การผลิตบิวทิลโอลิเอตโดยใช้ไลเปสตรึงรูป

นางสาวจิรนันท์ จันทร์ประเสริฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี
คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

BUTYLOLEATE PRODUCTION USING IMMOBILIZED LIPASE

Miss Jiranan Chanprasert

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering Program in Chemical Engineering
Department of Chemical Engineering
Faculty of Engineering
Chulalongkorn University
Academic Year 2011

Thesis Title	BUTYLOLEATE PRODUCTION USING IMMOBILIZED LIPASE
By	Miss Jiranan Chanprasert
Field of Study	Chemical Enginerring
Thesis Advisor	Associate Professor Muenduen Phisalaphong, Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……...Dean of the faculty of Engineering
(Associate Professor Boonsom Lerdhirunwrong, Dr.Ing.)

THESIS COMMITTEE

……...Chairman

(Associate Professor Bunjerd Jongsomjit, Ph.D.)

……...Thesis Advisor

(Associate Professor Muenduen Phisalaphong, Ph.D.)

……...Examiner

(Jirdsak Tscheikuna, Ph.D.)

……...External Examiner

(Associate Professor Sumimol Asavapisit, Ph.D.)

จิรนันท์ จันทร์ประเสริฐ : การผลิตบิวทิลโอลิเอตโดยใช้ไลเปสตรึงรูป (BUTYLOLEATE PRODUCTION USING IMMOBILIZED LIPASE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. เหมือนเดือน พิศาลพงศ์, 65 หน้า.

การผลิตเชื้อเพลิงชีวภาพได้รับความสนใจมากในขณะนี้ เนื่องจาก เป็นเชื้อเพลิงที่สามารถย่อยสลายได้และไม่เป็นพิษต่อสิ่งแวดล้อม ใน การศึกษานี้บิวทิลโอลิเอตถูกเตรียมโดยระบบที่ไม่มีการเติมตัวทำละลายโดย ปฏิกิริยาเอสเทอริฟิเคชันของกรดโอเลอิกและบิวทานอลโดยใช้โนโวไซม์ 435 เป็นตัวเร่งปฏิกิริยา ทางชีวภาพ โดยระบบแบบกะ โดย มีค่าการเปลี่ยนแปลงของกรดไขมันอิสระ ถึง 91.0% ที่อุณหภูมิในการเกิดปฏิกิริยา 45°C สัดส่วนโดยโมลาร์ของกรดโอเลอิกต่อบิวทานอล ที่ 1:2 ความเข้มข้นของตัวเร่งปฏิกิริยา ที่ 5% โดยน้ำหนักของกรด ไขมันอิสระ ความเร็วรอบใน การเขย่าที่ 250 รอบต่อนาที และ ระยะเวลาในการ ทำปฏิกิริยา ที่ 24 ชั่วโมง การแยกน้ำที่เกิดขึ้นในระหว่างการเปิดปฏิกิริยาเอสเตอริฟิเคชั่นด้วยเอนไซม์โดยการเติมโมเลกูลาร์ซีฟ สามารถเพิ่มค่าเปลี่ยนแปลงของกรดไขมันอิสระเป็น 96.1% โนโวไซม์ 435 ที่ถูกนำมาใช้ซ้ำ 5 รอบยังมีความว่องไวโดยที่สูญเสียความว่องไวในการเร่งปฏิกิริยาเพียงเล็กน้อยเท่านั้น จากการศึกษาทางจลพลศาสตร์ของปฏิกิริยาที่อุณหภูมิ 35- 60°C พบว่าเป็นปฏิกิริยาอันดับสอง ซึ่งมีค่าพลังงานกระตุ้นเท่ากับ 13.64 กิโลจูลต่อโมล ผลิตภัณฑ์นี้มีความเหมาะสมที่จะนำมาใช้เป็นน้ำมันหล่อลื่นชีวภาพและสารเติมแต่งเชื้อเพลิงดีเซลได้

ภาควิชา……วิศวกรรมเคมี…..ลายมือชื่อนิสิต.....

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

ปีการศึกษา .....2554.....

5370407021 : MAJOR CHEMICAL ENGINEERING
KEYWORDS : BUTYLOLEATE / OLEIC ACID / NOVOZYM 435/ BIOLUBRICANTS

JIRANAN CHANPRASERT: BUTYLOLEATE PRODUCTION USING IMMOBILIZED LIPASE. ADVISOR: ASSOC. PROF. MUENDUEN PHISALAPHONG, Ph.D., 65 pp.

Biofuels has received significant attention recently as a biodegradable and nonpolluting fuel. In this study, butyloleate was prepared in solvent-free system by esterification of oleic acid with butanol using immobilized lipase Novozym 435 as biocatalyst in a batch system. The conversion of free fatty acid (FFA) could reach 91.0% at reaction temperature of 45°C, oleic acid/butanol molar ratio of 1:2, Novozym 435 loading based on FFA weight of 5%, a shaking rate of 250 rpm, and a reaction period of 24 h. The removal of water that was produced during the enzymatic esterification by the addition of molecular sieves could enhance the FFA conversion to 96.1%. Novozym 435 having been used for five cycles still remained active with only slightly loss of catalytic activity. From kinetic study of the reaction at temperatures of 35- 60°C, the reaction appeared to be second order, at which the activation energy (Ea) was 13.64 kJ/mol. This product could reasonable be used as biobased industrial material in biolubricants and diesel fuel additives.

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

ACKNOWLEDGEMENTS

This research presented in this thesis is completed with the aid and encouragements from many people. The auther would like to take this prospect to thank the following people for their contributions to this research.

Firstly, The auther would like to express her deepest gratitude to my advisor, Associate Professor Dr. Muenduen Phisalaphong for her inspiration, continuous guidance, useful discussions, and supervision all the way through her thesis work and study.

Special appreciation is addressed to National Research Council of Thailand (NRCT) under financially supported to this work.

Gratefully thanks to all of her thesis committee, Associate Professor Dr. Bunjerd Jongsomjit, as a chairman, and Dr. jirdsak Tscheikuna, Associate Professor Suwimol Asavapisit, as the members of the thesis committee.

Many thanks are also addressed to Mrs. Wanna Sririnnuch and Mrs. Wanwimon Mekboonsonglarp (Scientific and Technological Research Equipment Centre, Chulalongkorn University) for their kind assistance in commencing Nuclear Magnatic Resonance (NMR).

The auther wish to thanks to all the member of the Biochemical Engineering Research Laboratory and all her friends and staffs in the Department of Chemical Engineering, Chulalongkorn University for their assistance, support, and encouragement.

Last but not least, the auther would like to express her highest gratitude to her parents who always pay attention, support, inspiration and love for all the way throughout my life and study.

CONTENTS

Page

Page

LIST OF TABLES

Table **Page**

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER I

INTRODUCTION

1.1 Motivation

Currently, energy problem is one of the most important issues and continually affects all human beings directly. In Thailand, fossil fuel resources such as oil, gas, and coal are limited. Therefore, the current research in chemical engineering is focused on developing alternative energy sources for the near future. Biodiesels and biolubricants from vegetable oils or waste oils are as parts of the research for the evolution of renewable energy sources in Thailand.

Biolubricants are environmentally compatible products due to their low toxicity and good biodegradability. Biolubricants are derived from vegetable oils such as palm oil, soy bean oil, sunflower oil, peanut oil, olive oil or animal fat with alcohols. The most commonly processes are performed by transesterification or esterification reaction. Many kinds of alcohols could be used, such as propanol, butanol, hexanol, octanol and 2-ethyl-1-hexanol. The biolubricant synthesis is classified as chemical or enzymatic production. The chemical processes are usually used alkaline (NaOH or KOH) or acid (H_2SO_4) as catalyst. For the enzymatic processes, lipase can be used as biocatalyst for transesterification or esterification. However, the high cost of enzymes often makes the enzymatic process economically unattractive (Noureddini et al., 2005). The use of immobilized enzyme has some industrial and economical advantages such as ease recovery and re-use, greater stability of the enzyme, and possibility of continuous operation (Oliveira et al., 2000).

Application of butanol has been attempted to keep an option ready for ethanol substitution. Advantages of butanol over ethanol such as lower vapor pressure, higher miscibility with gasoline and diesel (lower affinity for water), and higher stoichiometric air/fuel ratio, thereby improving combustion and reducing CO emissions, are the driving forces for replacing ethanol with butanol (Mehta et al.,2010).

This research focuses on the development of butyloleate production from oleic acid and butanol by using an immobilized enzyme, Novozym 435.

1.2 Research objective

The purpose of this research work is to study butyloleate production from oleic acid and butanol catalysed by lipase (Novozym 435).

1.3 Research scopes

In this research work, Novozym 435, which is Lipase B from *Candida antarctica*, EC 3.1.1.3 (S.M. Chemical Supplier Co., Lyd.) a nonspecific lipase immobilized on macroporous acrylic resin, was used as a biocatalyst. The optimal conditions for butyloleate production from oleic acid and butanol were determined. The range of the operating conditions were: reaction time at 0-48 h, stirring rate at 200, 250, 300 and 350 rpm, molar ratio of free fatty acid and butanol at 1:1 to 1:4 and the operating temperature at 35-60°C. Further more, the reusability of Novozym 435 was investigated and the effect of water on butyloleate production was examined.

1.4 Expected benefit

The expected benefit of this study is to obtain useful information for better understanding of production of butyloleate from oleic acid and butanol by lipase-catalysis.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Bio-lubricant

Pure, natural lubricants manufactured by environmental-safe processes have gained more and more attention recently, since they do not contain toxic compounds and are biological degradable (Dormo *et al*., 2004). Lubricant oil is used to reduce friction between two surfaces, for instance, in car engine. To apply for specific uses, lubricant oil properties were modified by certain techniques or methods, such as esterification, hydrolysis, ring-opening and epoxidation.

2.2 Butanol

Butanol (C_4H_9OH) is the primary alcohol with 4 carbons. It is now recognized as a solvent, an intermediate in chemical synthesis and important transport fuels. Butanol could be produced by solvent oganic clostridia via the Acetone-Butanol-Ethanol (ABE) fermentation (Qureshi *et al*., 1999). The history of the ABE fermentation stretches back to the early 1900s and it was once only second to ethanol as the largest industrial fermentation. Its demise was ultimately triggered by the availability of cheaper alternatives from the petrochemical industry (Qureshi *et al*., 2010). The search for a sustainable biofuel has now established biobutanol as an important alternative transportation fuel.

2.3 Fatty acid

Fatty acids are molecules consisting of long chains of carboxylic acid, which is an organic acid consisting of carbon, oxygen, and hydrogen atoms. The "backbone" of the fatty acid chain is a string of carbon atoms; the oxygen and hydrogen atoms "protrude" out from the carbon backbone. Most fatty acids are chains of between four and 28 carbon atoms. Fatty acids are either saturated or unsaturated. Saturated fatty acids contain exclusively single bonds between carbon atoms; whereas unsaturated fatty acids contain some double bonds between the carbon atoms.

For commercial use, it has been necessary to look for cheap raw materials to produce biofuel. The use of waste cooking oil to obtain biodiesel using a chemical catalyst was reported (Thanh *et al*., 2012). Waste cooking oils generally contain impurities, water and free fatty acids. However, it was shown that biodiesel from waste cooking oil could be used in different types of diesel engines with no loss of efficiency and significant reduction in the emissions of particulate matter, carbon monoxide and total hydrocarbons with respect to conventional diesel obtained from fossil fuel. An essential feature of biodiesel is that its fatty acid composition corresponds to that of its parent oil or fat. Thus, biodiesel fuels derived from different sources can have significantly varying fatty acid profiles and properties (Knothe, 2005).

There are several industrial application possibilities for fatty acid esters, as natural compounds. Oleic acid (cis-9-octadecenoic acid) is one of the most important fatty acids in nature from plant oils. Its esters produced by enzyme catalysis can be applied as lubricant (Dormoa *et al*., 2004).

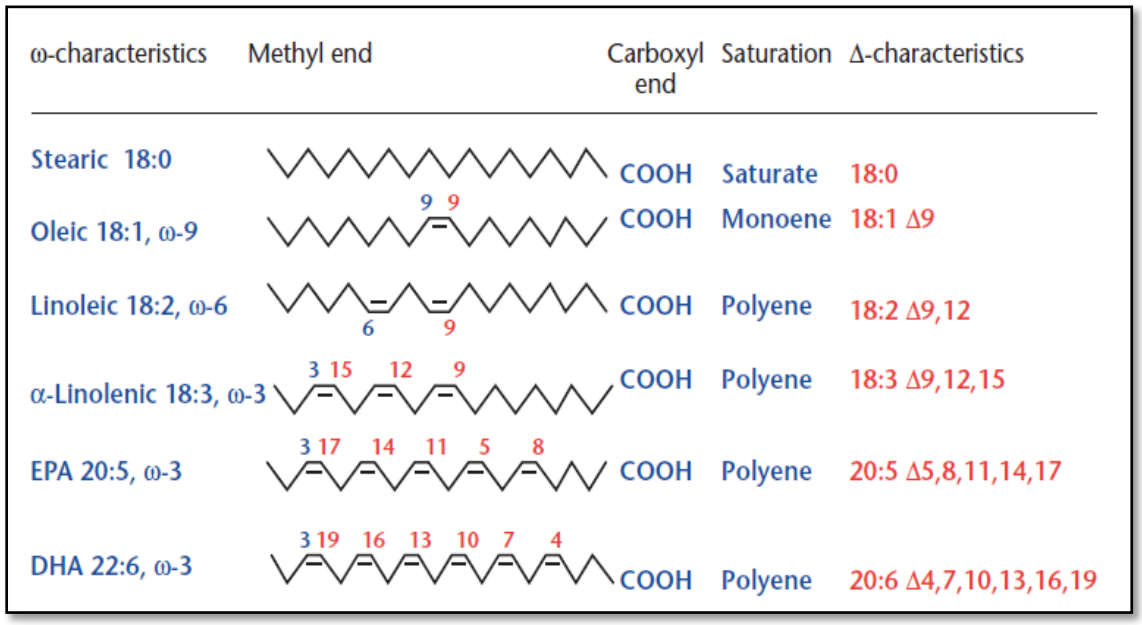

Figure2.1 The structures of fatty acid with a methyl end and a carboxyl (acidic) end (Rustan and Drevon, 2005).

