ภาวะที่เหมาะสมในการตรึงไลเพสจาก *Candida rugosa* เพื่อการผลิตไบโอดีเซล

นางสาว กิ่งแก้ว พิริยะคณานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

OPTIMAL IMMOBILIZATION CONDITIONS

OF LIPASE FROM *Candida rugosa* FOR BIODIESEL PRODUCTION

Miss Kingkaew Piriyakananon

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2008

Copyright of Chulalongkorn University

512048

Thesis Title   Optimal immobilization conditions of lipase
                      from *Candida rugosa* for biodiesel production
By   Miss Kingkaew Piriyakananon
Field of Study   Biotechnology
Advisor   Associate Professor Tikamporn Yongvanich
Co-Advisor   Pakorn Winayanuwattikun, Ph.D.

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

.... Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.... Chairman

(Associate Professor Piamsook Pongsawasdi, Ph.D.)

.... Advisor

(Associate Professor Tikamporn Yongvanich)

.... Co-Advisor

(Pakorn Winayanuwattikun, Ph.D.)

.... Examiner

(Kittinan Komolpis, Ph.D.)

.... External Examiner

(Jittima Charoenpanich, Ph.D.)

กิ่งแก้ว พิริยะคณานนท์ : ภาวะที่เหมาะสมในการตรึงไลเพสจาก *Candida rugosa* เพื่อการผลิตไบโอดีเซล (OPTIMAL IMMOBILIZATION CONDITIONS OF LIPASE FROM *Candida rugosa* FOR BIODIESEL PRODUCTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทิฆัมพร ยงวณิชย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : อ. ดร. ปกรณ์ วินะยานุวัติคุณ, 137 หน้า.

ไบโอดีเซลหรือ เมทิลเอสเทอร์ของกรดไขมัน เป็นแหล่งพลังงานที่ไม่เป็นพิษ สามารถย่อยสลายทางชีวภาพ และสร้างขึ้นใหม่ได้ ในปัจจุบัน การผลิตไบโอดีเซลโดยใช้เอนไซม์ไลเพสเป็นตัวเร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชันมีความน่าสนใจมากขึ้น เนื่องจาก การแยกผลิตภัณฑ์ร่วมกลีเซอรอล และกระบวนการทำให้ไบโอดีเซลบริสุทธิ์ทำได้ง่าย ไลเพสจากเชื้อ *Candida rugosa* (CRL) มีการนำมาใช้อย่างกว้างขวาง แต่เอนไซม์มีราคาแพงจึงเป็นอุปสรรคในการนำมาใช้ ดังนั้นเพื่อเป็นการลดต้นทุน ในงานวิจัยนี้จึงทำการตรึงรูปเอนไซม์ไลเพสจากเชื้อ *Candida rugosa* บนตัวค้ำจุนที่มีสมบัติไฮโดรโฟบิกทั้งหมด 7 ชนิด โดยตัวค้ำจุนที่เลือกได้คือ Sepabeads EC-OD และภาวะที่เหมาะสมสำหรับการตรึง CRL บน Sepabeads EC-OD คือค่าความเป็นกรดด่าง 6, ความแรงของไอออน 500 มิลลิโมลาร์, ปริมาณเอนไซม์ 8 มิลลิกรัมต่อมิลลิลิตร, อุณหภูมิ 30 อาศาเซลเซียส, ระยะเวลาที่ใช้ในการตรึง 30 นาที และใช้เทอร์เชียรีบิวทานอล เป็นสารเพิ่มประสิทธิภาพในการตรึง หลังจากนั้นหาภาวะที่เหมาะสมสำหรับปฏิกิริยาทรานส์เอสเทอริฟิเคชันที่เร่งด้วย CRL ตรึงรูปบน Sepabeads EC-OD ได้ดังนี้ การเติมเมทานอลแบบ 6 ขั้น , อัตราส่วนของเมทานอลต่อน้ำมัน 3 ต่อ 1, ปริมาณเอนไซม์ 30 เปอร์เซ็นต์ ของน้ำหนักน้ำมัน, ปริมาณน้ำ 5 เปอร์เซ็นต์โดยปริมาตรต่อน้ำหนักน้ำมัน, ระยะเวลาในการทำปฏิกิริยา 12 ชั่วโมง ที่อุณหภูมิ 40 องศาเซลเซียส เมื่อคัดเลือกน้ำมันจากเมล็ดพืชที่ไม่ใช้ในการบริโภค และของเหลือใช้ทางการเกษตร พบว่ามีน้ำมัน 6 ชนิดจากทั้งหมด 9 ชนิด ที่มีคุณสมบัติทางกายภาพผ่านมาตรฐาน เพื่อใช้เป็นสารตั้งต้นสำหรับปฏิกิริยาทรานส์เอสเทอริฟิเคชัน โดยเร่งด้วยไลเพสตรึงรูปทางการค้า 2 ชนิด เปรียบเทียบกับ CRL ที่ตรึงรูปบน Sepabeads EC-OD พบว่าน้ำมันจากเมล็ดพืช 3 ชนิด ได้แก่ มะละกอ เงาะ และ สบู่ดำ ให้ผลผลิตไบโอดีเซลที่สูงใกล้เคียงกันประมาณ 80 % เมื่อเร่งด้วยไลเพสตรึงรูปทั้ง 3 ชนิด ขั้นตอนสุดท้ายทดสอบการนำ CRL ตรึงรูปกลับมาใช้ซ้ำทั้งปฏิกิริยาทรานส์เอสเทอริฟิเคชันและไฮดรอลิซิส พบว่าผลผลิตไบโอดีเซลที่ได้ลดลงอย่างมากในการใช้ครั้งที่ 2 และ 3 ในทางตรงกันข้ามพบว่าการทำงานของเอนไซม์ตรึงรูปในปฏิกิริยาไฮดรอลิซิสคงที่หลังใช้แล้ว 10 ครั้ง

จากผลการทดลองทั้งหมด ชี้ให้เห็นว่า CRL ที่ตรึงรูปบน Sepabeads EC-OD ที่ได้ สามารถเร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชัน ในการผลิตไบโอดีเซลได้อย่างมีประสิทธิภาพเทียบเท่ากับเอนไซม์ตรึงรูปทางการค้า

สาขาวิชา.........เทคโนโลยีชีวภาพ......... ลายมือชื่อนิสิต ....

ปีการศึกษา 2551 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก ....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ....

v

4972223423   : MAJOR BIOTECHNOLOGY

KEYWORDS : LIPASES / IMMOBILIZATION / TRANSESTERIFICATION / BIODIESEL

KINGKAEW PIRIYAKANANON: OPTIMAL IMMOBILIZATION CONDITIONS OF LIPASE FROM *Candida rugosa* FOR BIODIESEL PRODUCTION. ADVISOR : ASSOC. PROF.TIKAMPORN YONGVANICH, CO-ADVISOR : PAKORN WINAYANUWUTTIKUN, Ph.D., 137 pp.

Biodiesel or fatty acid methyl ester is a non-toxic, biodegradable and renewable energy source. Recently, biodiesel can be produced by lipase catalyzed transesterification and become more attractive since the by product, glycerol can be easily recovered and the purification process for biodiesel is simpler. *Candida rugosa* lipase (CRL) is one of the most frequently used enzymes. However, the cost of enzyme remains a barrier. To reduce the cost, CRL were immobilized on 7 types of hydrophobic supports and Sepabeads EC-OD was finally selected. Afterwards, various optimal conditions for the immobilization of CRL on Sepabeads EC-OD were investigated. The results were as follows; pH 6, 500 mM ionic strength, 8 mg/ml enzyme loading at 30 °C for 30 min and t-butanol as the adjuvant. The immobilized lipase was later applied for the catalysis of transesterification between palm oil and methanol. The optimal transesterification by the immobilized CRL on Sepabeads EC-OD were investigated. The results were as follows; 6-step addition mode of methanol, 3 to 1 molar ratio of methanol to palm oil, 30% enzyme loading by oil weight and 5% water content (v/w of oil) for12 hours at 40°C. Then, 6 from 9 types of non-edible and waste plant oils were selected from standard physical properties to be used as the substrates for the production of biodiesel by transesteriification catalyzed by 2 commercial immobilized lipase and CRL on SepabeadsEC-OD in comparison. The results showed that seed oils of 3 species; papaya, rambutan and physic nuts could be highly converted to biodiesel in comparable yield when the reactions were catalyzed by all 3 types of enzymes (approximately 80 %). Finally, the enzymes were tested for reusability in both transesterification and hydrolysis. For the biodiesel production, the activity considerably decreased after the 2nd-3rd cycle. In contrast, the relative hydrolytic activities of the immobilized lipase could be well maintained over ten repeated cycles. Overall results indicate that the obtained immobilized CRL on SepabeadsEC-OD can catalyze the transesterification for the production of biodiesel as efficiently as the commercial enzymes.

