25	40.7	30.4	28.2	22.3	14.5
0.5	73.5	43.5	40.7	34.9	21.6
1	85.7	75.5	65.0	55.6	50.8
3	88.2	82.2	81.1	80.3	77.3
6	88.0	83.5	82.8	82.6	82.0
9	88.0	83.7	83.2	83.0	82.3
12	88.3	84.3	84.0	83.9	82.7
24	88.5	85.2	84.7	84.3	83.4

Table A-8 Determination of the kinetic constants and FFA conversion on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: various temperature, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm.

Temperature (^{o}C)	% conversion at 24 h	k ($h^{-1}(kmol/m^3)^{-1}$)	R^2
35	84.99	0.452	0.945
40	87.63	0.596	0.877
45	88.69	0.686	0.986
55	85.60	0.667	0.842
60	84.53	1.071	0.858

Table A-9 Determination of the kinetic constants and FFA conversion on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 40 ^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm.

Times	% conversion at 24 h	k (h-1(kmol/m3)-1)	R2
1	87.4	0.607	0.940
2	81.2	0.384	0.947
3	80.4	0.294	0.981
4	80.2	0.226	0.994
5	79.3	0.171	0.926

Table A-10 Determination of the kinetic constants on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 45 ^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm.

Times	% conversion at 24 h	k ($h^{-1}(kmol/m^3)^{-1}$)	R^2
1	88.5	0.673	0.991
2	85.2	0.406	0.966
3	84.7	0.302	0.994
4	84.3	0.227	0.998
5	83.4	0.120	0.956

Table A-11 Determination of the activation energy on the Novozym 435 catalyzed alcoholysis of oleic acid. Conditions: 35-45 ^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435and 250 rpm.

Temp (^{o}C)	1/T (1/K)	k ($h^{-1}(kmol/m^3)^{-1}$)	lnk
35	0.00324	0.452	-1.19
40	0.00319	0.596	-0.885
45	0.00314	0.686	-0.760
55	0.00305	0.667	-0.797
60	0.00300	1.071	-0.887

Table A-12 The removal of water produced of Novozym 435 catalyzed alcoholysis in oleic acid. Conditions: 45^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435, 250 rpm and molecular sieve 17 g.

Reaction time (h)	% conversion	
	Without molecular sieve	With molecular sieve
0	0.00	0.00
0.25	43.6	44.3
0.5	74.2	76.9
1	86.6	87.4
3	87.8	91.9
6	88.4	94.5
9	88.4	94.5
12	88.6	94.7
24	88.9	94.7

Table A-13 Comparison analysis of the FFA conversion by titration and H'-NMR

Conditions	% conversion	
	Titration	H'-NMR
5 % (w/w) Novozym 435, 45^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 250 rpm and reaction time at 24 h	88.7 ± 0.43%	86.5 %
10 % (w/w) Novozym 435, 45^{o}C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 250 rpm and reaction time at 24 h	90.6 ± 0.55	88.5%
[PrOH] : [Oleic acid] = 3:1, 5 % (w/w) Novozym 435, 45^{o}C, 40 g oleic acid, 250 rpm and reaction time at 24 h	85.4 ± 0.12%	84.7%
300 rpm, [PrOH]:[Oleic acid] = 2:1, 5 % (w/w) Novozym 435, 45^{o}C, 40 g oleic acid and reaction time at 24 h	87.5 ±0.79%	85.5%
35 ^{o}C, [PrOH]:[Oleic acid] = 2:1, 5 % (w/w) Novozym 435, 40 g oleic acid, 250 rpm and reaction time at 24 h	85.0 ± 2.48%	84.0%

The final FFA conversions obtained by titration method from Table 4.6 were compared to FFA conversion calculated using data from NMR; the results are relatively comparable. The result analyzed by NMR was found to be in good agreement with the obtained results analyzed by the titration method.

APPENDIX B

CALCULATE PROPYL ESTEER AND PROPYL ESTER PERCENT CONVERSION

Table B-1 Properties of reactants

Reactants	Molar mass(g/g mol)	Density (g/cm^3)
Oleic acid	282.5	0.895
Propanol	60.1	0.803
Isopropanol	60.1	0.786

Molar ratio of propanol to oleic acid

Molecular ratio of propanol to oleic acid = $\frac{N_{PrOH}}{N_{oleic}}$

$$\left(\frac{\text{Mass (g)}}{\text{MW (g/g mol)}}\right)_{\text{oleic acid}} = \left(\frac{\text{Mass (g)}}{\text{MW (g/g mol)}}\right)_{\text{propanol}}$$

$$\text{Mass of propanol (g)} = \left(\frac{\text{Mass (g)}}{\text{MW (g/g mol)}}\right)_{\text{oleic acid}} \times (MW_{\text{propanol}}\ (\text{g/g mol}))$$

$$\text{Volume of propanol} = \frac{\text{Mass of propanol (g)}}{\text{density of propanol } \left(\frac{\text{g}}{\text{cm}^3}\right)}$$