Table 2.1 Common biological saturated fatty acid

Symbol	common name	structure	Source
12:0	Lauric acid	$CH_3(CH_2)_{10}COOH$	Palm kernel oil
14:0	Myristic acid	$CH_3(CH_2)_{12}COOH$	Oil of nutmeg
16:0	Palmitic acid	$CH_3(CH_2)_{14}COOH$	Palm oil
18:0	Stearic acid	$CH_3(CH_2)_{16}COOH$	Beef tallow
20:0	Arachidic aicd	$CH_3(CH_2)_{18}COOH$	acid Liver

Table 2.2 Common biological unsaturated fatty acids

Symbol	common name	structure	Source
16:1	Palmitoleic acid	$CH_3(CH_2)_5CH=CH-(CH_2)_7COOH$	Whale oil
18:1	Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	Olive oil
18:2	Linoleic acid	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$	Soybean oil, safflower oil
20:4	arachidonic acid	$CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2COOH$	Fish oils, linseed oil

2.4 Enzymatic catalyst

Enzymatic fuels production from raw vegetable oils has been extensively studied for many authors in the past years.

Different lipases, such as *Candida antarctica, Pseudomonas cepacia* and *Thermomyces lanuginosus* have been employed as biocatalysts in the production of biodiesel from vegetable oil. These enzymes can be used free or immobilized. Several researchers have reported that the commercially available Novozym 435 (*C. antarctica* lipase B immobilized on acrylic resin) was the most effective catalyst among tested lipases for biodiesel production (Maceiras *et al*., 2009).

Table 2.3 Enzymatic and non-enzymatic methods for biodiesel fuel production (Fukuda *et al*., 2001).

Conditions	**Alkali-catalysis process**	**Lipase-catalysis process**
Reaction temperature	60-70°C	30-40°C
Free fatty acids in raw material	Saponified products	Methyl esters
Water in raw materials	Interference with the reaction	No influence
Yield of methyl esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst	Cheap	Relatively expensive

2.5 Immobilized enzyme

Currently Immobilized enzymes are the object of interest. The number of applications of immobilized enzymes is increasing (Katchalski-Katzir et al., 1993). However, experimental investigations have produced unexpected results such as a significant reduction or even an increase in activity compared with soluble enzymes. Immobilization of macromolecules can be generally defined as a procedure leading to their restricted mobility. A classification of immobilization methods according to different chemical and physical principles is shown in Figure 2.2

2.5.1 Advantages of immobilized enzymes

1) The enzyme can be packed into columns and used over a long period.

2) The enzyme is easily removed.

3) The products can be easily separated and the feedback inhibition can be reduced.

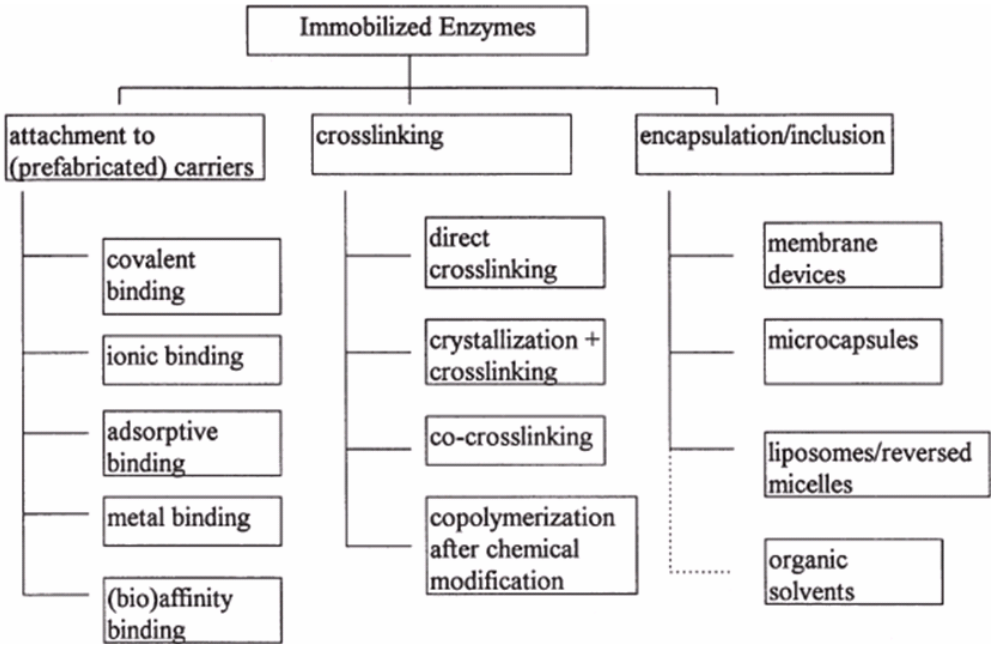

Figure 2.2 Classification of immobilization methods (Tischer et al.,1999)**.**

2.6 Esterification Reaction

The classic synthesis the esterification, which carboxylic acids (RCOOH) and alcohols (R'OH) react together to form compounds known as esters and water.

$$RCOOH + R'OH \leftrightarrow RCOOR' + H_2O \qquad (2.1)$$

The biolubricant synthesis is classified as chemical or enzymatic production. The chemical processes are usually used alkaline (NaOH or KOH) or acid (H_2SO_4) as catalyst, whereas in bioprocesses, lipases are used as enzyme (biocatalyst) to catalyze transesterification or esterification. Although transesterification using an alkaline as catalyst may give high productivity of biolubricant, the reaction has several

drawbacks. It is energy intensive and difficult to recovery glycerol. On the other hand, no removal of glycerol is required under biolubricant synthesis by esterification of free fatty acids. Moreover, free fatty acids, which are byproducts of refining of crude plant oil products, are cheaper raw materials for the synthesis of biolubricant than refined or crude oils.

2.7 Kinetic of reaction

2.7.1 The reaction rate constant

In the chemical reaction, one of the reactants that is disappearing as a result of the reaction is the basic of calculation a species A. Usually chosen the limiting reactant is basic for calculation. The rate of a reaction can be expressed in terms of the concentrations of the various species.

For a general reaction rate form: A + B → C + D, where substance A and B are reacting to produce C + D.

$$\text{Rate} = k(T)[A]^m[B]^n$$

In this equation, k is the rate constant for the reaction; [A], [B] is the concentration of A, B; m is the order of the reaction with respect to A and n is the order of the reaction with respect to B

2.7.2 The Arrhenius equation

Quantitatively the relationship between the temperature term and the rate of reaction term is determined by the Arrhenius Equation.

$$k(T) = Ae^{-Ea/RT}$$

Where, A = pre-exponential factor or frequency factor

Ea = activation energy, J/mol or cal/mol

R = gas constant = 8.314 J/mol.K = 1.987 cal/mol.K

T = absolute temperature, K

The activation energy is determined experimentally by carrying out the reaction at several different temperatures. After taking the natural logarithm of the Arrhenius equation, the modified equation is usually of the form:

$$\ln k = \ln A - \frac{Ea}{RT}$$

The activation energy can be determined from a straight line whose slope from a plot of ln k versus 1/T.

2.8 Optimization of the enzymatic esterification reaction

2.8.1 The effect of reaction time

The effect of reaction time, time course of methanolysis reaction of waste frying oil (WFO) in series of experiments involving different methanol to oil molar ratio have been carried out. It indicates that the optimum reaction time is 4 h since after that time there is no change in the methyl esters yield (Maceiras *et al*., 2009). Under the study of effect of reaction time of esterification of palm fatty acid distillation (PFAD) in supercritical methanol, the yield increased steadily with increased reaction time up to 30 min, providing the yield of 95%. After that, the conversions remained nearly constant (Yujareon *et al*., 2009).

2.8.2 The effect of enzyme loading

Under the study of effect of lipase loading on the esterification, Initial rate increased with increasing the lipase concentration till 12% (w/w of substrates), and with further increase of biocatalyst concentration, a decrease in initial rate was observed (Sabeder *et al*., 2006). Under the study of enzymatic production of biodiesel from waste frying oil with methanol, it was found that methyl esters content was increased by increasing lipase loading for lipase concentrations below 10%, and then was decreased for the higher lipase loading concentrations more than 10% (Maceiras *et al*., 2009).

2.8.3 The effect of molar ratios of oil/alcohol

It is well known that one of the most important parameters in enzymatic esterifications is acid/alcohol molar ratio. The optimal molar ratio of acid/alcohol for esterification has been determined. The highest acid conversion of ester by lipase enzyme was achieved at 1:2 molar ratio (Dormo *et al*., 2004). The effect of PFAD to methanol molar ratio varied from 1:1 to 1:12 under the condition: reaction time of 30 min and temperature of 250°C and 300°C was investigated. The maximum yield of FAMEs was 74% and 95% with molar ratio 1:6 at 250°C and 300°C, respectively (Yujareon *et al*.,2009).

2.8.4 The effect of temperature

To study the effect of temperature on lipase activity, reaction was performed at temperature from 30°C to 110°C. The conversion after 72 h increased with increasing temperature to 60°C (78%) (Sabeder *et al*., 2006). The studied in series of experiments in the range of 30 °C to 60°C, the concentration of product increased in higher temperatures (Dormo *et al*., 2004). The effect of reaction temperature of inlet PFAD to methanol was carried out between 250 to 300 °C. The yield of FAMEs slightly increased when the system temperature increased (from 64% to 73%) (Yujareon *et al*., 2011).

2.9 Literature review

There are many researches for the development of suitable technologies for biodiesel production. Varieties of oil, alcohols, catalysts and reaction conditions have been used for the research studies.

Kose *et al*.,(2002) studies immobilized *Candida antractica* lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. The refined cotton seed oil of Turkish origin with primary and secondary alcohols was investigated in the presence of an immobilized enzyme form *Candida antractica*, commercially called Novozym.435. The optimum conditions of Methanolysis were as follow: 30% enzyme based on oil weight; oil/alcohol molar ratio 1:4; temperature: 50°C and reaction time: 7 h. Maximum methyl esters (ME) yield at 91.5% was obtained. At the same conditions, cotton seed oil could be converted with short-chain primary and secondary alcohols to its corresponding ester with conversions between 72% and 94%. The results indicated that alcoholysis products of cotton seed oil could be used as valuable intermediates in oleochemistry

Koszorz *et al*.,(2004) studied enzymatic esterification of oleic-acid and i-amyl alcohol using a pervaporation membrane reactor with immobilized lipase enzyme, Novozym 435. Computer calculations performed with the use of this model showed

that there exists a range of pervaporation process parameters which prohibits the reaction from proceeding.

Dormo *et al*.,(2004) studied the manufacture of an environmental-safe biolubricant from fusel oil by enzymatic esterification in solvent-free system. Experiments were carried out, and the effects of water content, temperature, substrate concentration and the molar ratio of oleic acid and alcohols were investigated. To eliminate the negative effect of the water produced in the reaction, an integrated system constructed with a pervaporation unit for water removal was used. The 99.8% conversion was achieved under optimal conditions.

Li *et al*.,(2006) studied lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. Combined use of Lipozyme TL IM and Novozym 435 was proposed further to catalyze the methanolysis and the highest biodiesel yield of 95% could be achieved under the optimum conditions (*tert*-butanol /oil volume ratio 1:1; methanol/oil molar ratio 4:1; 3% Lipozyme TL IM and 1% Novozym 435 based on the oil weight; temperature 35^{o}C; 130 rpm, 12 h). There was no obvious loss in lipase activity even after being repeatedly used for 200 cycles with *tert*-butanol as the reaction medium.

Berrios *et al*.,(2007) studied the kinetics of the esterification of free fatty acids (in sunflower oil) with methanol. The results showed the first-order kinetic law for the forward reaction and a second-order one for the reverse reaction. The energy of activation for the forward reaction decreased with increasing catalyst concentration from 50,745 to 44,559 J/mol. FFA can be effectively removed by esterification which using a 5% sulphuric acid concentration relative to FFA, a methanol oleic acid mole ratio of 60:1, speed of rotation 250 RPM, 60°C.

Dizge *et al*.,(2008) reported that the optimum pH for immobilized *T. lanuginosus* enzyme (with 80% immobilization yield) was 6.0, which was the same pH as for the free enzyme. The optimal conditions for processing 20 g of refined canola oil with methanol were: 430mg lipase, 1:6oil/methanol molar ratio, 0.1g water and 40°C. Maximum methylesters yield was 90% of which enzymatic activity remained after 10 batches, when tert-butanol was adopted to remove by-product

glycerol during repeated use of the lipase. The immobilized lipase was stable and maintained catalytic activity during the repeated uses.

Hernandez-Martın and Otero,(2008) studies different enzyme requirements for the synthesis of biodiesel: Novozym®435 and Lipozyme®TL IM. Loss of lipase activity induced by the nucleophile was found greater with methanol than ethanol, and was greater for Lipozyme®TL IM than for Novozym®435. The optimum volume of ethanol depended on the loading of solid biocatalyst and was higher for preparations of Novozym®435 than for Lipozyme®TL IM. Quantitative conversions to biodiesel could be obtained in only 7 h at 25 °C with 50% (w/w) Novozym®435 in a single-step process. Under such conditions, Novozym®435 retains 85% of its initial activity after nine cycles of reaction under optimum conditions in a batch reactor.