Field of Study : ......Biotechnology......   Student's Signature Kingkaew Piriyakananon
Academic Year :.........2008............   Advisor's Signature ....
                                            Co-Advisor's Signature Pakorn Winayanuwuttikun

ACKNOWLEDGEMENTS

CONTENTS

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

°C	degree of celcius	min.	minute
cm	centimeter	ml	milliliter
cSt	centistoke	mM	millimolar
et al.	et alibi (latin), and others	mol	mole
g	gram	μg	microgram
hr.	hour	μl	microliter
kg	kilogram	N.D.	non-detectable
L	liter	nm	nanometer
m	meter	ppm	part per million
M	Molar	psi	pound (force) per square inch
max	maximum	s	second
mg	milligram	v/v	volume by volume
min	minimum	w/w, wt	weight by weight

CHAPTER I

INTRODUCTION

1.1 Statement of purpose

The growth in consumption of petroleum oil throughout the world has caused urgent economic, security, and environmental problems. One of the best ways to reduce our dependence on petroleum oil is to develop renewable fuels such as biodiesel (Guan *et al.*, 2008). Biodiesel is a natural substitute of diesel fuel that comes from renewable sources (Rosa *et al.*, 2009), that can be produced from a range of organic feedstock including fresh or waste vegetable oils, animal fats, and oilseed plants (Jeong *et al.*, 2009 and Patil and Deng, 2009). Biodiesel has significantly lower emissions than petroleum-based diesel when it is burned, whether used in its pure form or blended with petroleum diesel. It does not contribute to a net rise in the level of carbon dioxide in the atmosphere and leads to minimize the intensity of greenhouse effect (Antolín *et al.*, 2002 and Vicente *et al.*, 2004). In addition, biodiesel is better than diesel fuel in terms of sulfur content, flash point, aromatic content and biodegradability. Vegetable oils are becoming promising alternative to diesel fuel because they are renewable in nature and can be produced locally and environmental friendly as well. Many researchers have been searching for cheaper plant oils to be used as alternative feedstock for biodiesel production. Few sources have been identified such as waste cooking oil (Wang *et al.*, 2006) and oils from non-edible oil-producing plants such as physic nut (Modi *et al.*, 2007 and Berchmans and Hirata, 2008), cotton seed (Kose *et al.*, 2002, Royon *et al.*, 2007 and Demirbas A., 2008), rubber seeds (Ramadhas *et al.*, 2005) and pumpkin (Schinas *et al.*, 2009).

The general method to produce biodiesel fuel is by transesterification of vegetable oil with methanol in the presence of either alkaline or strong acid catalysts. The transesterification reaction can be represented as

$$
\begin{array}{ccccccc}
\text{CH}_2\text{-OOC-R}_1 & & & & \text{R}_1\text{-COO-R'} & & \text{CH}_2\text{-OH} \\
| & & & \xrightarrow{\text{Catalyst}} & & & | \\
\text{CH-OOC-R}_2 & + & 3\,\text{R'OH} & \rightleftharpoons & \text{R}_2\text{-COO-R'} & + & \text{CH-OH} \\
| & & & & & & | \\
\text{CH}_2\text{-OOC-R}_3 & & & & \text{R}_3\text{-COO-R'} & & \text{CH}_2\text{-OH} \\
\text{Triglyceride} & & \text{Alcohol} & & \text{Alkyl ester} & & \text{Glycerol}
\end{array}
$$
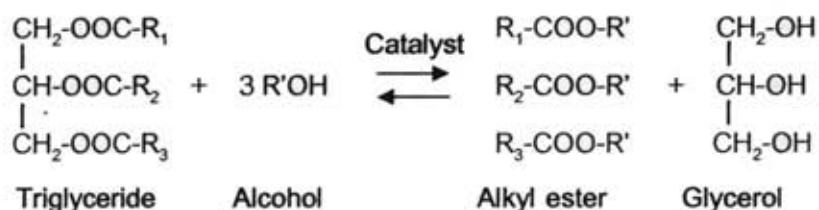
The production of biodiesel by transesterification process employing alkali catalyst has been industrially accepted for its high conversion and reaction rate (Lu *et al.*, 2007 and Shao *et al.*, 2008). However, the reaction has several drawbacks. It is energy intensive and recovery of glycerol is difficult. The acidic or alkaline catalyst has to be removed from the product, alkaline wastewater requires treatment (Zeng *et al.*, 2006), and free fatty acid and water interfere with the reaction. Recently, enzymatic transesterification has attracted much attention for biodiesel production as it produces high purity product and enables easy separation from the byproduct, glycerol (Ranganathan *et al.*, 2007 and Dizge and Keskinler., 2009)

Lipases, known as glycerol ester hydrolases (EC. 3.1.1.3), is one of the most extensively used enzymes that catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids (Chang *et al.*, 2008 and Lei *et al.*, 2009). Depending on the nature of substrate and reaction conditions, lipases can catalyze a wide range of enantio- and regioselective reactions such as hydrolysis, esterifications, transesterifications, aminolysis and ammoniolysis (Deng *et al.*, 2005, Chang *et al.*, 2008, Dizge *et al.*, 2008 and Lei *et al.*, 2009). Lipases are widely spread in plants, animals and microorganisms (Cihangir and Sarikaya, 2004 and Deng *et al.*, 2005). Microbial lipases are most interesting and more useful than lipase derived from plants or animals because of the greater and available varieties of catalytic activities, the possible high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations, and rapid growth of microorganisms on inexpensive media (Ibrahim C.O., 2008 and Fang *et al.*, 2009). Lipases are produced by a widespread number of microorganisms, including bacteria such as *Bacillus* sp. *Pseudomonas* sp. fungi such as *Aspergillus* sp. *Rhizopus* sp. and yeast such as *Candida* sp. Among them, *Candida rugosa* lipase (CRL) is one of the most commonly used (Rahman

et al., 2005) enzyme in organic solvent owing to its high activity in hydrolysis, esterification, transesterification and aminolysis. Due to the wide variety of environmental conditions, lipases are often easily inactivated, difficult to be separated from the reaction system for reuse and high cost. Utilization of lipase as a catalyst for biodiesel production is a clean technology due to its non-toxic and environmental friendly nature. Byproduct glycerol, can be easily recovered without complex processing and free fatty acid contained in waste oils and fats can be completely converted to methyl ester (Mamoru *et al.*, 2001 and Kösa *et al.*, 2002) Only mild operating conditions are required compared with chemical method (Wang *et al.*, 2006). However, due to the wide variety of environmental conditions, lipases are often easily inactivated, difficult to be separated from the reaction system for reuse resulting in higher cost than alkaline or acidic catalysts. Consequently, the further industrial applications of lipases are limited. The synthetic utility of lipase can be greatly improved by immobilization which has become a widely used technique to overcome practical problem in the use of crude lipase (Lei *et al.*, 2009). Many methods have been developed for immobilization of enzymes onto supports. Physical methods, especially adsorption may have a higher commercial potential than other methods because adsorption is simple, less expensive, and high catalytic activity can still be retained (Gitlesen *et al.*, 1997, Xu *et al.*, 2006 and Chang *et al.*, 2007). From transesterification process, by product glycerol is hydrophilic and insoluble in the oil so it is easily adsorbed on to the surface of the immobilized lipase leading to negative effect on lipase activity and operational stability (Wang *et al.*, 2006). Therefore, one way to address this problem, the enzyme can be applied on hydrophobic carriers such as macroporous polymers by adsorption due to the special characteristics of lipases, for example, avoids product contamination and allows biocatalyst recovery, reuse and continuous operation, enzyme activity stability, thermal stability and operational lifetime of lipase can be enhanced (Yang *et al.*, 2006). One account of the relatively high surface hydrophobicity of lipase, simple adsorption of lipase on suitable hydrophobic support has been the more popular strategy over covalent conjugation methods (Petkar *et al.*, 2006). There are varieties of support materials such as chitosan (Amorim *et al.*, 2003, Feresti and Ferreira, 2007 and Ye *et al.*, 2007), silica (Blanco