Calculate catalyst

$$\text{Mass of catalyst} = \text{Mass of oleic acid} \times \frac{\%\ \text{emzyme based on oleic acid weight}}{100}$$

Calculate of the percentage FFA conversion by titration

Weight of FFA = KOH (mL) x [KOH] x 0.2824

$$\text{weight of FFA per one gram sample} = \frac{\text{KOH (ml)x[KOH]x28.24614}}{\text{weight of sample}}$$

$$\%\ \text{conversion} = \frac{(\text{initial weight} - \text{weight at time})}{\text{initial weight}} \text{ x } 100$$

Calculate of the percentage FFA conversion by H'-NMR

$$\%\ \text{conversion} = \frac{\text{integration of methoxy group per one mole equivalent}}{\text{integration of methylene group per one mole equivalent}} \text{ x } 100$$

Example

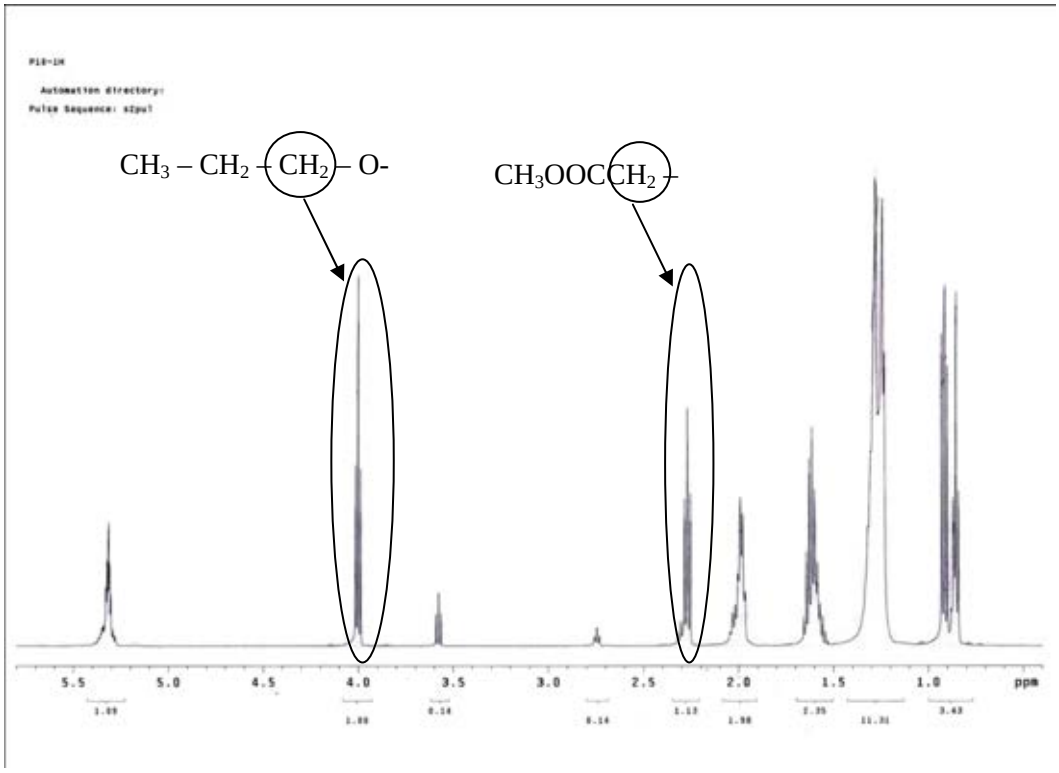

Figure B-1 H'-NMR spectrum of the reaction mixture of oleic acid and propanol Condition: 10 % (w/w) Novozym 435, 45°C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 250 rpm and reaction time at 24 h

$$\%\ \text{conversion} = \frac{1/2}{1.13/2} \text{ x } 100 = 88.5\%$$

Calculate kinetic constant (k)