Jeong and Park,(2008) studied lipase-catalyzed transesterification of rapeseed oils for biodiesel production with *tert*-butanol. The application if Novozym 435 was determined to catalyze the transesterification process and a conversion of 76.1% was achieved at the selected conditions (reaction temperature 40^{o}C, methanol/oil molar ratio 3:1, 5% (w/w) Novozym 435 based on the oil weight, water content 1% (w/w), and reaction time of 24 h). Under these reaction conditions, a conversion of approximately 76.1% was achieved. It has also been determined that rapeseed oil can be converted to fatty acid methyl ester using this system and the results of this study contribute to the basic data relevant to the development of continuous enzymatic process

Liu *et al*.,(2009) studies the effect of two crucial parameters, type of biocatalysts (Novozym 435, Lipozyme TLIM and Lipozyme RMIM), and different alcohols (methanol, ethanol, propanol, isopropanol, isobutanol, isoamyl alcohol and fusel oil-like alcohol mixture), on conversion rate. The results showed that each lipase presented a different kinetic pattern depending on the monohydric alcohols in solvent-free and tert-butanol systems. It was indicated that a possible use of fusel oil-like mixture as a raw material for biodiesel production was a promising procedure. In addition, a reaction kinetics model was developed for the methanolysis of waste baked duck oil using combined lipases of Novozym 435 and Lipozyme TLIM in

solvent-free system. The kinetic parameters were estimated by fitting experimental data and deduced to be a pseudo-third-order reaction and the activation energy was 31.65 kJ/mol.

Maceiras *et al*.,(2009) studied the enzymatic reaction of waste frying oil with methanol using Novozym 435 as catalyst. The reaction parameters such as the molar ratio of alcohol to oil, enzyme loading and reaction time were investigated. The optimum reaction conditions were at the molar ratio of 25:1 (methanol to oil), 10% (based on oil weight) enzyme loading and reaction time of 4 h at 50°C with biodiesel yield of 89.1%.

Sonare and Rathod,(2010) showed that the highest activity of Lipozyme RM IM for transesterification was at mild reaction temperature of 45 °C. In solvent free system, inactivation of enzyme by methanol and glycerol was observed. Tertiary butanol was found to be a good solvent which solubilized both triglycerides and glycerol. The activity of immobilized lipase was highly increased in comparison with that of the solvent free system because its activity sites became more effective. Immobilized enzyme could be repeatedly used without troublesome method of separation and the decrease in its activity was not largely observed.

Pinnarat and Savage,(2010) studies the esterification of excess ethanol with oleic acid at sub- and supercritical reaction conditions. The ethyl esterification of oleic acid without added catalyst can be done at mild subcritical reaction conditions. Moreover, the reaction does not require the excess of ethanol, and it can work in the presence of modest amounts of water in the feed stream. The kinetics of esterification were determined: the forward and reverse rate constants at five different temperatures and the corresponding Arrhenius parameters.

Alenezi *et al*., (2010) studied non-catalytic esterification of free fatty acids (FFA) with supercritical methanol. Effects of temperature, stirring rate and reaction time intervals were investigated. The yield of FAME was found to increase with an increase in temperature, and with an increase in the molar ratio of methanol to FFA. The yield of FAME was not affected by the increase of stirring rate more than 850 rpm. The activation energy value for the forward reaction was 72 kJ/mol.

Zhang *et al*.,(2010) studies biodiesel from palm oil and dimethyl carbonate catalyzed by immobilized-lipase in solvent-free system. The effects of the reaction conditions (type of lipases, molar ratio of DMC and palm oil, amount of catalyst, reaction temperature and time) on the yield of FAMEs were investigated. The yield of FAMEs could reach 90.5% at 55°C for 24 h with the molar ratio of DMC to oil 10:1 and the catalyst amount of 20% Novozym 435 (based on the oil weight). There was no obvious loss in the FAMEs yield after Novozym 435 having been used for eight cycles.

CHAPTER III

EXPERIMENTS

This chapter consists of experimental systems and procedures used in this research, which is divided into 5 main parts:

1. Materials and chemicals

2. Enzymatic esterification

3. Equipments

4. Optimization of process parameter

5. Butyoleate Analysis

3.1 Materials and chemicals

Novozym 435 (Lipase B from *C. antractica*, EC 3.1.1.3, a nonspecific lipase immobilized on macroporous acrylic resin) used as biocatalyst was purchase from S.M. Chemical suppliers Co., Ltd**.** Molecular sieve was used in this study purchased from local suppliers in Thailand.

All chemicals used in this work (n-butanol, isobutanol, n-propanol, oleic fatty acid) were analytical grade, and were purchased from local suppliers in Thailand.

3.2 Enzymatic esterification reaction

The esterification was carried out in 250 mL Erlenmayer flasks. The reaction containing of 40 g of oleic fatty acid and butanol at a molar ratio of 1:1, 1:2, 1:3 or 1:4. The reaction was catalyzed by Novozym 435 at 2.5%, 5% or 10% (w/w of oil). The mixtures were incubated at 35, 45, 55 or 60°C under a constant shaking at 200, 250, 300 or 350 rpm. Samples of 6 mL were taken from the reaction mixture at 1, 3, 6, 9, 12 and 24 h of the reaction. The residual alcohol and water were removed from the sample by evaporation before the FFA concentration 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 Equipments

3.3.1. Sciencetific balance

3.3.2. Micropipette

3.3.3. Heater

3.3.4. Burette

3.3.5. Incubator shaker (Innova 4000, ALT, Connecticut, USA)

3.3.6. Centrifuge (5100, Kubota, Fujioka, Japan)

3.4 Optimization of process parameter

To study the optimal conditions for enzymatic esterification of oleic acid with butyl alcohol, effects of the following conditions and kinetics of the reaction were investigated.

3.4.1. Effect of temperature

3.4.2. Effect of stirring rate

3.4.3. Effect of molar ratio of oleic acid/butanol

3.4.4. Effect of enzyme loading

3.4.5. Effect of water removal

3.4.6. Repeated use of the immobilized Novozym 435

3.4.7. Kinetics of esterification reaction.

3.5 Butyoleate Analysis

The content of fatty acid was obtained by titration. Percentage of oleic acid conversion was determined by the titration with 0.025M KOH solution using phenolphthalein as the indicator. Concentration of oleic acid could be calculated from the titration volume of the KOH solution. The data was used to calculate the FFA conversion. The accuracy of data from the titration method was confirmed by data from NMR analysis.

NMR is a useful method for determining the molecular structure of a chemical as a whole. The result of merging data from infrared spectroscopy (to determine the function of a compound) and NMR (provides information on the number of each type of hydrogen) is sufficient to determine concentrations of known compounds and to investigate unknown structure as well (Arbain and Salimon, 2010).

CHAPTER IV
RESULTS AND DISCUSSION

Experimental results were obtained from the esterification of oleic acid with butanol by the *Candida antractica* B lipase, commercially known as Novozym 435. Effects of reaction time, temperature, enzyme loading, molar ratio of acid to butanol, speed of agitation and kinetic of esterification reaction in solvent free system were determined. Results and discussions are presented in 3 parts:

4.1 Butyloleate production using immobilized lipase

4.2 Effects of reaction conditions on esterification reaction

4.2.1 Effect of reaction time

4.2.2 Effect of enzyme loading

4.2.3 Effect of acid/alcohol molar ratio

4.2.4 Effect of speed of agitation

4.2.5 Effect of reaction temperature

4.2.6 Effect of alcohol type

4.2.7 Effect of water removal

4.3 Reusability of enzyme

4.4 Kinetics of enzymatic esterification of reaction

4.1 Butyloleate production using immobilized lipase

In this study, the immobilized lipase, Novozym 435 was applied for the butyloleate production. The reaction of oleic acid and butanol at a molar ratio of 1:2 was carried out in 250 mL Erlenmayer flasks. The reaction was catalyzed by 5% lipase (by weight of oleic acid). The mixtures were incubated at 45°C under constant shaking at 250 rpm. Butanol was evaporated from the samples followed by centrifuging to separate the product for analysis.

4.2 Effects of reaction conditions on esterification reaction

4.2.1 Effect of reaction time

The effect of reaction time on the esterification of oleic acid and butanol catalyzed by immobilized lipase Novozym 435 was determined under the reaction conditions: molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; reaction temperature, 45°C and a stirring rate of 250 rpm. From Figure 4.1, it was found that the conversion of FFA was increased quickly with increasing reaction time during 0-6 h and it was then gradually increased until it reached the maximum at 24 h. The maximal conversion at 91.0% was obtained.

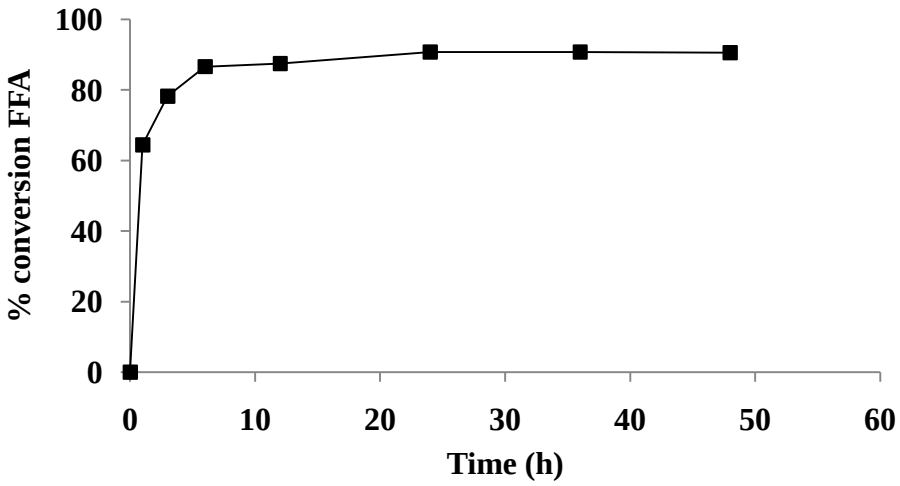

Figure 4.1 Effect of reaction time on the conversion of FFA. Reaction conditions: molar ratio of butanol/oleic acid molar ratio, 2:1; Novozym 435 based on acid weight, 5%; reaction temperature, 45°C; a stirring rate of 250 rpm, and reaction period of 48 h.

4.2.2 Effect of enzyme loading

The catalyst loading is an essential economical factor for industrial application. Thus, the effect of enzyme loading was examined. To study this effect, the percentage of enzyme loading was varied from 2.5% to 10% (w/w) with respect to fatty acid weight. Reaction temperature was fixed at 45°C and oleic acid to butanol molar ratio was 1:2. From Figure 4.2, it was found that the conversion of FFA increased with increasing of enzyme loading and reached the maximum of about 91.0% when the concentration of catalyst was 5-10%.

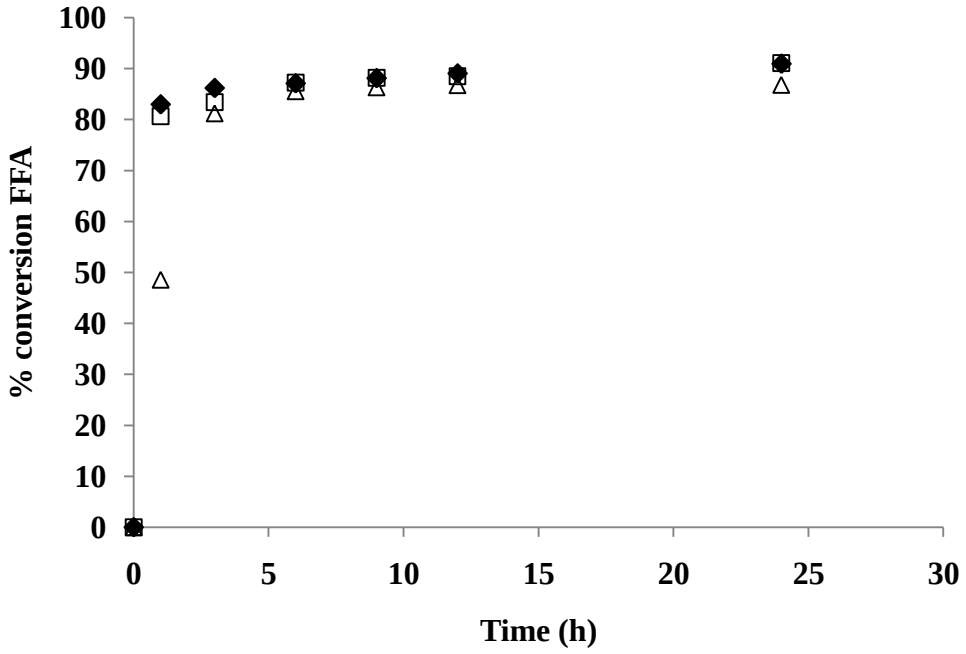

Figure 4.2 Effect of enzyme loading on the conversion of FFA. Reaction conditions: molar ratio of butanol/oleic acid molar ratio, 2:1; Temperature 45°C; a stirring rate of 250 rpm, and reaction period of 24 hr. (□) 10% ; (♦) 5%; (Δ) 2.5% (w/w) of fatty acid.