et al., 2004 and Blanco *et al.*, 2007), $CaCO_3$ (Rosu *et al.*, 1998 and Ghamgui *et al.*, 2004) that can be used for lipase immobilization and greatly improved the stability of enzyme. They also showed great potential for the production of biodiesel with the 70.2% conversion rate remained after 19 consecutive batches of reusages (Yang *et al.*, 2006). When an immobilization of *Candida rugosa* lipase by adsorption on bentonite was studied (Yeşiloğlu Y, 2005), the half –life of the immobilized enzyme was about 45 min, whereas for the soluble free lipase was 17 min at 50°C. Moreover, previous studies have shown that many factors can affect the activity recovery and reusability of enzymes in immobilization process. Some of the most important factors are the properties of the enzyme molecule, concentration of enzyme, temperature, ionic strength, pH, choice of support, the selection of an immobilization strategy, water and solutes present (Cruz *et al.*, 2009).

Since the immobilization efficiency of the enzyme depends on various factors, this study focussed on the optimal conditions for immobilization of lipase from *Candida rugosa* on hydrophobic supports for the transesterication of palm oil with methanol. Subsequently, the optimal conditions (addition mode, molar ratio of oil:methanol, enzyme loading, water content, time and temperature) by the optimized immobilized lipases for the conversion to biodiesel were investigated.

1.2 Objectives of this research

The aim of this study was to investigate the optimal conditions for immobilization of lipase from *Candida rugosa* on hydrophobic support for the production of biodiesel.

1.3 Scopes of the investigation

1.3.1 To select the appropriate hydrophobic support for the immobilization

1.3.2 To determine the optimal conditions for the immobilization

1.3.3 To select the potential feedstocks from non-edible and waste plant oils to be used as the optimal substrates for the production of biodiesel by transesterification.

1.3.4 To determine the optimal conditions for transesterification catalyzed by obtained immobilized lipase for the production of biodiesel

1.3.5 To compare the yield of biodiesel from transesterification catalyzed by 2 commercial lipases, Novozyme435 and Lipozyme RMIM with the obtained immobilized *Candida rugosa* lipase

1.3.6 To determine the stability of immobilized lipase

1.4 Expected results

This research will provide potential feedstocks from non-edible and waste plant oils to be used as the optimal substrates for the production of biodiesel by transesterification. The present work also offers the type of hydrophobic support suitable for the optimal immobilization of lipase from *Candida rugosa*. The yield of biodiesel from optimized transesterification will also be compared with the commercial lipases.

1.5 Thesis organization

This thesis consists of five chapters as follows: Chapter 1 is the introduction. Chapter 2 gives the theoretical and literature reviews. Chapter 3 comprises material and methods. The results can be found in Chapter 4 and the final chapter contains the discussion and conclusion.

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 BIODIESEL

The raw materials for the production of biodiesel, fatty acid alkyl ester, include biological sources such as vegetable oil, animal fat and recycle cooking grease or oil. Biodiesel can be produced by several processes and the property is similar to petrodiesel as shown in Table 2-1.

Table 2-1. Comparative properties of diesel and biodiesel

Fuel properties	Diesel	Biodiesel
Fuel Standard	ASTM D975	ASTM D6751
Lower Heating Value, Btu/gal	~129,050	~118,176
Kinematic Viscosity, at $40^{\circ}C$	1.3 - 4.1	4.0 – 6.0
Specific Gravity Kg/l, at $60^{\circ}F$	0.85	0.88
Density, lb/gal, at $15^{\circ}C$	7.079	7.328
Water and Sediment, vol%	0.05 max	0.05 max
Carbon, wt%	87	77
Hydrogen, wt%	13	12
Oxygen, by dif. wt%	0	11
Sulfur, wt%	0.05 max	0.0 to 0.0024
Boiling Point, $^{\circ}C$	180 to 340	315 to 350
Flash Point, $^{\circ}C$	60 to 80	100 to 170
Cloud Point, $^{\circ}C$	-15 to 5	-3 to 12
Pour Point, $^{\circ}C$	-35 to -15	-15 to 10
Cetane Number	40 - 55	48 – 65
Lubricity SLBOCLE, grams	2000-5000	>7,000
Lubricity HFRR, microns	300 – 600	<300

From Dwivedi *et al.*, 2006

2.1.1 Sources of raw materials

The feedstocks employed in biodiesel production are generally classified into vegetable oils, animal fats, and waste oils (Jeong et al., 2009). Only oils from plants which are renewable, potentially inexhaustible source of energy and abundant or can be collected in a large amount with an appreciable quantity of oil are feasible to use for biodiesel preparation (Winayanuwattikun et al., 2008).

Commonly accepted biodiesel raw materials include the oils from soybean (Watanabe et al., 2002, Noureddini et al., 2005 and Rosa et al., 2008), canola (Dizge and Keskinler, 2008), rapeseed (Kamini and Lefuji, 2001), sunflower (Antolín et al., 2002, Rashid et al., 2008 and Dizge et al., 2009) and palm oil. Moreover, there are several non- edible and waste plant oil seed species, such as physic nut (Tamalampudi et al., 2008, Tiwari et al., 2007 and Oliveira et al., 2009), cotton seed (Köse et al., 2002 and Qian et al., 2008), pumpkin (Winayanuwattikun et al., 2008 and Schinas et al., 2009) etc., which could be utilized as sources for production of oil.

2.1.2 The production of biodiesel

Previous studies have shown that triglycerides hold promise as alternative diesel engine fuels. Some natural glycerides contain higher levels of unsaturated and saturated fatty acids. They can not be used as fuel in a diesel engine in their original from. The high viscosity, acid composition, and free fatty acid content of such oil, as well as gum formation due to oxidation and polymerization during storage and combustion, carbon deposits, and lubricating oil thickening are some of the more obvious problems (Darnoko et al., 2000, Komers et al., 2001 and Demirbas A., 2003). Consequently, considerable effort has gone into developing vegetable oil derivatives that approximate the properties and performance of hydrocarbon-based diesel fuels. Problems encountered in substituting triglycerides for diesel fuels are mostly associated with their high viscosity, low volatility and

polyunsaturated character. There are various ways to produce biodiesel (Demirbas A., 2009) as follows:

2.1.2.1 Direct use and blending

Vegetable oil can be directly mixed with diesel fuel and may be used for running an engine. The blending of vegetable oil with diesel fuel were experimented successfully by various researchers. A diesel fleet was powered with a blend of 95% filtered used cooking oil and 5% diesel in 1982. A blend of 20% oil and 80% diesel was found to be successful (Narayan *et al.*, 1996). It has been proved that the use of 100% vegetable oil was also possible with some minor modifications in the fuel system. The high fuel caused the major problems associated with the use of pure vegetable oils as fuel viscosity in compression ignition engines. Micro-emulsification, pyrolysis and transesterification are the remedies used to solve the problems encountered due to high fuel viscosity (Ramadhas *et al.*, 2004).