Example

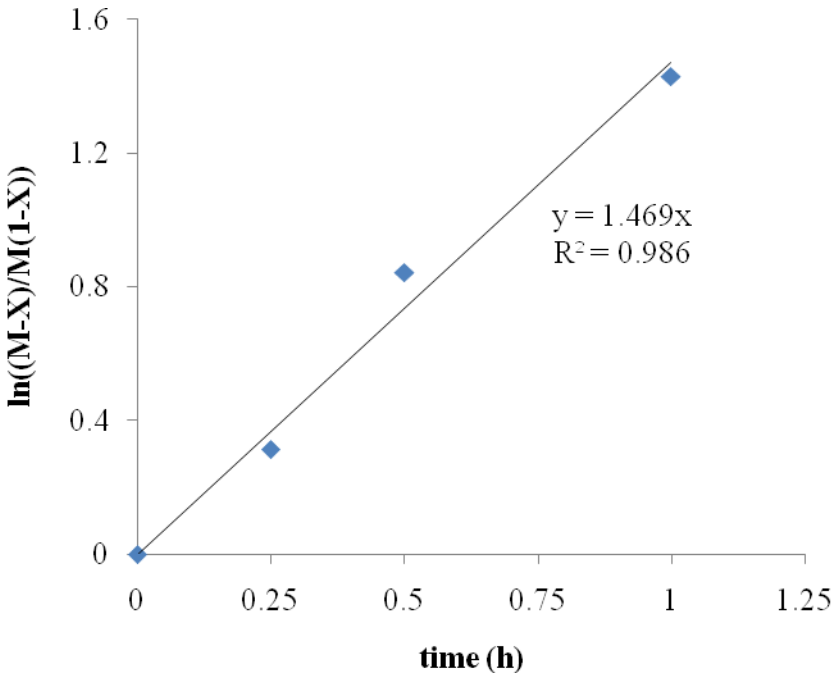

Figure B-2 The kinetic constants of Novozym 435 catalyzed alcoholysis in oleic acid. Conditions: 45°C, [PrOH]:[Oleic acid] = 2:1, 40 g oleic acid, 5% (w/w) Novozym 435 and 250 rpm

The conversions from various time were used to calculate the kinetic constants of the initial rate (0-1 h) from plot of $\ln((M-X_{oleic\ a})/M(1-X_{oleic\ a}))$ and time as showed in Figure 4.15, the slope of this graph is 1.481 and the kinetic constants (k) is found to be 1.469/(2.14(2-1)) = 0.686 $(h^{-1}(kmol/m^3)^{-1})$

From $\ln\frac{(M-X_{oleic\ a})}{M(1-X_{oleic\ a})} = [Oleic\ a]_0\,(M-1)\,k\,t$

Slope is $[Oleic\ a]_0\,(M-1)\,k = 1.469$

$k = \frac{slope}{[Oleic\ a]_0\,(M-1))}$

k = 1.469/(2.14(2-1)) = 0.686 ($h^{-1}(kmol/m^3)^{-1}$)

Where M is Molar ratio of propanol to oleic acid

$[Oleic\ a]_0$ is initial concentration of oleic acid

Calculate activation energy (E_a)

Example

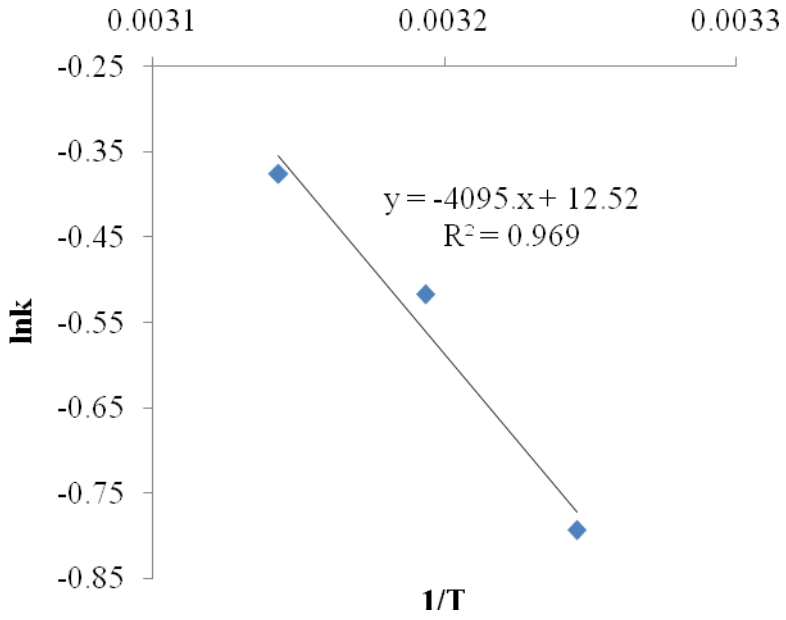

Figure B-3 Second order reaction rate in Arrhenius plot during the esterification reaction of oleic acid and propanol at various temperatures.

From $lnk = lnA - E_a/RT$

$lnA = 12.52$

$A = 2.74\times10^5\ h^{-1}(kmol/m^3)^{-1}$

$-E_a/R = -4095.0\ h^{-1}(kmol/m^3)^{-1}$

$E_a = 4095.0 \times 8.314 = 34{,}045.8$ J/mol = 34.05 kJ/mol

Where Ea is the activation energy

R is the molar gas constant (8.314 J/mol K)

A is the frequency factor

VITA

Ms. Karnjana Sena was born on June 24, 1987 at Nakhon Sri Thamarat Province. She finished her high school education in 2005 from Triam Udom Suksa school of the south. She received her Bachelor Degree of Engineering (Chemical Engineering) from Faculty of Engineering, Thammasat University in 2009 and continued her study in the Master degree program of Chemical Engineering, Faculty of Engineering, Chulalongkorn University.

Her academic publication is as follows:

Karnjana Sena, Muenduen Phisalaphong **"Propyloleate Production using Immobilized Lipase"** Proceedings of the conference, The 4th AUN/SEED-Net Regional Conference on Biotechnology: EMERGING BIOTECHNOLOGY FOR GREEN ENGINEERING, 26-27 January 2012 (Bangkok, Thailand).