In general, initial rate increased with increasing biocatalyst concentration. However, extra loading of lipase beyond the maximal level could cause a decrease in the initial rate (Sabeder et al., 2006). Maceiras et al. (2009) reported that methyl esters yield increased with lipase concentrations up to 10% (w/w) of oil, after that the yield decreased. Similarly, Zhang et al. (2010) reported that the FAMEs yield increased

with increasing catalyst amount. The maximum FAMEs yield was 90.2% at 20% (w/w) enzyme loading. However, FAMEs yield was not increased with the addition of enzyme loading further than 20% (w/w). It was suggested that too much catalyst loading could cause the mixture too viscous, leading to a problem of mixing (Zhang, 2010). In this work, slightly increase of FFA conversion was observed with the increase of enzyme loading from 5 to 10% (w/w) of oleic acid. Considering the economical point of view, the enzyme loading at 5% (w/w) of oleic acid was used in the further study.

4.2.3 Effect of fatty acid/alcohol molar ratio

One of the most important parameters in enzymatic esterification is the molar ratio of fatty acid/alcohol. Therefore, it is necessary to find the optimum proportion for maximum production. In this study, the reaction was carried out with various molar ratio of oleic acid to butanol (1:1, 1:2, 1:3, 1:4) with 5% (w/w) enzyme loading at reaction temperature of 45°C.

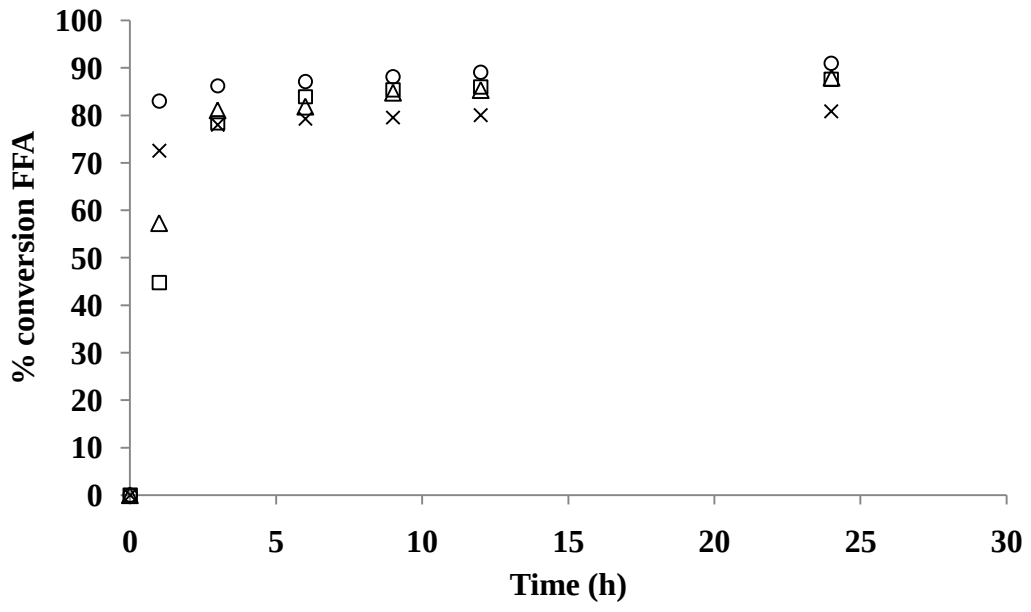

Figure 4.3 Effect of oleic acid / butanol molar ratio on the conversion of FFA. Reaction conditions: 5% (w/w) enzyme loading; temperature of 45°C; stirring rate of 250 rpm, and reaction period of 24 h. (x) 1:1; (○)1:2; (Δ) 1:3; (□) 1:4 acid/alcohol molar ratio.

As seen in Figure 4.3, the conversion yield of FFA increased as the oleic acid to butanol molar ratio increased from 1:1 to 1:2. The maximum ester conversion (91.0%) was obtained at the molar ratio of 1:2. After that, the additional increase of butanol caused slightly decrease of the conversion. The similar result has been reported by Dormo et al. in 2004. The higher ester conversion was obtained in shorter period of time at the molar ratios of acid to alcohol in the range of 1:2 to1:5 compared to 1:1 and the highest acid conversion was achieved at the molar ratio of 1:2. In 2010, Pinnarat and Savage also reported that the highest conversion yield of ethyl ester was obtained at the 1:3 ratio of oleic acid to ethanol. Further increasing of alcohol than that, the conversion yield was found slightly decreased. Furthermore, from the report of Alenezi et al. in 2010, the optimal molar ratio of methanol to FFA for esterification was 1.6:1 with the maximum yield of 97%. Since esterification reaction is reversible reaction, increasing alcohol concentration is one way to shift the reaction toward the ester synthesis. However, the use of too high amount of alcohol might slow down reaction rates due to inhibition effect of alcohol on enzyme activity.

4.2.4 Effect of speed of agitation

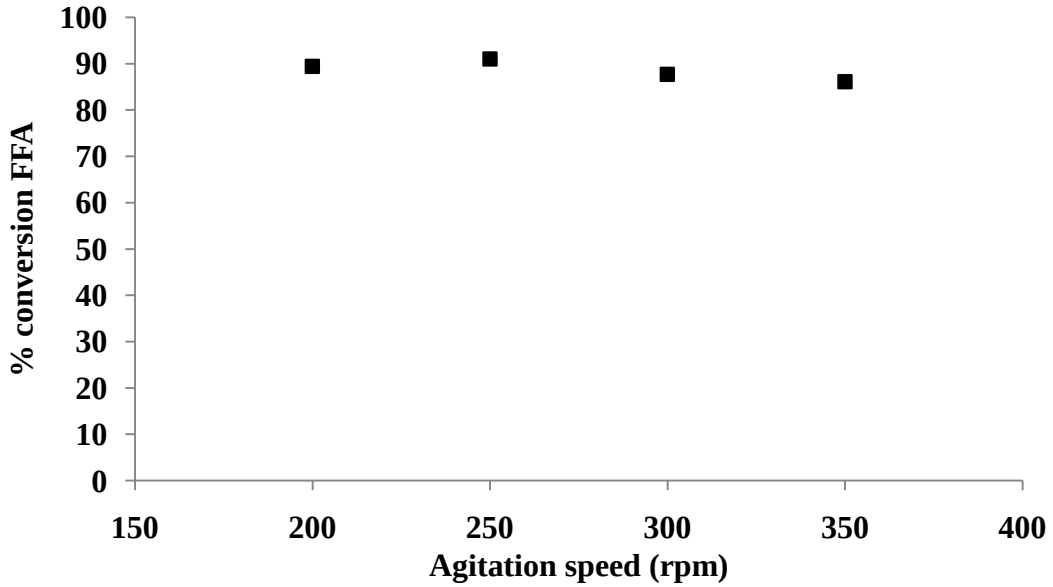

Figure 4.4 Effect of speed of agitation on the conversion of FFA using immobilized lipases with the molar ratio of oleic acid to butanol of 1:2, reaction temperature of 45°C and reaction time of 24 h.

The effect of speed of agitation on the conversion was examined by using immobilized enzyme (Novozym 435) as biocatalysts in esterification of oleic acid and butanol at the reaction temperature of 45°C, oleic acid/butanol molar ratio (w/w) of 1:2 with 5% enzyme loading. Tests were conducted at various agitation speeds from 200-350 rpm. As shown in Figure 4.4, the highest ester conversion of 91.0% was obtained at the speed of agitation of 250 rpm. The final FFA conversion relatively decreased with the increase of agitation speeds higher than 250 rpm. Generally, the overall rate of reaction is dependent on mixing behavior of the system and catalytic activity as well. Since lipase is a biocatalyst, shearing force at too high agitation speed could considerably cause the loss of catalytic activity.

4.2.5 Effect of reaction temperature

As enzymes are very sensitive to temperature, the effect of temperature on conversion of FFA was determined at the system temperature between 35 to 60 °C with the oleic acid to butanol molar ratio of 1:2, 5% (w/w) of Novozym 435 and reaction time of 24 h. As shown in Figure 4.5, the FFA conversion increased with increasing temperature. The initial rates at reaction temperature of 45-60°C were significantly higher than the rate at 35°C. The final highest percentage conversion of FFA (91.0%) was obtained at 60°C. However, only slightly differences in FFA conversion and reaction rate were observed when the reaction temperature was varied from 45 to 60 °C.

Dormo et al. (2004) previously reported the influence of reaction temperature on the synthesis of isoamyl-oleate. The final concentration of isoamyl-oleate was increased with increasing temperature. According to the study of an environmental-safe biolubricant from fusel oil by enzymatic esterification in solvent-free system by Garcia's group in 2000, it was reported that the conversion could be increased with increasing temperature. The further study of temperature effect on biodiesel conversion rate showed that the conversion increased rapidly within first 5 h, and slightly increased with temperature (Liu et al., 2010).

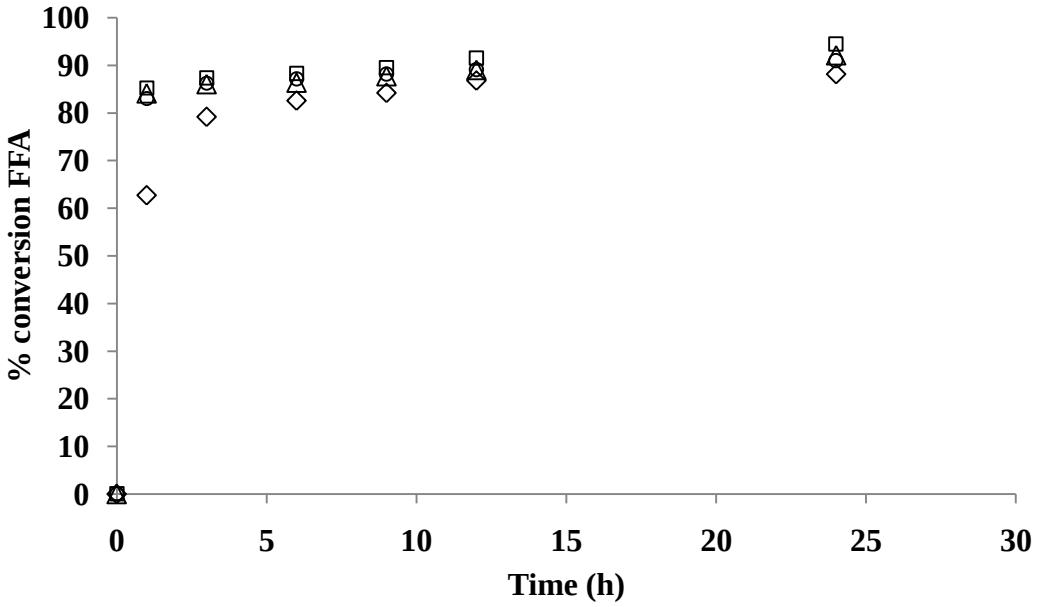

Figure 4.5 Effect of reaction temperature on the conversion of FFA. Reaction conditions: molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm, and reaction period of 24 h. (◊) 35°C; (○) 45°C; (Δ) 55°C; (□) 60°C.

4.2.6 Effect of alcohol type

In order to investigate the effect of alcohol structure on butyloleate conversion by Novozym 435, the esterification reaction of oleic acid and n-butanol (straight chain) was performed in comparison to that of iso-butanol (branch chain) under the optimal controlled conditions: reaction temperature of 45°C, 5% (w/w) enzyme loading, 250 rpm of stirring rate, and acid to alcohol molar ratio of 1:2.

The esterification reaction by Novozym 435 with the branch chain alcohol (iso-butanol) showed slightly higher initial rate than that using the straight chain alcohol (n-butanol). However, the final conversion yields of the 1st run by using iso-butanol and n-butanol were about at the same level (91.0%). For the 2nd, 3rd, 4th and 5th run, the FFA conversions of both systems slightly decreased. No difference in conversions was observed with the different chemical structures of alcohols.

Dormo et al. (2004) studied the effect of chain length of alcohols in fusel oil on the esterification reaction and reported that the ester rates of oleic acid with isoamyl alcohol were larger than that with ethanol, indicating the influence of the structure and length of the alcohol molecule on the reaction rate. Variation in results should depend upon the types of substrates, catalysts and operating conditions.

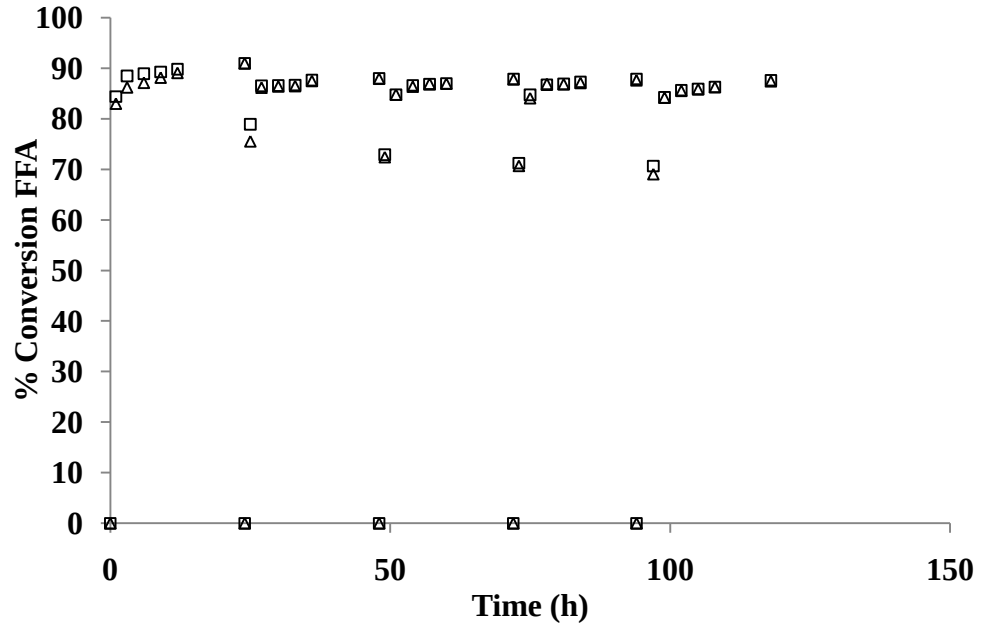

Figure 4.6 The effect of alcohol type. Reaction conditions: oleic acid/alcohol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; temperature of 45°C and reaction period of 24 h. Various type of alcohol (□) iso-butanol; (Δ) butanol.