2.1.2.2 Pyrolysis

Pyrolysis refers to chemical change caused by application of heat to get simpler compounds from a complex compound. The process is also known as cracking. Vegetable oils can be cracked to reduce viscosity and improve cetane number. The products of cracking include alkanes, alkenes, and carboxylic acids. Soyabean oil, cottonseed oil, rapeseed oil and other oils are successfully cracked with appropriate catalysts to get biodiesel (Ma and Hanna, 1999). By using this technique good flow characteristics were achieved due to reduction in viscosity. Disadvantages of this process include high equipment cost and need for separate distillation equipment for separation of various fractions. Also the product obtained was similar to gasoline containing sulfur which makes it less eco-friendly (Ma and Hanna, 1999)

2.1.2.3 Microemulsion

To solve the problem of high viscosity of vegetable oil, micro emulsions with solvents such as methanol, ethanol (Demirbas A., 2009), propanol (Ranganathan et al., 2008) and butanol have been used. A micro emulsion is defined as the colloidal equilibrium dispersion of optically isotropic fluid microstructures with dimensions generally in the range of 1–150 nm formed spontaneously from two normally immiscible liquids and one or more ionic or non-ionic amphiphiles. These can improve spray characteristics by explosive vaporization of the low boiling constituents in the micelles. All micro emulsions with butanol, hexanol and octanol will meet the maximum viscosity limitation for diesel engines.

2.1.2.4 Transesterification

The most popular method of producing biodiesel is the transesterification of vegetable oils. Biodiesel obtained by transesterification process is a mixture of mono-alkyl esters of higher fatty acids. Transesterification is the alcoholysis of triglyceric esters resulting in a mixture of mono-alkyl esters and glycerol and the sequence of processes. The high viscosity component, glycerol, is removed and hence the product has low viscosity like the fossil fuels. The mixture of these mono-alkyl esters can therefore be used as a substitute for fossil fuels. The transesterification process can be done in a number of ways such as using an alkali catalyst, acid catalyst, biocatalyst, heterogeneous catalyst or using alcohols in their supercritical state. In the alkali process, sodium hydroxide (NaOH) or potassium hydroxide (KOH) (Leung and Guo, 2006, Sharma and Singh, 2008 and Tiwari et al., 2007) is used as a catalyst along with methanol or ethanol. Initially, during the process, alcoxy is formed by reaction of the catalyst with alcohol and the alcoxy is then reacted with any vegetable oil to form biodiesel and glycerol. Glycerol, being denser settles at the bottom and

biodiesel can be decanted. This process is the most efficient and least corrosive of all the processes and the reaction rate is reasonably high even at a low temperature of 60 $^{\circ}$C. There may be risk of free acid or water contamination and soap formation is likely to take place which makes the separation process difficult (Ma and Hanna, 1999; Fukuda et al., 2001; Barnwal and Sharma, 2005). The second conventional way of producing biodiesel is the application of an acid catalyst instead of a base. Any mineral acid can be used to catalyze the process. The most commonly used acids are sulfuric acid (Ramadhas et al., 2004, Ghadge and Raheman, 2005, Tiwari et al., 2007 and Guan et al., 2009), sulfonic acid (Guerreiro et al., 2006) and ferric sulfate (Wang et al., 2007). Although yield is high, the acids, being corrosive, may cause damage to the equipment and the reaction rate was also observed to be low.

It has been recently found that enzymes such as lipase can be used to catalyze transesterification process by immobilizing them in a suitable support. The advantage of immobilization is that the enzyme can be reused without separation. Also, the operating temperature of the process is low (approximately 50 $^{\circ}$C) compared to other techniques as shown in Table 2-2.

Table 2-2. Comparison between alkali-catalyzed and lipase-catalyzed methods for biodiesel production

	alkali-catalyzed process	lipase-catalyzed process
Reaction temperature	60-70 $^{\circ}$C	30-40 $^{\circ}$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

From Fukuda *et al.*, 2001

2.2 Lipase

Lipase, triacylglycerol acylhydrolases (EC 3.1.1.3), constitutes a group of enzyme whose natural catalytic function is to hydrolyze ester bonds in tri-, di-, and monoglycerides (Villeneuve *et al.*, 2000, Cihangir and Sarikaya, 2004 and Châabouni *et al.*, 2008) and variety of compounds containing carboxylic ester moieties that are not acylglycerols. Lipase and phospholipase natural substrates are insoluble in water and their activity use maximum only at the lipid-water interface (Villeneuve *et al.*, 2000). This unique property known as interfacial activation may differ for each specific lipase. From the EC number of lipase, 3.1.1.3, whose components indicate the following groups of enzymes:

E.C.3.	Hydrolases
E.C.3.1.	Acting on ester bonds
E.C.3.1.1.	Carboxylic ester hydrolases
E.C.3.1.1.3	Triacylglycerol lipases

2.2.1 Source of lipase

Lipases from a large number of plant, animal and microorganism (Villeneuve *et al.*, 2000, Deng *et al.*, 2005 and Ibrahim, C.O., 2008) sources have been purified

to homogeneity. Lipases isolated from different sources have a wide range of properties depending on their sources with respect to positional specificity, fatty acid specificity, thermostability, pH optimum, etc (Hasan *et al.*, 2006). Microbial enzymes are often more useful than enzymes derived from plants or animals (Ibrahim, C.O., 2008) because of their production is more convenient and safer, the great variety of catalytic activities available, the high yields possible, tend to have neutral or alkaline pH optima, ease of genetic and environmental manipulation to increase the yield of cells, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media (Hasan *et al.*, 2006).

2.2.2 Lipase produced by microorganisms

Both intracellular and extracellular lipases are produced by microorganism. Lipases are produced by a widespread number of microorganisms, bacteria, fungi and yeast. In particular, lipases produced by bacteria such as *Pseudomonas sp* (Karadzic et al., 2006). *Burkholderia cepacia* and fungi belonging to the genera *Penicillium*, *Rhizopus* and *Rhizomucor* and yeast such as *Candida sp.* are well-known industrial lipase producers.

The yeast *Candida rugosa* (previously named *Candida cylindracea*) is a major source of commercial lipase (CRL) broadly employed by various laboratories and industrial applications and classified as a non-specific lipase with respect to the position of the fatty acid chain released from the glycerol molecule. However, recently it has been recognised that CRL is indeed a heterogeneous mixture of different proteins since *C. rugosa* synthesises and secretes multiple lipase isoenzymes differing in biochemical properties and in substrate specificity (Maria and Gago, 1999).

2.2.3 Lipase property

Lipolytic reactions occur at the lipid–water interface, implying that the kinetics cannot be described by Michaelis–Menten equations, as these are valid

only if the catalytic reaction takes place in one homogenous phase. Lipolytic substrates usually form equilibrium between monomeric, micellar and emulsified states, resulting in the need for a suitable model system to study lipase kinetics. The monolayer technique has been used extensively and, more recently, an oil-drop technology has been put forward in which lipase kinetics are monitored by automatic analysis of the profile of an oil drop hanging in water; the decrease in the interfacial tension between the oil and water caused by lipase hydrolysis is measured as a function of time. The best-known phenomenon emerging from early kinetic studies of lipolytic reactions became known as 'interfacial activation', describing the fact that the activity of lipases is enhanced towards insoluble substrates that form an emulsion. Lipases, in contrast to esterases, were therefore defined as carboxylesterases acting on emulsified substrates.

2.2.4 Enzymatic reaction of lipase

Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface and do not hydrolyze dissolved substrates in the bulk fluid (Vakhlu *et al.*, 2005). A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases. Lipases display little activity in aqueous solutions containing soluble substrates (Sharma *et al.*, 2001).

Lipases have been used in numerous applications of which the most important one is in the production of fine chemicals and oleochemicals. The products include fatty acids from hydrolysis of lipids, esters or glycerides via esterification reactions and modified lipids and structural triglycerides via the transesterification reactions, namely acidolysis, alcoholysis and interesterification (Ibrahim C.O., 2008) (Figure 2-1).