4.2.7 Effect of water removal

Fatty acid butyl ester and water are the products, generated during the reversible esterification reaction. In order to enhance the equilibrium conversion of ester synthesis, water should be removed from the system during the reaction. Therefore, in this work, 40% (w/w) alkali alumino silicate (molecular sieve type 4A) based on fatty acid weight was added into the system to adsorb water during the reaction. The specifications of the molecular sieve type 4A is shown in Appendix C.

The reaction was conducted for 24 h at 45°C with the enzyme loading of 5% (w/w), oleic acid to butanol molar ratio of 1:2 and the speed of agitation of 250 rpm.

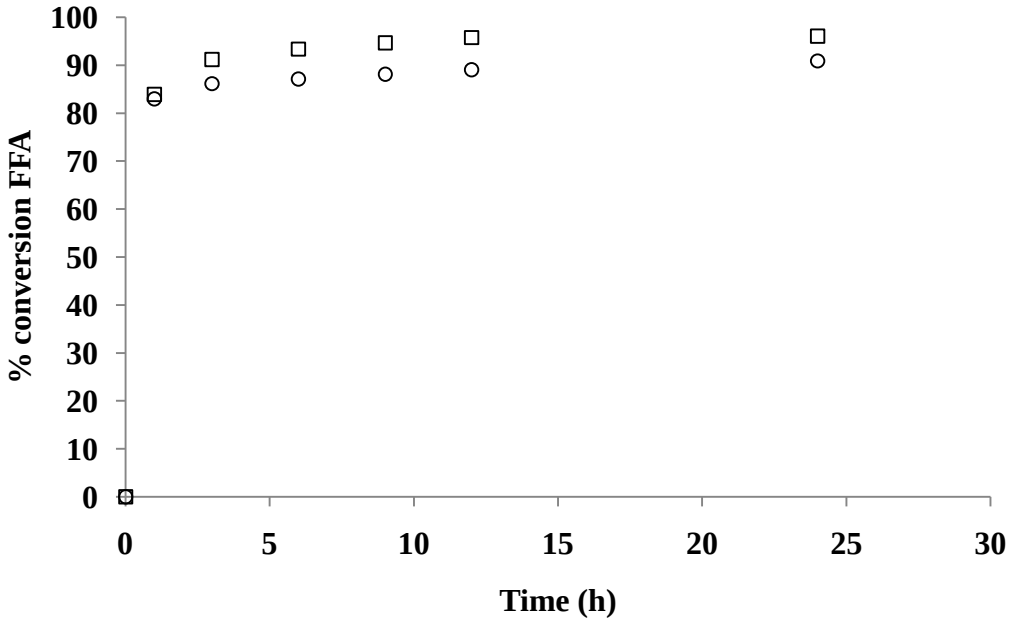

Figure 4.7 Effect of molecular sieve on FFA conversion in 24 h of the reaction. Reaction conditions : 5% enzyme loading, 250 rpm of stirring rate, 1:2 of molar ratio of oleic acid to butanol at 45°C ; (□) with molecular sieve; (○) without molecular sieve.

Figure 4.7 illustrates the influence of water removal on the FFA conversion. It was found that with the addition of molecular sieve during the reaction, about 5.0% butyloleate increased compared to that of the system without the molecular sieve addition. The similar result has been reported by Jeong and Park in 2004 on the study of the effect of molecular sieves as water absorbers on enzyme-catalyzed rapeseed oil methanolysis. The removal of water with molecular sieves during the reaction could raise the enzymatic synthesis of sorbitan acrylate using Novozym 435, with the exception of the inherent water content of the enzymes (Subkerd, 2008).

4.3 Reusability of enzyme

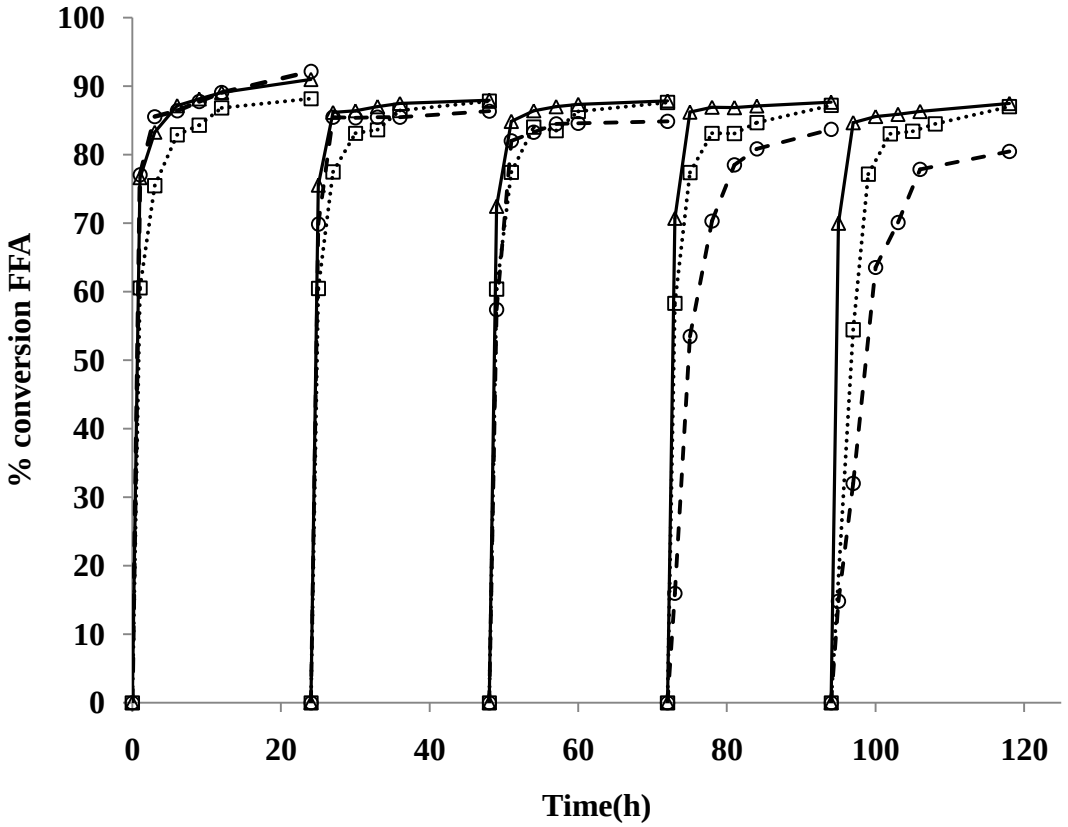

Figure 4.8 Repeated use of Novozym 435; Reaction conditions: molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; cycle times 24 h; variable temperature from (□)35°C; (Δ)45°C; and (○)55°C.

In order to reduce the cost of the enzyme utilization, which would help lower the production cost, the repeated use of the immobilized enzyme is required. In this research**,** the repeated use of Novozym 435 was determined by varying at three different temperatures (35°C, 45°C, 55°C) while keeping the speed of agitation at 250 rpm, enzyme loading at 5% (w/w), oleic acid to butanol molar ratio of 1:2 and cycle time of 24 h.

As shown in Figure 4.8, on the 1st run, the final conversion yield increased with increasing temperature. The initial rates of the reactions at the temperatures of

45°C and 55°C were significantly higher than that of 35°C. Overall, after the rapid increase of conversion in the initial 6 h, the butyloleate conversion slowly increased and reached the equilibrium within 24 h. In consideration of the FFA conversion for the production of oleic acid butyl ester under the repeated use of Novozym 435 for five cycles, it was found that the catalytic activity of Novozym 435 was greatly reduced under the controlled reaction temperature of 55°C compared to that of 35°C and 45°C. The final FFA conversion of the 5^{th} run of 55°C was reduced to about 87.3% of the initial one. On the other hand, under the operated temperature at 35°C and 45°C, it was found that the catalytic activity of Novozym 435 remained almost unchanged after the five repeated uses, in which less than 3.0% of the FFA conversion was loss. Therefore, Novozym 435 was stable under the esterification at controlled temperature of 35-45°C.

According to Shah's study on stability of immobilized lipase (*Pseudomonas cepacia* from Amano Enzyme Inc. (Nagoya, Japan) in 2007, after four times of the repeated uses, the rapid decline in activity was observed. Maceiras et al. (2009) reported the recycle of Novozyme 435 for four times without loss of any activity on the synthesis of methyl ester. On the other hand, Zhang et al. (2010) reported the decrease of yield of FAMEs after the lipase (Novozym435) was used for eight cycles. Mengyu et al. (2009) also reported that the catalyst ($Fe_2(SO_4)_3$/C) showed a significant loss of activity with the first recycling, and then showed a slower gradual loss after that. Therefore, the reusability of immobilized lipase was not only dependent on the type of enzymes but also depending on operating conditions.

4.4 Kinetics of enzymatic esterification of reaction

The esterification reaction of oleic acid and butanol can be represented by the following equation:

$$C_4H_{10}O + C_{18}H_{34}O_2 \rightarrow C_{22}H_{42}O_2 + H_2O$$

Butanol Oleic acid Butyl Oleate Water

The rate equation for second order reaction, is given by

$$R = \frac{-d[\text{Oleic â}]}{dt} = k\,[\text{Oleic â}]\,[\text{Butanol}] \qquad (1)$$

Since $[\text{Oleic â}] = [\text{Oleic â}]_0{}^2(1-X_{\text{oleic â}})^2$ (2)

Where [Oleic â] denotes the concentration of oleic acid; $[\text{Oleic â}]_0$ is the initial concentration of oleic acid and $X_{\text{Oleic â}}$ is conversion of oleic acid.

$$[\text{Butanol}] = [\text{Butanol}]_0 (1-X_{\text{Butanol}}) \quad (3)$$

$$[\text{Butanol}] = [\text{Oleic â}]_0 (M-X_{\text{oleic â}}) \quad (4)$$

Where, M is molar ratio of butanol/oleic acid.

By substitution of oleic acid concentration and butanol concentration in Eq. (2) and Eq. (4) into Eq. (1) and integration, the rate constant (k) for the second order reaction could be determined from Eq.(5)

$$\text{Ln}\frac{(M-\text{Xoleic â})}{M(1-\text{Xoleic â})} = [\text{Oleic a}]_0 (M-1)k\,t \quad (5)$$

Where, k is the rate constant of the reaction.

4.4.1 Kinetics of the repeated use of Novozym 435

The kinetic rates of the repeated use of Novozym 435 obtained from the experimental data at oleic acid/butanol molar ratio of 1:2, 5% Novozym 435 and at the operating temperature of 45°C, were correlated with Eq.(5). The rate constant (k) of esterification reaction could be determined based on decreased amount of fatty acid. Figure 4.9 shows the plot of the rate constants (k) for the reaction.

The result illustrated in Fig. 4.9 demonstrates that Novozym 435 was quite stable and effective in the esterification reaction. The operational stability of the enzyme was maintained at >90% yield up to 5 cycles. It was found that the k values were gradually decreased with the repeated use.

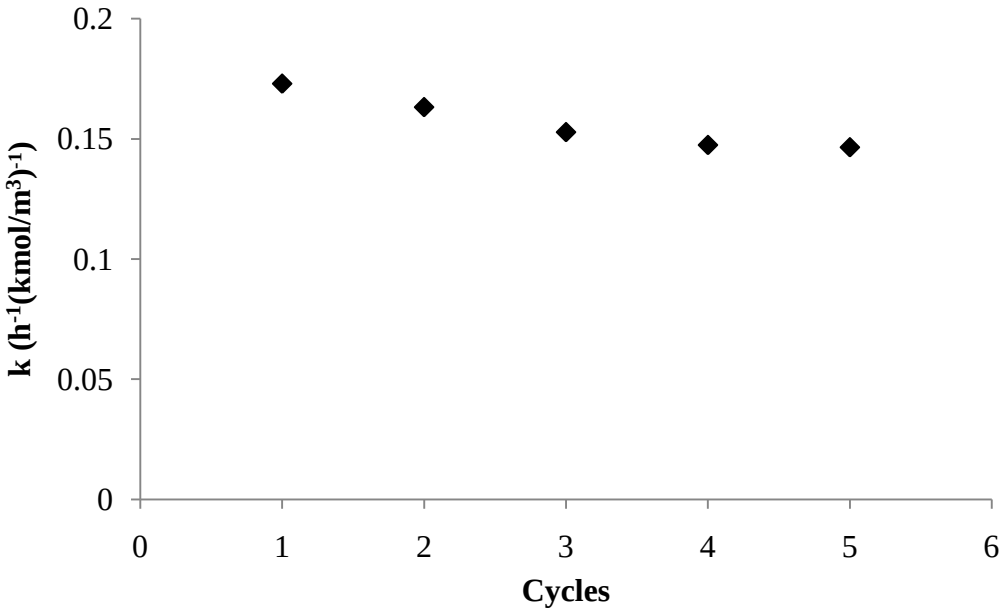

Figure 4.9 The rate constants (k) of the repeated use of Novozym 435. Reaction conditions: molar ratio of butanol/oleic acid, 2:1; Novozym 435 based on acid weight, 5%; reaction temperature, 45°C and a stirring rate of 250 rpm.