2.2.4.1 Hydrolysis

$$\underset{\substack{\|\\R\text{-}C\text{-}O\text{-}R'}}{\overset{O}{}} + H_2O \longrightarrow \underset{\substack{\|\\R\text{-}C\text{-}OH}}{\overset{O}{}} + HO\text{-}R'$$

2.2.4.2 Esterification

$$\underset{\substack{\|\\R\text{-}C\text{-}O\text{-}R'}}{\overset{O}{}} + HO\text{-}R' \longrightarrow \underset{\substack{\|\\R\text{-}C\text{-}O\text{-}R'}}{\overset{O}{}} + H_2O$$

2.2.4.3 Transesterification

2.2.4.3.1 Acidolysis

$$\underset{\substack{\|\\R_1\text{-}C\text{-}O\text{-}R_1'}}{\overset{O}{}} + \underset{\substack{\|\\R_2\text{-}C\text{-}OH}}{\overset{O}{}} \longrightarrow \underset{\substack{\|\\R_2\text{-}C\text{-}O\text{-}R_1'}}{\overset{O}{}} + \underset{\substack{\|\\R_2\text{-}C\text{-}OH}}{\overset{O}{}}$$
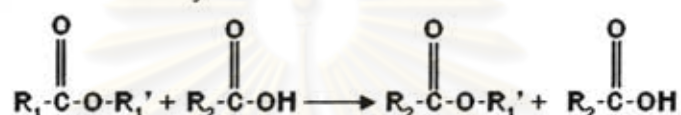
2.2.4.3.2 Alcoholysis

$$\underset{\substack{\|\\R_1\text{-}C\text{-}O\text{-}R_1'}}{\overset{O}{}} + HO\text{-}R_2 \longrightarrow \underset{\substack{\|\\R_1\text{-}C\text{-}O\text{-}R_2'}}{\overset{O}{}} + HO\text{-}R_1$$

2.2.4.3.3 Interesterification

$$\underset{\substack{\|\\R_1\text{-}C\text{-}O\text{-}R_1'}}{\overset{O}{}} + \underset{\substack{\|\\R_2\text{-}C\text{-}O\text{-}R_2}}{\overset{O}{}} \longrightarrow \underset{\substack{\|\\R_1\text{-}C\text{-}O\text{-}R_2'}}{\overset{O}{}} + \underset{\substack{\|\\R_2\text{-}C\text{-}O\text{-}R'_1}}{\overset{O}{}}$$

2.2.4.3.4 Aminolysis

$$\underset{\substack{\|\\R_1\text{-}C\text{-}O\text{-}R_1'}}{\overset{O}{}} + H_2N\text{-}R_2 \longrightarrow \underset{\substack{\|\\R_1\text{-}C\text{-}NH\text{-}R_2'}}{\overset{O}{}} + HO\text{-}R'_1$$
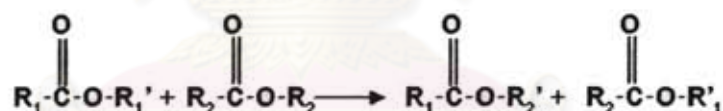
Figure 2-1 Different reactions catalyzed by lipase in aqueous and non-aqueous solutions.

Due the wide variety of environmental condition, lipases are often easily inactivated and difficult to be separated from the reaction system for reuse. Consequently, the further industrial applications of lipase are limited. By an appropriate choice of the immobilization process, operational costs of industrial

processes involving lipase can be significantly reduced (Hung *et al.*, 2003 and Chang *et al.*, 2008)

2.2.5 Applications of lipases

Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals (Cihangir and Sarikaya, 2004, Dandavate and Madamwar, 2007 and Rajendran *et al.*, 2008). Lipase can be used to accelerate the degradation of fatty waste (Masse *et al.*, 2001) and polyurethane.

2.3 Immobilization

Research and development work provided a bewildering array of support materials and methods for immobilization. Much of the expansion may be attributed to developments provide specific improvements for a given application. Surprisingly, there have been few detailed and comprehensive comparative studies on immobilization methods and supports. Therefore, no ideal support material or method of immobilization has emerged to provide a standard for each type of immobilization. Selection of support material and method of immobilization is made by weighing the various characteristics and required features of the enzyme and cell application against the properties, limitations, characteristics of the combined immobilization support. A number of practical aspects should be considered before embarking on experimental work to ensure that the final immobilized enzyme or cell preparation is fit for the planned purpose or application and will operate at optimum effectiveness. This part provides some background to assist in choice evaluation for support and method of immobilization.

2.3.1 Choice of support

The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. Ideal support properties

include physical resistance to compression, hydrophilicity, inertness toward
enzymes ease of derivatization, biocompatibility, resistance to microbial attack, and
availability at low cost.

Supports can be classified as inorganic and organic according to their
chemical composition (Table 2-3). The organic supports can be subdivided into
natural and synthetic polymer.

Table 2-3. Classification of supports

Organic

Natural polymers

- Polysaccharides: cellulose, dextrans, agar, agarose, chitin, alginate

- Protein: collagen, albumin

- Carbon

Synthetic polymers

- Polystyrene

- Other polymer: polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl
  and allyl-polymers

Inorganic

Natural minerals: bentonite, silica

Processed materials: glass (nonporous and controlled pore), metals, controlled pore metal oxides

---

The physical characteristics of the matrices (such as mean particle diameter,
swelling behavior, mechanical strength, and compression behavior) will be of major
importance for the performance of the immobilized. In particular, pore parameters

and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Nonporous supports show few diffusional limitations but have a low loading capacity (Öztürk *et al.*, 2007). Therefore, porous supports are generally preferred because of the greater protection from the environment. Porous supports should have a controlled pore distribution in order to optimize capacity and flow properties. In spite of the many advantages of inorganic carriers (e.g., high stability against physical, chemical, and microbial degradation), most of the industrial applications are performed with organic matrices.

2.3.2 Methods of enzyme immobilization

Enzyme immobilization technology may be an effective means to perform enzyme reuse and to improve its activity and stability (Chang *et al.*, 2008). A number of method for immobilization of enzymes have been reported in the literatures review such as adsorption onto an insoluble material, covalent linking to an insoluble carrier, entrapping enzymes within the matrix and encapsulation in gel bead. The relative merits each are discussed briefly below.

2.3.2.1 Adsorption

Immobilization by adsorption is the simplest method and involves reversible surface interactions between enzyme/cell and support material. The forces involved are mostly electrostatic, such as Van der Waals interactions, hydrophobic interactions, hydrogen bonds, ionic bonds (Villeneuve *et al.*, 2000), although hydrophobic bonding can be significant. These forces are very weak, but sufficiently large in number to enable reasonable binding. For example, it is known that yeast cells have a surface chemistry that is substantially negatively charged so that use of a positively charged support will enable immobilization. Existing surface chemistry between the enzyme/cell and support is utilized so no chemical activation/modification is required and little damage is normally done to

enzyme or cells in this method of immobilization. The procedure consists of mixing together the biological components and a support with adsorption properties, under suitable conditions of pH, ionic strength and others, for the period of incubation, followed by collection of the immobilized material and extensive washing to remove nonbound biological components.

2.3.2.2 Covalent linkage

This method of immobilization involves the formation of a covalent bond between the enzyme and support material. The bond is normally formed between functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of the enzyme. A number of amino acid functional groups are suitable for participation in covalent bond formation. Those that are most often involved are the amino group (NH_2) of lysine or arginine, the carboxyl group (CO_2H) of aspartic acid or glutamic acid, the hydroxyl group (OH) of serine or threonine, and the sulfydryl group (SH) of cysteine.

It is important to choose a method that will not inactivate the enzyme by reacting with amino acids at the active site. So, if an enzyme employs a carboxyl group at the active site for participation in catalysis, it is wise to choose a reaction that involves amino groups for the covalent bond with the support. Chemical methods of crosslinking normally involve covalent bond formation between the enzyme by means of a bi- or multifunctional reagent, such as glutaraldehyde toluene and diisocyanate. However, the toxicity of such reagents is a limiting factor in applying this method to living cell and many enzymes.

2.3.2.3 Entrapment

Immobilization by entrapment differs from adsorption and covalent binding in that enzyme molecules are free in solution, but restricted in movement by the lattice structure of a gel. The porosity of the gel lattice is controlled to ensure that the structure is tight enough to prevent leakage of enzyme or cell, yet at the same time allow free movement of substrate and product. Inevitably, the support will act as a barrier to mass transfer, and although this can have serious implications for reaction kinetics, it can have useful advantages since harmful cells, protein and enzyme are prevented from interaction with the immobilization biocatalyst. Entrapment can be achieved by mixing an enzyme with a polyionic polymer material and then cross linking the polymer with multivalent cations in an ion-exchange reaction to from a lattice structure that traps the enzyme or cell (ionotropic gelation). Alternatively, it is possible to mix the enzyme with chemical monomers that are then polymerized to from a cross linked polymeric network, trapping the enzyme in the interstitial spaces of the lattice. The latter method is more widely used, and a number of acrylic monomers are available for the formation of hydrophilic copolymers.