4.4.2 Effect of temperature on kinetic constants

Figure 4.10 illustrates the increase of rate constant of the reaction (k) with the temperature as shown in Figure 4.10. The influence of temperature on the reaction rates could be described by the Arrhenius equation (Eq. 6).

$$k = k_0 e^{-Ea/RT} \quad (6)$$

or $$\ln(k)=\ln(k_0) - Ea/RT \quad (7)$$

The Arrhenius plot of ln(k) versus the inverse temperature, 1/T is shown in Figure 4.11. The negative slope from the Arrhenius plot can be used to determine the activation energy, Ea. The activity energy of enzymatic esterification reaction of oleic acid was found to be 13.64 kJ/mol.

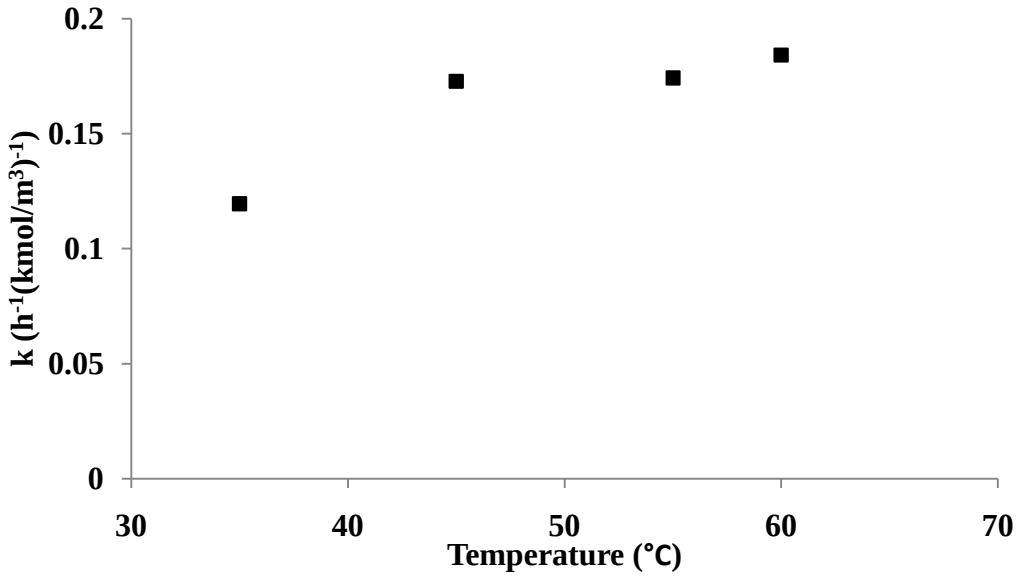

Figure 4.10 The rate constants (k) of the enzymatic esterification as a function of reaction temperature. Reaction conditions: Oleic acid to butanol molar ratio 1:2, 5% Novozym 435, 250 rpm and temperature 35, 45, 55 and 60°C.

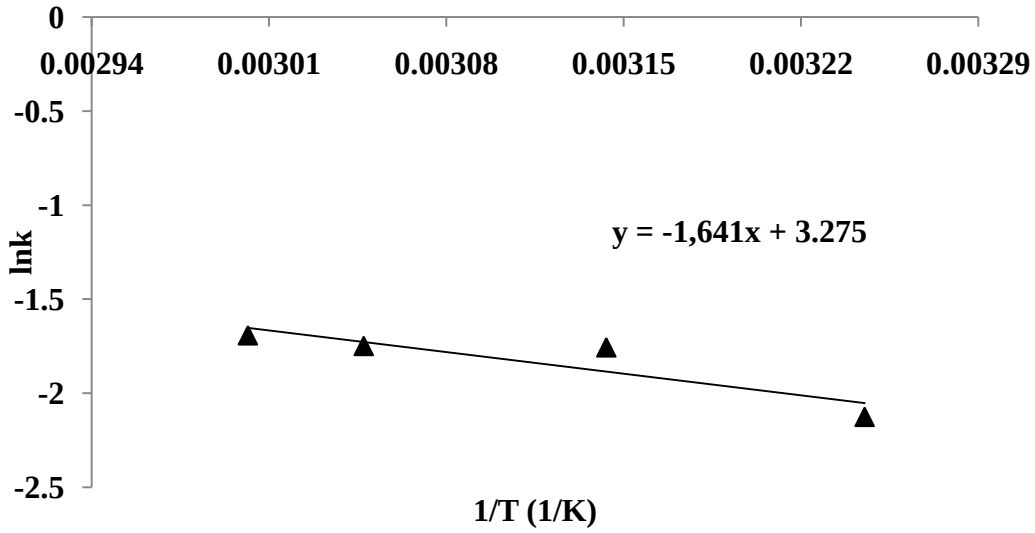

Figure 4.11 Temperature dependence of rate constants (k) of the enzymatic esterification catalyzed by Novozyme 435. Reaction conditions: Oleic acid to butanol molar ratio 1:2, 5% Novozym 435, 250 rpm and temperature 35, 45, 55 and 60 °C.

Under noncatalytic esterification with ethanol at subcritical reaction conditions, the activation energy (Ea) for forward reaction (k_1) and reverse reaction (k_{-1}) were reported at 56±2 kJ/mol and 66±14 kJ/mol, respectively (Pinnarat and Savage, 2010). The Ea values of 25–60 kJ/mol was reported for sulfuric acid-catalyzed esterification of FFA with methanol (Berrios et al., 2007 and Aranda et al., 2008). The further study of the esterification of myristic acid with methanol in presence of triglycerides using sulfated zirconia prepared by solvent-free method as the heterogeneous catalyst reported the activation energy of reaction at 22.51 kJ/mol (Rattanaphra et al., 2011). The Ea value obtained from the enzymatic esterification catalyzed by Novozyme 435 in this study was lower compared to those of esterification using chemical catalysts.

4.4.3 Determination of the kinetic constants in the repeated use of Novozym 435 at different temperatures.

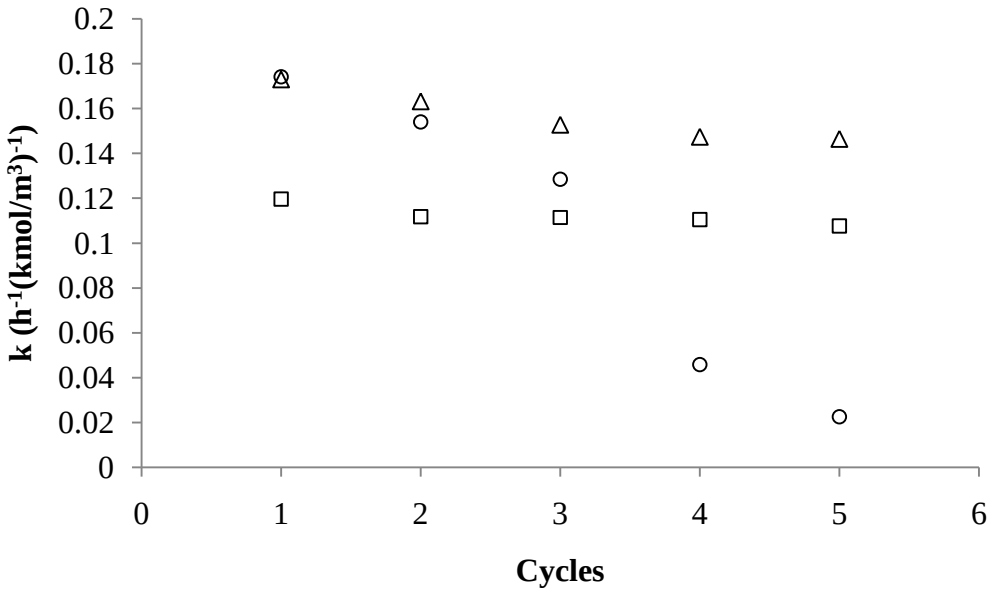

Figure 4.12 The rate constants (k) of the enzymatic esterification on the repeated use of Novozym 435 at different reaction temperatures. Reaction conditions: Oleic acid to butanol molar ratio 1:2, 5% Novozym 435, 250 rpm and reaction temperature at (□) 35 °C, (Δ) 45 °C and (○) 55 °C.

Figure 4.12 demonstrates the kinetic constants (k) on reusability of the enzyme Novozym 435 by varying at three different temperatures (35°C, 45°C, 55°C), under the speed of agitation of 250 rpm, enzyme loading 5% (w/w), oleic acid to butanol molar ratio of 1:2 and cycle time 24 h. The highest decline of k with the number of the repeated use was observed at 55°C, whereas, the k values at 35°C and 45°C was gradually decreased with the number of the repeated use. The k values at 45°C was considerably higher than the k values at 35°C from the 1^{st} run to the 5^{th} run and higher than the k values at 55 °C from the 2^{nd} run to the 5^{th} run**.** Therefore the optimal temperature for the repeated use of Novozym 435 in this reaction was 45°C.

CHAPTER V
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study described the development of the butyloleate production from oleic acid and butanol catalysed by Novozym 435, a nonspecific lipase immobilized on macroporous acrylic resin. The optimal conditions and reusability of Novozym 435 for the esterification are investigated.

For the butyloleate production, the optimal conditions and kinetics of esterification reaction using Novozym 435 could be concluded as follows:

1) The butyloleate could be produced by esterification reaction from oleic fatty acid with butanol using Novozym 435 as catalyst.

2) The optimal conditions for the esterification reaction were: the reaction temperature of 45°C, the oleic / butanol molar ratio of 1:2, 5% enzyme loading, 250 rpm of stirring rate and 10-24 h of reaction time. The maximum butyloleate concentration at 91.0% conversion was obtained.

3) The removal of water generated during the enzymatic esterification reaction by adding molecular sieves could enhance the conversion yield.

4) Novozym 435 was stable and effective in the esterification reaction at 45°C. The operational stability of the enzyme was maintained >87% yield up to 5 cycles. The repeated use of Novozym 435 on the esterification at 55°C - 60°C resulted in the significant reduction of catalytic activity of the enzyme.

5) The reaction kinetics of enzymatic esterification of oleic acid with butanol agrees with second order reaction. The activation energy of enzymatic reaction was approximately 13.64 kJ/mol.

5.2 Recommendation

Although the effects of enzyme loading, the reaction temperature, the stirring rate, the molar ratio of acid to alcohol have been studied in this research, some other interesting points still could be further investigated. These are some recommendations:

1) The study to develop the process of esterification reaction from batch mode to continuous mode.

2) The study of reused immobilized lipase should be taken to investigate the cause of the loss in the enzymatic activity.

REFERENCES

Alenezi, R., Leeke, G.A., Winterbottom, J.M., Santos, R.C.D., Khan, A.R. Esterification kinetics of free fatty acids with supercritical methanol for biodiesel production. *Energy Conversion and Management* 51 (2010): 1055-1059.

Aranda, D.A.G., Santos, R.T.P., Tapanes, N.C.O., Lamos, A.L.D., and Antunes, O.A. C. Acid-Catalyzed Homogeneous Esterification Reaction for Biodiesel Production from Palm Fatty Acids. *Catal Lett* 122 (2008): 20–25.

Arbain, N.H. and Salimon, J. Synthesis and characterization of ester trimethylolpropane based jatropha curcas oil as biolubricant base stocks. *Journal of Science and Technology* 2 (2010): 2.

Berrios, M., Siles, J., Martın, M.A., Martın, A. A kinetic study of the esterification of free fatty acids (FFA) in sunflower oil. *Fuel* 86 (2007): 2383-2388.

Cardoso, A.L., Neves, S.C.G., and Silva, M.J.D. Esterification of Oleic Acid for Biodiesel Production Catalyzed by $SnCl_2$: A Kinetic Investigation. *Energies* 1 (2008): 79-92.

Chen, Y., Xiao, B., Chang, J., Fu, Y., Lv, P., and Wang, X. Synthesis of biodiesel from waste cooking oil using immobilized lipase in fixed bed reactor. *Energy conversion and management* 50 (2009): 668–673.

Dizge, N., and Keskinler, B. Enzymatic production of biodiesel from canola oil using immobilized lipase. *Biomass and bioenergy* 32 (2008): 1274-1278.

Dormo, N., Belafi-Bako, K., Barthab, L., Ehrensteinc, U., Gubicza, L. Manufacture of an environmental-safe biolubricant from fusel oil by enzymatic esterification in solvent-free system. *Biochemical Engineering Journal* 21 (2004): 229–234.

Fukuda, H., Kondo, A., and Noda, H. Biodiesel Fuel Production by Transesterification of Oils. *Journal of bioscience and bioengineering* 92 (2001): 405-416.

Garcia, T., Sanchez, N., Martinez, M., and Aracil, J. Enzymatic synthesis of fatty esters Part I. Kinetic approach. *Enzyme and Microbial Technology* 25 (1999): 584–590.

Hernandez-Martın, E., and Otero, C. Different enzyme requirements for the synthesis of biodiesel: Novozym®435 and Lipozyme®TL IM. *Bioresource Technology* 99 (2008): 277–286.

Jain, S., Sharma, M.P., and Rajvanshi, S. Acid base catalyzed tranesterification kinetics of waste cooking oil. *Fuel Processing Technology* 92 (2011): 32-38.

Joeng, G., and Park, D. Lipase-Catalyzed Transesterification of Rapeseed Oil for Biodiesel Production with *tert* –Butanol. *APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY* 148 (2008): 131-139.

Katchalski-Katzir, E. Immobilized enzymes — learning from past successes and failures. *Trends in Biotechnology* 11 (1993): 471-478.

Knothe, G. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Processing Technology* 86 (2005): 1059 – 1070.