2.3.2.4 Encapsulation

Encapsulation of enzyme and or cell can be achieved by enveloping the biological components within various forms of semi permeable membranes. It is similar to entrapment in that the enzymes and cell are free in solution, but restricted in space. Large proteins or enzymes cannot pass out of or into the capsule, but small substrates and products can pass freely across the semipermeable membrane. Many materials have been used to construct microcapsules varying from 10-100 μm in diameter; for example, nylon and cellulose nitrate have proven popular. The problems associated with diffusion are more acute and many result in rupture of the membrane if

products from a reaction accumulate rapidly. A further problem is that the immobilized cell or enzyme particle may have a density fairly similar to that of the bulk solution with consequent problems in reactor configuration, flow dynamics, and so on.

Since most enzymes applied for industrial uses need to be immobilized in order to be reusable and therefore reduce the cost of the operation, there are many methods to immobilize enzyme. Each method provides different advantages and disadvantages depending upon its nature as shown in Table 2-4.

Table 2-4. Comparative methods for the immobilization of enzyme

Characteristics	Adsorption	Covalent binding	Entrapment	Membrane confinement
Preparation	Simple	Difficult	Difficult	Simple
Cost	Low	High	Moderate	High
Binding force	Variable	Strong	Weak	Strong
Enzyme leakage	Yes	No	Yes	No
Applicability	Wide	Selective	Wide	Vary wide
Running problems	High	Low	High	High
Matrix effects	Yes	Yes	Yes	No
Large diffusional barriers	No	No	Yes	Yes
Microbial protection	No	No	Yes	Yes

Reference: http://www.org /ippagele/ipdate/2004/05/file/e200405-1101.pdf

After immobilization of lipase, changes were observed in enzyme activity, optimum pH, affinity to substrate and stability.

2.4 Literature reviews

The extent of immobilization changes depended on the source of enzyme, the type of support and the method of immobilization. (Bayramoğlu and Arica, 2008)

2.4.1 Review of lipase immobilization

From previous literature, studies on different support materials and protocols for lipase immobilization and the effects of operational conditions on the enzyme activity and enantioselectivity are also explored.

Takaç and Bakkal immobilized *Candida rugosa* lipase on Amberlite XAD 7 and immobilization under the best reaction conditions in achieving high activity. The authors found that pH 6 and 45°C were obtained for immobilization *C.rugosa* lipase adsorbed on Amberlite XAD 7 was used in the hydrolysis of racemic Naproxen methyl ester (Takaç and Bakkal, 2007).

Huang and Cheng immobilized alkali lipase from *Penicillium expansum* on biomodal ceramic foam and determined on the preferable immobilization conditions. The optimal conditions for immobilization of lipase were found at pH 8, 12 g lipase/g support, 4 hr immobilizing time and $20\,^{\circ}$C immobilizing temperature (Huang and Cheng, 2008).

Chang *et al.* immobilized *Candida rugosa* lipase by adsorption on Celite. The immobilization conditions and characterization of the immobilized enzyme were investigated. The optimum immobilization conditions were as follows: immobilization time 59.1 min, immobilization temperature $10.7\,^{\circ}$C and enzyme support ratio 0.5 (w/w): the highest specific activity obtained was 18.16 U/mg-protein with activity yield of 34.1% (Chang *et al.*, 2007).

Yeşiloğlu immobilized *Candida rugosa* lipase by non-covalently on bentonite. The authors found that the optimum pH of bentonite-immobilized lipase was 7.5, which is slightly higher than the free enzyme, the value of the pH 7.0 (Yeşiloğlu Y., 2005).

Gitlesen *et al.* immobilized different lipases by adsorption on polypropylene powder. The adsorption of lipase from *Candida rugosa* lipase was better than other sources of lipase. Adsorption of a crude lipase from *Candida rugosa* was fast and equilibrium was reached in 30 and 100 min for protein and lipase activity adsorption respectively (Gitlesen *et al.*, 1997).

Lei *et al.* immobilized porcine pancreas lipase by covalent on magnetic microspheres. The authors found that the activity yield was up to 63% ($\pm 2.3\%$) and enzyme loading of 39 (± 0.5) mg/g support. The resulting immobilized lipase had higher optimum temperature compared with those of free lipase and exhibited better thermal, broader pH stability and excellent reusability (Lei *et al.*, 2009).

Yang *et al.* immobilized lipase from *Candida* sp. 99-125 by physical adsorption onto macroporous resins (such as NKA-9, AB-8, H103 and D4020). They reported that NKA-9 was an appropriate support because 98.98% degree of immobilization was obtained. As lipase was adsorbed on NKA in heptane to produce biodiesel, the batch conversion rate reached 97.3% when the three step methanolysis protocol was used (Yang *et al.*, 2006).

Ye *et al.* immobilized lipase from *Candida rugosa* by adsorption and chemical bonding onto chitosan-modified poly (acrylonitrile-co-maleic acid) membrane surface. The activity retention of the immobilized lipase on the chitosan-tethered membrane by adsorption (54.1%) is higher than that by chemical bonding (44.5%) (Ye *et al.*, 2006).

Feresti and Ferreira studied the immobilized lipase from *Candida rugosa*, *Pseudomonas fluorescens* and *Candida antarctica* B onto chitosan and glutaraldehyde pretreated chitosan powder. The prepared biocatalysts were assayed in the direct esterification of oleic acid and ethanol to produce the ethyl oleate. The immobilization of lipase from *Candida antarctica* B onto untreated chitosan powder led to 75% conversion of the fatty acid in 24 hr of reaction. They concluded that *Candida antarctica* B was the most active (Feresti and Ferreira, 2007).

2.4.2 Review of transesterification catalyzed by lipase

Authors/year	Oil/enzyme	Acyl acceptor	Conversion (%)	Technique employed
Watanabe et al. (2000)	Vegetable oil, Novozyme 435	Methanol	90-93	Stepwise addition of methanol
Samukawa et al. (2000)	Soybean oil, Novozyme 435	Methanol	97	Stepwise addition methanol and preincubation of enzyme in methyl oleate and soyabean oil
Ban et al. (2001)	Vegetable oil, Rhizopus oryzae	Methanol	90	Stepwise addition of methanol and application of glutaraldehyde for stability of enzyme
Iso et al. (2001)	Triolein, Pseudomonas flourescens	Butanol	90	Butanol was used as an acyl acceptor and no solvent was used
Shimada et al. (2002)	Waste cooking oil, Novozyme 435	Methanol	90	Stepwise addition of methanol
Bako et al. (2002)	Sunflower oil, Novozyme 435	Methanol	97	Stepwise addition of methanol and removal of glycerol by dialysis
Du et al. (2004)	Soy bean oil, Novozyme 435	Methyl acetate	92	A novel acyl acceptor, methyl acetate which had no inhibitory effects was used

Authors/year	Oil/enzyme	Acyl acceptor	Conversion (%)	Technique employed
Xu *et al.* (2004)	Soy bean oil, Novozyme 435	Methanol	98	Stepwise addition of methanol and removal of glycerol using the solvent, iso-propanol
Li *et al.* (2006)	Rapeseed oil, Novozyme 435 & Lipozyme TL IM	Methanol	95	Combined use of Lipozyme TL IM and Novozyme 435 along with tert-butanol as solvent
Royon *et al.* (2007)	Cotton seed oil, Novozyme 435	Methanol	97	tert-Butanol was used as a solvent
Modi *et al.* (2007)	Jatropha oil, Novozyme 435	Ethyl acetate	91.3	Ethyl acetate having no inhibitory effects was used
Hama *et al.* (2007)	Soy bean oil, *Rhizopus oryzae*	Methanol	90	Stepwise addition of methanol in a packed bed reactor
Dizge and Keskinler (2009)	Canola oil, *Thermomyces lanuginosus*	Methanol	90	Stepwise addition of methanol
Lu et al. (2008)	Glycerol trioleate, *Candida* sp. 99-125	Methanol & CCl_4	90.58	Three step methanolysis in different solvents systems
Dizge *et al.* (2009)	Sunflower oil, *Thermomyces lanuginosus*	Methanol	97	Stepwise addition of methanol and flow rate of substrate

Authors/year	Oil/enzyme	Acyl acceptor	Conversion (%)	Technique employed
Halim, S.F.A. and Kamaruddin, A.H. (2008)	Waste cooking palm oil, Novozyme 435	Methanol & *tert*-butanol	88	Optimal condition in batch system
Watanabe *et al.* (2002)	Soybean oil, *Candida antarctica*	Methanol	93.8	Stepwise addition of methanol
Lu *et al.* (2007)	Lard, *Candida sp.* 99-125	Methanol	87.4	n-hexane was used as a solvent
Kōsa *et al.* (2002)	Cotton seed, *Candida antarctica*	Isoamyl alcohol	94	New alcohol type on the alcoholysis
Noureddini *et al.* (2005)	Soybean oil, *Pseudomonas cepacia*	Methanol & Ethanol	67 & 65	-
Kamini, N.R. and Iefuji,H. (2001)	Vegetable oil, *Cryptococcus spp.* S-2	Methanol	80.2	Amount of water in reaction
Nie *et al.* (2006)	Salad oil, *Candida sp.* 99-125	Methanol	96	Optimal conditions in a fixed bed reaction
Gao *et al.* (2009)	Chinese tallow kernel oil, lipase	Methanol	93.86	-

CHAPTER III
MATERIALS AND METHODS

3.1 Equipments

Autoclave	(Ta Chang Medical instrument, Taiwan)
Balance	(Sartorius, Germany)
Centrifuge tubes	(Oxygen scientific, U.S.A.)
Digital Balance	(Satorious, Germany)
Desiccator	(Sigma-Aldrich, U.S.A.)
Gas Chromatography	(Shimadzu, Japan)
Heater	(Chatcharee holding, Thailand)
High performance liquid chromatography	(Shimudzu, Japan)
Magnetic stirrer	(KIKA Labortechnik, Malaysia)
Magnetic bar	(Lio Lab Limited Partnership)
Microcentrifuge	(Global Medical Instrumentation, USA)
Microplate spectrometer	(BMG Labtech, Germany)
Microrefrigerated centrifuge: model 5417	(Eppendrof, UK)
pH meter	(Model 250, Denver Instrument)
Rotatory evaporator	(BÜCHI Labortechnik AG, Switzerland)
UV-VIS spectrophotometer	(Thermo scientific, UK)
Vacuum pump	(Scientific industries, USA)
Vortex	(Scientific industries,)
Whatman No.1	(Whatman, England)
Water bath	(T.S. Instrument, Thailand)

3.2 Chemicals

Acetone	(Carlo erba, Italy)
Acylglycerols standard (triolein, diolein and monoolein)	(Sigma, USA)
Amberlite XAD 2	(Rohm and Haas company, U.S.A.)
Amberlite XAD 4	(Rohm and Haas company, U.S.A.)
Amberlite XAD 7	(Rohm and Haas company, U.S.A.)

Amberlite XAD 16	(Rohm and Haas company, U.S.A)
Amberlite XAD 761	(Rohm and Haas company, U.S.A)
Bovine serum albumin, BSA	(Merck, Germany)
Bradford's reagent	(Biorad,U.S.A)
Butanol	(Carlo erba, Italy)
Candida rugosa lipase type VII	(Sigma,U.S.A.)
Di-potassium hydrogen phosphate	(Scharlau,Spain)
Eicosane	(Aldrich,Germany)
Ethanol	(Lab scan, Thailand)
Ethyl acetate	(Carlo erba, Italy)
Ethylene glycol	(Lab scan, Thailand)
Formic acid	(Lab scan, Thailand)
Glacial acetic acid	(Labscan, Thailand)
Hexane	(Labscan, Thailand)
Isopropanol: CH_3CHOH	(Lab scan,Thailand)
Lipozyme RM IM	(Novo Nordisk, Denmark)
Methanol	(Labscan, Thailand)
Novozym 435	(Novo Nordisk, Denmark)
Oleic acid	(Sigma, USA)
Oleic acid methyl ester (oleate)	(Sigma, USA)
Palm oil	(Morakot industry, Thailand)
Potassium di-hydrogen phosphate (KH_2PO_4)	(Merck, Germany)
p-nitrophenyl palmitate:	(Sigma, U.S.A.)
Sepabeads EC-BU	(Mitsubishi chemical corporation, Italy)
Sepabeads EC-OD	(Mitsubishi chemical corporation, Italy)
Silica gel plate (silica gel 60 F254)	(Merck, Germany)
Sodium carbonate	(Scharlau,Spain)
Sodium dodecyl sulfate, SDS	(Sigma, U.S.A.)
Sodium hydroxide	(Merck, Germany)
Sulfuric acid	(RIEDEL-DE-HAEN, Germany)
Tris (hydroxymethyl) aminomethane	(Scharlau,Spain)

Triton x-100	(Scharlau,Spain)
t-butanol	(Carlo erba, Italy)
Tween-80	(Lab scan, Thailand)

3.3 Data analysis

Statistical analysis program	(Graph Pad InStat3)
Graph analysis program	(Graph Pad Prism4)
GC data analysis program	(GC-solution solfware version 2.30.00 su6)
HPLC data analysis program	(LC solution software)

3.4 Research methodology

All experiments were performed at least in triplicates and the results were presented as mean values. The research methodology is as follows:

3.4.1 Immobilization of *Candida rugosa* lipase

3.4.1.1 Preparation of supports

Supports were prepared by suspending 1 g of support powder in 3 ml methanol. The suspension was kept stirred at 350 rpm at room temperature. After 30 min, methanol was removed from the reaction and supports were washed with 20 mM phosphate buffer pH 7.5 and kept stirred at 350 rpm at room temperature for 30 min 3 times. Then, the supports were separated into 2 groups, namely, one group was pretreated by drying at $45^{\circ}C$ and the other was immediately used for immobilization.

3.4.1.2 Immobilization of enzyme

The enzyme solution was prepared by dissolving 200 mg crude *Candida rugosa* lipase in 3 ml 20 mM phosphate buffer solution, pH 7.5. The solution was centrifuged at 5,000 rpm, $4^{\circ}C$ for 15 min to remove insoluble components. After centrifugation, supernatant was removed and the protein content of *Candida rugosa* lipase was determined by Bradford method. Protein content was compared with standard curve of BSA shown in Appendix C. Finally, the volume of lipase solution was calculated for 3 mg/ml of protein. The

supernatant was then brought in contact with 1 g of support. The lipase-support was stirred at 350 rpm for 6 hr at room temperature. After incubation, the solution was removed from immobilized enzyme and washed with 3 ml of 20 mM phosphate buffer pH 7.5 for 5 min until no enzyme was detected. The protein content in the solution was then measured.

The amount of adsorbed protein was calculated from the difference in protein content before and after adsorption. All solutions were quantitated for protein contents by Bradford method as described in section 3.4.5.2. Then, the immobilized lipases were dried at room temperature in desiccators and the enzyme was finally assayed for activities as described in section 3.4.5.1.

For industrial applications, enzymes have always been immobilized onto insoluble or solid supports, it is important to choose proper support for enzyme immobilization, since its interaction with enzyme may have remarkable influence on the stability and activity of the enzyme.

3.4.2 Support selection

In order to select a support that is suitable for hydrolytic activity, 7 types of supports were used as follows: Amberlite XAD 2, Amberlite XAD 4, Amberlite XAD 7, Amberlite XAD 16, Amberlite XAD 761, SepabeadsEC-OD and Sepabeads EC-BU.

3.4.3 Optimal condition for the immobilization

There are many factors affecting the activity recovery and reusability of enzymes in immobilization process. Some of the most important factors are the properties of the enzyme molecule, choice of support, concentration of enzyme, temperature and other. In addition , the factors studied were as follows: pH, ionic strength, protein loading, time of immobilization, temperature and adjuvant.