Kose, O., Tuter, M., Aksoy, H.A. Immobilized *Candida Antarctica* lipase catalyzed alcoholysis of cotton seed oil in a solvent-free medium. *Bioresource Technology* 83 (2002): 125–129.

Koszorz, Z., Nemestothy, N., Ziobrowski, Z., Belafi-Bako, K., and Krupiczka, R. Influence of pervaporation process parameters on enzymatic catalyst deactivation. *Desalination* 162 (2004): 307-313.

Li, L., Du, Wei., Liu, D., and Wang, L. Lipase-catalyzed tranesterification of rapeseed oils for biodiesel production with novel organic solvent as the reaction medium. *Journal of Molecular Catalysis B: Enzymatic* 43 (2006): 58-62.

Liu, Y., Tanb, H., Zhang, X., Yana, Y., and Hameed, B.H. Effect of monohydric alcohols on enzymatic transesterification for biodiesel production. *Chemical Engineering Journal* 157 (2010): 223-229.

Maceiras, R., Vega, M., Costa, C., Ramos, P., and Marquez, M.C. Effect of methanol content on enzymatic production of biodiesel from waste frying oil. *Fuel* 88 (2009): 2130-2134.

Mahesh, N., Varma and Giridhar, M. Synthesis of Biodiesel from castor oil and Linseed Oil in Supercritical Fluids. *Ind. Eng. Chem. Res.* 46 (2007): 1-6.

Mehta, R.N., Chakraborty, M., Mahanta, P., and Parikh, P.A. Evaluation of Fuel Properties of Butanol−Biodiesel−Diesel Blends and Their Impact on Engine Performance and Emissions. *Ind. Eng. Chem. Res.* 49 (2010): 7660-7665.

Mengyu, G., Deng, P., Li, M., En, Y., and Jianbing, H. The Kinetics of the Esterification of Free Fatty Acids in Waste Cooking Oil Using $Fe_2(SO_4)_3$/C Catalyst. *Chinese Journal of Chemical Engineering* 17 (2009): 83-87.

Miao, X., Li, R., and Yao, H. Effective acid-catalyzed tranesterification for biodiesel production. *Energy Conversion and Management* 50 (2009): 2680-2684.

Nie, K., Xie, F., Wang, F., and Tan, T. Lipase catalyzed methanolysis to produce biodiesel: Optimization of the biodiesel production. *Journal of Molecular Catalysis B: Enzymatic* 43 (2006): 142–147.

Noureddini, H., Gao, X., and Philkana, R.S. Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. *Bioresource Technology* 96 (2005): 769-777.

Oliveira, A.C., Rosa, M.F., Aires-Barros, M.R., Cabral, J.M.S. Enzymatic esterification of ethanol and oleic acid - a kinetic study. *Journal of Molecular Catalysis B: Enzymatic* 11 (2011): 999 – 1005.

Ognjanovic, N., Bezbradica, D., and Knezevic-Jugovic, Z. Enzymatic conversion of sunflower oil to biodiesel in a solvent-free system: Process optimization and the immobilized system stability. *Bioresource Technology* 100 (2009): 5146 – 5154.

Pinnarat, T., and Savage, P.E. Noncatalytic esterification of oleic acid in ethanol. *The journal of Supercritical Fluids* 53 (2010): 53-59.

Qureshi, N., Meagher, M.M., Hutkins, R.W. Recovery of butanol from model solutions and fermentation broth using a silicalite/silicone membrane. *Journal of Membrane Science* 158 (1999): 115-125.

Qureshi, N., Saha, B.C., Dien, B., Hector, R.E., Cotta, M.A. Production of butanol (a biofuel) from agricultural residues: Part I–Use of barley straw hydrolysate. *Biomass and bioenergy* 34 (2010): 559-565.

Rattanaphra, D., Harvey, A.P., Thanapimmetha, A., and Srinophakun, P. Kinetic of myristic acid esterification with methanol in the presence of triglycerides over sulfated zirconia. *Renewable Energy* 36 (2011): 2679-2686.

Rustan, A.C. and Drevon, C.A. Fatty Acids: Structures and Properties., 23 SEP 2005.

Sabeder, S., Habulin, M., Knez, Z. Lipase-catalyzed synthesis of fatty acid fructose esters. *Journal of Food Engineering* 77 (2006): 880-886.

Shah, S., and Gupta, M.N. Lipase catalyzed preparation of biodiesel from Jatropha oil in a solvent free system. *Process Biochemistry* 42 (2007): 409-414.

Sonare, N.R., and Rathod, V.K. Transesterification of used sunflower oil using immobilized enzyme. *Journal of Molecular Catalysis B: Enzymatic* 66 (2010): 142-147.

Song, C., Qi, Y., Deng, T., Hou, X., and Qin, Z. Kinetic model for the esterification of oleic acid catalyzed by zinc acetate in subcritical methanol. *Renewable Energy* 35 (2010): 625-628.

Subkerd, S. Semi-continuous production ofbiodiesel from palm fatty acid using Novozym435. Master's thesis, Science Program in Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University, 2008.

Tischer, W., and Wedekind, F. Immobilized Enzymes: Methods and Applications. *Topics in Current Chemistry* 200 (1999): 95-126.

Thanh, L.T., Okitsu, K., Boi, L.V. and Maeda, Y. Catalytic Technologies for Biodiesel Fuel Production and Utilization of Glycerol: A Review. *Catalysts* 2 (2012): 191-222.

Yujaroen, D., Goto, M., Sasaki, M., Shotipruk, A. Esterification of palm fatty acid distillate (PFAD) in supercritical methanol: Effect of hydrolysis on reaction reactivity. *Fuel* 88 (2009): 2011-2016.

Zhang, L., Sun, S., Xin, Z., Sheng, B., and Liu, Q. Synthesis and component confirmation of biodiesel from palm oil and dimethyl carbonate catalyzed by immobilized- lipase in solvent-free system. *Fuel* 89 (2010): 3960-3965.

APPENDICES

APPENDIX A

CALCULATION

Table A-1 Properties of materials

Reactants	Molar mass(g/g mol)	Density (g/cm^3)
Oleic acid	282.40	0.895
Butanol	74.12	0.81
Isobutanol	74.12	0.802

A-1 Calculated molar ratio of butanol to oleic acid

$$\text{Molecular ratio of butanol to oleic acid} = \frac{N_{BuOH}}{N_{\text{oleic â}}}$$

$$\frac{\text{Mass (g) of oleic â}}{\text{Mw (g/g mol) of oleic â}} = \frac{\text{Mass (g) of butanol}}{\text{Mw (g/g mol) of butanol}}$$

$$\text{Mass of butanol(g)} = \frac{\text{Mass (g) of oleic â}}{\text{Mw (g/g mol) of oleic â}} \times \text{Mw (g/g mol) of butanol}$$

$$\text{Volume of butanol} = \frac{\text{Mass (g) of butanol}}{\text{Density of butanol } (g/cm^3)}$$

A-2 Calculated mass of catalyst

$$\text{Mass of catalyst} = \text{Mass of oleic acid} \times \frac{\text{\% enzyme loading (base on oleic â wt)}}{100}$$

A-3 Analyzed product from esterification

A.3.1. Calculated weight of oleic acid

Weight of oleic acid = Volume of KOH x Concentration of KOH x 0.2824

A.3.2. Calculated % conversion of free fatty acid

$$\% \text{ conversion of free fatty acid} = \frac{\text{Initial acid value} - \text{Acid value at time}}{\text{Initial acid value}} \times 100$$

A-4 Calculated activation energy (E_a)

Example

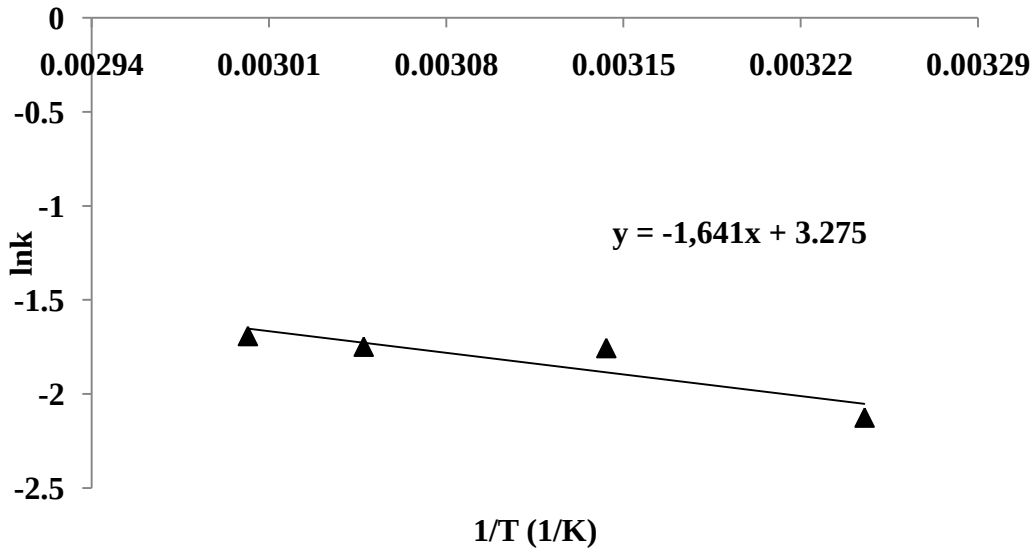

Figure A-1 Second order reaction rate in Arrhenius plot during the esterification of butanol of oleic acid at various temperatures.

From $\ln k = \ln k_0 - E_a/RT$

$\ln k_0 = 3.275$

$k_0 = 26.44$

slope is $-E_a/R = -1{,}641\ h^{-1}(kmol/m^3)^{-1}$

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

$E_a = (-1{,}641) \times (-8.314) = 13{,}643.27$ J/mol = 13.64 kJ/mol

where Ea is the activation energy

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

A is the frequency factor

APPENDIX B

EXPERIMENTAL DATA ANALYSIS

B-1 Experimental data of enzymatic esterification reaction time

Table B.1.1 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; reaction temperature, 45°C; a stirring rate of 250 rpm, and reaction period of 48 h.

Time (h)	% conversion FFA
0	0
1	83.00
3	86.22
6	87.13
12	89.08
24	90.97
36	90.97
48	90.92

Table B.1.2 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 2.5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	48.19	48.46	49.11	48.59	0.47
3	3	81.57	81.34	80.82	81.25	0.38
4	6	85.53	85.55	85.68	85.59	0.09
5	9	86.20	86.53	86.33	86.35	0.17
6	12	86.49	87.07	86.77	86.78	0.29
7	24	86.62	87.07	86.82	86.84	0.23

Table B.1.3 % conversion FFA (1st of cycles) at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	83.36	83.50	82.14	83.00	0.74
3	3	85.45	86.70	86.51	86.22	0.68
4	6	87.27	86.72	87.41	87.13	0.36
5	9	87.91	88.27	88.26	88.15	0.21
6	12	88.97	89.18	89.08	89.08	0.10
7	24	90.76	91.67	90.50	90.97	0.61

Table B.1.4 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 10%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	80.38	81.43	80.09	80.63	0.70
3	3	83.23	84.13	82.88	83.41	0.64
4	6	86.18	88.04	87.57	87.26	0.97
5	9	87.86	88.60	88.08	88.18	0.38
6	12	88.14	88.76	88.61	88.50	0.32
7	24	91.09	90.96	91.18	91.07	0.11

Table B.1.5 % conversion FFA (1^{st} cycles) at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 35°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	63.50	61.97	62.75	62.74	0.76
3	3	79.05	79.58	79.01	79.21	0.32
4	6	82.96	81.97	82.91	82.62	0.56
5	9	84.02	83.96	84.75	84.24	0.44
6	12	86.23	87.22	87.01	86.82	0.52
7	24	88.40	87.79	88.36	88.18	0.34

Table B.1.6 % conversion FFA (1st cycles) at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 55°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	83.51	84.34	84.51	84.12	0.53
3	3	86.74	86.09	85.31	86.05	0.72
4	6	86.33	86.86	85.90	86.34	0.48
5	9	87.36	88.14	87.66	87.72	0.39
6	12	88.58	89.66	88.82	89.02	0.57
7	24	92.09	92.18	92.07	92.11	0.06

Table B.1.7 % conversion FFA (1st cycles) at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 60°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	85.13	85.53	85.07	85.24	0.25
3	3	87.70	87.23	87.20	87.37	0.28
4	6	88.43	88.45	88.04	88.31	0.23
5	9	88.98	89.89	89.60	89.49	0.46
6	12	91.29	91.49	91.76	91.52	0.24
7	24	94.61	94.92	93.96	94.50	0.49

Table B.1.8 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 200 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	73.70	73.47	74.69	73.95	0.65
3	3	86.75	86.80	85.70	86.42	0.62
4	6	86.97	87.34	86.36	86.89	0.49
5	9	87.55	87.73	86.89	87.39	0.44
6	12	89.01	89.11	89.96	89.36	0.52
7	24	89.03	89.19	89.93	89.38	0.48

Table B.1.9 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 300 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	75.59	75.06	75.25	75.30	0.27
3	3	87.15	86.42	86.94	86.84	0.37
4	6	87.28	87.54	87.70	87.51	0.22
5	9	87.13	87.49	88.34	87.67	0.64
6	12	87.67	87.60	87.78	87.68	0.09
7	24	87.76	87.44	87.85	87.68	0.22

Table B.1.10 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 350 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	72.93	74.02	73.57	73.51	0.55
3	3	82.41	83.02	82.59	82.67	0.31
4	6	83.16	84.89	84.58	84.21	0.92
5	9	83.76	84.73	84.60	84.37	0.53
6	12	84.23	85.03	84.96	84.74	0.44
7	24	85.23	86.90	86.06	86.07	0.84

Table B.1.11 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:1; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	72.62	72.90	72.14	72.56	0.39
3	3	78.35	77.66	78.14	78.05	0.36
4	6	79.24	78.86	79.75	79.28	0.45
5	9	79.44	79.19	80.06	79.57	0.44
6	12	79.70	80.53	79.89	80.04	0.43
7	24	80.71	80.92	81.01	80.88	0.15

Table B.1.12 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:3; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	57.07	57.53	57.29	57.30	0.23
3	3	80.69	81.62	80.86	81.06	0.50
4	6	81.27	82.14	82.11	81.84	0.49
5	9	85.05	83.94	85.33	84.77	0.74
6	12	85.59	84.53	85.89	85.34	0.71
7	24	87.70	87.63	88.45	87.92	0.46

Table B.1.13 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:4; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	43.89	45.05	45.41	44.78	0.80
3	3	77.80	78.89	78.59	78.42	0.57
4	6	84.19	83.79	83.92	83.92	0.23
5	9	85.14	85.66	85.37	85.37	0.27
6	12	85.92	86.16	85.97	85.97	0.17
7	24	87.48	87.42	87.60	87.50	0.26

Table B.1.14 % conversion FFA for reusability of enzyme **(**2nd cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 35°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	60.53	59.87	60.68	60.36	0.43
3	3	76.73	77.46	78.21	77.47	0.74
4	6	83.03	83.16	83.22	83.14	0.09
5	9	83.81	83.67	83.47	83.65	0.17
6	12	86.64	86.23	86.40	86.42	0.21
7	24	87.72	88.37	87.31	87.80	0.53

Table B.1.15 % conversion FFA for reusability of enzyme **(**3rd cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 35°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	60.60	59.99	60.48	60.35	0.32
3	3	77.75	76.66	77.89	77.43	0.67
4	6	82.78	83.02	83.56	83.12	0.40
5	9	83.58	83.67	83.31	83.52	0.19
6	12	86.68	86.68	83.48	86.28	0.69
7	24	87.43	87.50	87.85	87.59	0.23

Table B.1.16 % conversion FFA for reusability of enzyme **(**4th cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 35°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	58.22	57.99	58.77	58.33	0.40
3	3	76.84	77.79	77.50	77.37	0.50
4	6	83.35	83.02	82.56	82.97	0.40
5	9	82.59	83.42	83.23	83.08	0.43
6	12	84.49	84.66	84.85	84.66	0.18
7	24	87.93	86.93	86.69	87.18	0.65

Table B.1.17 % conversion FFA for reusability of enzyme **(**5th cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 35°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	54.72	54.88	53.65	54.42	0.67
3	3	77.48	77.28	76.68	77.15	0.42
4	6	82.38	82.78	83.85	83.01	0.76
5	9	84.48	82.82	82.85	83.39	0.95
6	12	84.76	84.97	83.64	84.46	0.72
7	24	85.30	85.31	84.71	85.11	0.34

Table B.1.18 % conversion FFA for reusability of enzyme **(**2nd cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	75.08	75.82	75.76	75.55	0.41
3	3	85.82	86.39	86.32	86.17	0.31
4	6	86.15	86.59	86.48	86.41	0.23
5	9	86.77	87.04	87.14	86.98	0.19
6	12	87.18	87.59	87.70	87.49	0.28
7	24	87.91	87.74	88.17	87.94	0.21

Table B.1.19 % conversion FFA for reusability of enzyme **(**3rd cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	72.89	72.20	72.26	72.45	0.38
3	3	84.62	84.98	84.92	84.84	0.19
4	6	85.96	86.70	86.56	86.41	0.39
5	9	87.07	86.52	87.41	87.00	0.45
6	12	86.85	87.53	87.62	87.33	0.42
7	24	87.67	87.94	87.96	87.85	0.16

Table B.1.20 % conversion FFA for reusability of enzyme **(**4th cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	70.98	69.88	71.28	70.71	0.74
3	3	84.12	84.05	84.14	84.10	0.05
4	6	86.51	87.15	87.13	86.93	0.37
5	9	87.46	86.27	86.88	86.87	0.60
6	12	87.14	87.20	87.00	87.11	0.10
7	24	87.40	87.14	88.39	87.64	0.66

Table B.1.21 % conversion FFA for reusability of enzyme **(**5th cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	69.19	69.03	69.02	69.08	0.10
3	3	84.38	84.42	83.85	84.22	0.32
4	6	85.68	85.55	85.48	85.57	0.10
5	9	86.04	85.97	85.58	85.86	0.25
6	12	85.74	86.96	86.21	86.31	0.62
7	24	86.92	87.97	87.48	87.46	0.53

Table B.1.22 % conversion FFA for reusability of enzyme **(**2nd cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 55°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	69.86	70.59	69.04	69.83	0.78
3	3	85.05	85.89	85.41	85.45	0.42
4	6	84.97	85.92	85.26	85.39	0.49
5	9	84.88	85.98	85.46	85.44	0.55
6	12	85.40	85.82	85.19	85.47	0.32
7	24	86.56	86.03	86.52	86.37	0.30

Table B.1.23 % conversion FFA for reusability of enzyme **(**3rd cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 55°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	58.24	57.42	56.47	57.38	0.89
3	3	81.40	83.10	81.43	81.98	0.97
4	6	83.69	83.08	83.04	83.27	0.36
5	9	84.15	84.95	84.36	84.49	0.41
6	12	84.52	84.77	84.46	84.59	0.16
7	24	84.74	85.14	84.61	84.83	0.27

Table B.1.24 % conversion FFA for reusability of enzyme **(**4th cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 55°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	15.27	17.11	15.54	15.98	0.99
3	3	54.03	53.20	53.11	53.45	0.51
4	6	71.13	70.25	69.63	70.34	0.76
5	9	79.62	78.10	77.77	78.50	0.98
6	12	81.86	80.28	80.32	80.82	0.90
7	24	84.41	82.93	83.65	83.67	0.74

Table B.1.25 % conversion FFA for reusability of enzyme **(**5th cycle**s)** at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 55°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	15.09	15.02	14.21	14.77	0.49
3	3	32.49	31.65	31.84	31.99	0.44
4	6	63.77	63.89	63.03	63.56	0.47
5	9	71.11	69.51	69.63	70.08	0.89
6	12	77.20	77.99	78.27	77.82	0.55
7	24	80.80	79.57	81.00	80.46	0.78

Table B.1.26 % conversion FFA for adding molecular sieve at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				S.D.
		Ex1	Ex2	Ex3	Average	
1	0	0	0	0	0	0
2	1	84.00	83.79	83.95	83.91	0.11
3	3	91.19	91.30	91.10	91.19	0.10
4	6	94.09	93.74	92.48	93.44	0.85
5	9	94.82	94.58	94.67	94.69	0.12
6	12	95.86	96.55	95.11	95.84	0.72
7	24	95.79	96.83	95.64	96.09	0.65

Table B.1.27 % conversion FFA at molar ratio of oleic acid/butanol molar ratio, 1:2; Novozym 435 based on acid weight, 5%; a stirring rate of 250 rpm; reaction temperature, 45°C and reaction period of 24 h.

Sample No.	Time (h)	% conversion FFA				
		Cycle				
		1	2	3	4	5
1	0	0	0	0	0	0
2	1	84.43	78.95	72.04	71.23	70.68
3	3	88.51	86.55	84.81	84.78	84.27
4	6	88.97	86.61	86.65	86.75	85.68
5	9	89.31	86.71	86.87	86.92	85.94
6	12	89.85	87.68	87.04	87.36	86.33
7	24	91.01	88.00	87.87	87.65	87.47

Table B.1.28 % conversion FFA (a) Titration (b) H-NMR

Condition	% conversion FFA	
	Titration[a]	H'NMR[b]
Molar ratio, 1:2; 2.5% enzyme loading; 250 rpm; 45°C	86.84 ± 0.23	86.84
molar ratio, 1:2; 10% enzyme loading; 250 rpm; 45°C	86.84 ± 0.23	86.96
molar ratio, 1:2; 5% enzyme loading; 250 rpm; 35°C	88.18 ± 0.34	88.50
molar ratio, 1:1; 5% enzyme loading; 250 rpm; 45°C	80.88 ± 0.15	78.74
(5^{th} cycles) molar ratio, 1:2; 5% enzyme loading; 250 rpm; 45°C	87.46 ± 0.53	86.96
(5^{th} cycles) molar ratio 1:2; 5% enzyme loading; 250 rpm; 55°C	80.46 ± 0.78	80.65

APPENDIX C

C-1 Data sheet of Novozym 435

Novozym® 435

Valid from **2011-09-14**

Product Characteristics:

Declared enzyme	Lipase
Declared activity	10000 PLU/g
Colour	Off-white Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity.
Physical form	Immobilized Granulate
Approximate density (g/ml)	0.40
Carriers	Acrylic resin
Production organism	Aspergillus niger
Production method	Produced by submerged fermentation of a genetically modified micro organism. The enzyme protein, which in itself is not genetically modified, is separated and purified from the production organism.

Product Specification:

	Lower Limit	Upper Limit	Unit
Propyl Laurate Unit PLU	10000		/g
Loss on Drying 105 C	-	3	%

Packaging: See the standard packaging list for more information.

Recommended Storage:

Best before	When stored as recommended, the product is best used within 6 months from date of delivery.
Storage at customer's warehouse	0-25°C (32°F-77°F)
Storage Conditions	In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement.

Safety and handling precautions

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. Powdered enzymes are readily inhaled and should be handled only with specific precautions to prevent inhalation of dust. All equipment and handling procedures must be designed to control airborne dust. Personal respiratory protection is recommended in all cases where full dust control is not secured . All spills, however minor, should be removed immediately. Use respiratory protection. Major spills should be carefully shovelled into plastic-lined containers. Minor spills and the remains of major spills should be removed by vacuum cleaning or flushing with water (avoid splashing). Vacuum cleaners and central vacuum systems should be equipped with HEPA filters. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes. A Material Safety Data Sheet is supplied with all products. See the Safety Manual for further information regarding how to handle the product safely.

C-2 Data sheet of Molecular sieve 4A

FAX TRANSMITTAL
(Specifications)

Subject: **Specifications of 4A Molecular Sieve**

■ **Introduction**

Molecular Sieve type 4A is an alkali alumino silicate; it is the sodium form of the Type A crystal structure. 4A molecular sieve has an effective pore opening of about 4 angstroms (0.4nm).

type 4A molecular sieve will adsorb most molecules with a kinetic diameter of less than 4 angstroms and exclude those larger. Such adsorbable molecules include simple gas molecules such as oxygen, nitrogen, carbon dioxyde and straight chain hydrocarbons. Branched chain hydrocarbons and aromatics are excluded.

Technovation International Molecular Sieve 4A

■ **Typical Chemical Formula**

$Na_2O \cdot Al_2O_3 \cdot 2SiO_2 \cdot 4.5 H_2O$

■ **$SiO_2 : Al_2O_3 \approx 2$**

■ **Technical Parameter**

Item	Unit	Technical data			
Shape		Pellet		Sphere	
Diameter	mm	1.5-1.7	3.0-3.3	1.7-2.5	3.0-5.0
Size ratio up to grade	%	≥98	≥98	≥96	≥96
Bulk density	g/ml	≥0.68	≥0.68	≥0.68	≥0.68
Wear ratio	%	≤0.20	≤0.20	≤0.20	≤0.20
Crushing strength	N	≥30/cm	≥45/cm	≥30/piece	≥70/piece
Static water adsorption	%	≥22	≥22	≥22	≥22
Static methanol adsorption	%	≥15	≥15	≥15	≥15
Water content, as shipped	%	≤1.5	≤1.5	≤1.5	≤1.5

■ **Application**

■ Drying and removing of CO_2 from natural gas, LPG, air, inert and athmospheric gases, etc.

- Removal of hydrocarbons, ammonia and methanol from gas streams (ammonia syn gas treating)
- Special types are used in the air break units of buses, trucks and locomotives.
- Packed in small bags, it may be used simply as a packaging desiccant.

■ Regeneration

molecular sieve Type 4A can be regenerated by either heating in the case of thermal swing processes; or by lowering the pressure in the case of pressure swing processes.
To remove moisture from a 4A molecular sieve, a temperature of 250-280¡C is required. A properly regenerated molecular sieve can give moisture dew points below -100¡C.
The outlet concentrations on a pressure swing process will depend on the gas present, and on the conditions of the process

■ Size

4A - Zeolites are available in beads of 1-2 mm, (10x18 mesh) 2-3 mm, (8x12 mesh) , 2.5-5 mm, (4x8 mesh) and as powder, and in pellet 1.6¡, 3.2¡.

■ Attention

- To avoid damp and pre-adsorption of organic before running, or must to be reactivated.

Technovation International Co.,Ltd.

29/3 M.2 Nakniwat 48, Ladprao, Ladprao, Bangkok 10230
Tel. 0-2932-8375 Fax. 0-2932-8376

VITAE

Miss Jiranan Chanprasert was born in August 24th, 1987 in Chonburi, Thailand. She finished high school from Chonkanyanukoon School, Chonburi and received Bachelor's Degree in Chemical Engineering from the King Mongkut's Institute of Technology, Ladkrabang in 2009. After that, she continued her Master's Degree in Chemical Engineering at the Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University in 2011.

Publications

Chanprasert, J., and Phisalaphong, M., Butyloleate Production using Immobilized Lipase. The 4th AUN/SEED-Net Regional Conference on Biotechnology, Emerging Biotechnology for Green, 26-27 January 2012 (Bangkok, Thailand).