3.4.3.1 Effect of pH on immobilization

The effect of pH on the immobilization of lipase was studied at different pH values of buffer ranging from 4.0 to 10.0. The stock solution of buffer was prepared as 1 M buffer solution. All these buffer solutions were diluted to 20 mM before use which were used to dissolve crude lipase for immobilization method. The immobilized lipase was assayed by the method described in section 3.4.5. The result was expressed as immobilization efficiency.

3.4.3.2 Effect of ionic strength on immobilization

After the optimal pH was obtained, the following concentrations of buffer at that pH were prepared: 10, 50, 100, 250, 500, 750 and 1000 mM from the 1M stock solution to dissolve crude lipase. The immobilized lipase was assayed by the method described in section 3.4.5. The result was expressed as immobilization efficiency.

3.4.3.3 Effect of protein loading on immobilization

The effect of protein loading on the immobilization lipase was assayed by adjusting protein loading values such as 1, 3, 6, 8 and 10 mg/ml. Amount of proteins in lipase solution were prepared by dissolving commercial crude *Candida rugosa* lipase in the proper buffer solution from section 3.4.3.1 and ionic strength from section 3.4.3.2. The immobilized lipase was assayed by the method described in section 3.4.5. The result was expressed as immobilization efficiency.

3.4.3.4 Effect of time and temperature on immobilization

After the optimal pH, ionic strength and enzyme loading were obtained as described in section 3.4.3.1-3.4.3.3, the effect of time and temperature were investigated. The times of immobilization were performed by checking the residual activity of lipase solution as follows; 100 µl of lipase solution was taken for each time of immobilization for 10 hours at various temperatures namely, 10,

20, 25, 30, 40, 50 and 60 $^\circ$C. The results were expressed as the percentage

of the residual activity of the activity at room temperature (25 $^\circ$C).

From the experiment described above, after the optimal period of time

for the immobilization of was obtained, the effect of temperature on activity of

immobilized lipase was examined by checking activity of immobilized lipase

from each temperature of the immobilization. The assay methods for the free

and immobilized lipase are described in section 3.4.5. The result was

expressed as immobilization efficiency.

3.4.3.5 Effect of adjuvants on immobilization

After the optimal pH, ionic strength, enzyme loading, time and

temperature were obtained as described in section 3.4.3.1-3.4.3.4, the effect of

adjuvants was investigated.

3.4.3.5.1 Screening of adjuvant concentration

Adjuvants were divided into 2 groups namely, alcohol and

detergents. The alcohol group consisted of methanol, ethanol, iso-

propanol, n-butanol and t-butanol whereas the detergents were as

follows: SDS, tween-80, ethylene glycol and triton X-100. In addition, the

effect of type and concentration of adjuvants were also studied. The

concentration of alcohols used were 0, 1, 2.5, 5, 10, 20 and 30 %(v/v)

and those of detergents were 0, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 10 and 20

% (v/v). The experimental procedure to determine catalytic activity of

enzyme solutions containing adjuvants were as follows: 200 µl soluble

lipase from section 3.4.1.2 containing the corresponding amount of

adjuvants were incubated at temperatures for the period of optimal

times described in section 3.4.3.4 and was assayed according to the

protocol described about hydrolysis of pNPP as described in section

3.4.5.1.

3.4.3.5.2 Screening of adjuvant types

When the proper concentration of each adjuvant from the method described above was obtained, the effect of the type of adjuvant on the immobilization of *Candida rugosa* lipase was then determined. The suitable concentrations of adjuvants were added in to the enzyme solution for 2 min before contact with support and expressed as immobilization efficiency of lipase. Transesterification was performed to confirm the activity of immobilized lipase .The yield of fatty acid methyl ester was determined by high performance liquid chromatography as described in section 3.4.11.3.

3.4.4 Transesterification and hydrolysis catalyzed by immobilized *Candida rugosa* lipase

After the optimal conditions for immobilization of *Candida rugosa* lipase were obtained as described section 3.4.3.1- 3.4.3.5, the activity of immobilized lipase was investigated. The lipase activities were determined by using immobilized lipase catalyzed transesterification and hydrolysis. The conditions for transesterification were as follows; 20 % (w/w of oil) enzyme, one to three mole ratio of oil to methanol, three addition steps of methanol at 0, 3, and 6 hours in 20 ml screw-capped vial. The reactions were carried out at 40 $^{\circ}$C for 24 hours by magnetic stirrer. The conditions for hydrolysis were as follows; 20 % (w/w of oil) enzyme and 100 % (w/w of oil) water were mixed well in the reaction at 40 $^{\circ}$C for 9 hours by magnetic stirrer. The yields of fatty acid methyl ester and fatty acid were determined by HPLC analysis as described in 3.4.11.3.

3.4.5 Enzyme activity and protein assay

3.4.5.1 Enzyme activity assay

Activity of the free and immobilized lipase was assayed using 0.5% (w/v) p-nitrophenyl palmitate (pNPP) in ethanol as substrate. The reaction mixture consisting of 0.25 ml of 50 mM Tris-HCl buffer, pH 8 containing immobilized lipase or 25 μl of free lipase was initiated by adding 0.25 ml of

substrate and mixed for 5 min at room temperature. The reaction was terminated by adding 0.5 ml 0f 0.25 M Na_2CO_3 followed by centrifugation at 14,000 rpm at $4°C$ for 5 min. The increase in the absorbance at 410 nm produced by the release of p-nitrophenol in the enzymatic hydrolysis of p-NPP was then measured.

One international unit (IU) of lipase was defined as the amount of enzyme needed to liberate 1μmol of p-nitrophenol per minute using p-nitrophenyl palmitate as substrate. Calculation for the unit of enzyme activity was described in Appendix D.

3.4.5.2 Protein assay

The amount of protein content before and after immobilization was determined by Bradford protein assay method. The reaction mixture consisted of 5 μl of sample containing 300 μl of Bradford reagent in 96 well plates and was incubated at room temperature for 5 min, and later measured for the absorbance at 595 nm. Standard curve was prepared to determine concentration of protein using bovine serum albumin (BSA) at the concentration of 0.1-0.6 mg/ml. The amount of protein bound to the enzyme carriers was determined as the difference between the initial and residual protein concentrations in the supernatants. The calculation method was shown in Appendix C.

3.4.5.3 Immobilization efficiency

The efficiency of immobilization was calculated in terms of lipase activity, specific activity, protein loading and activity yields. The calculation method was shown in Appendix D.

3.4.6 Screening of raw materials for feedstock

3.4.6.1 Extraction of seed oil samples

Prior to the extraction process, plant seeds were dried overnight at 100 $^\circ$C in an oven to remove excess moisture. The dried seeds were then weighed and ground into fine particles of small size. The oil was then extracted using soxhlet extraction procedure. 15 g of plant seeds were packed in a thimble and the oil was extracted with 250 ml n-hexane for 6 hr. After extraction, the solvent was removed in a rotary evaporator at 60 $^\circ$C, under moderate vacuum. The extracted oil was then measured to calculate the content of oil in the plant seeds. Yield was calculated on dry weight basis. The calculation method was shown in Appendix E.

3.4.6.2 Fatty acid composition analysis

3.4.6.2.1 Transesterification of plant oil by chemical catalysis

The direct transesterification of plant oils triglycerides was performed. One gram of oil was used in the transesterification experiments. Sodium hydroxide (final concentration of 10% w/w based on the mass of oil) was used as an alkaline catalyst. Methanol was employed in a 3:1 alcohol to oil molar ratio. The transesterification reactions were performed in 20 ml screw-capped vials, mechanically stirred and heated in a water bath for 24 hours at 55°C. After completion of reaction, samples were taken from the reaction and centrifuged at 12,000 rpm 30 min to separate upper ester layer and a lower glycerol layer. The upper layer was analyzed by gas chromatography to determine the yield of fatty acid alkyl esters as described in section 3.4.11.1. All transesterification tests were conducted in triplicates.

3.4.6.2.2 Characterization of oil

The quality of oil is expressed in terms of the physicochemical properties such as saponification value, iodine value, cetane number and viscosity (η) by equation shown in Appendix E.

3.4.7 Transesterification catalyzed by immobilized *Candida rugosa* lipase

Transesterification reactions were carried out in 20 ml screw-capped vial containing 1 g of palm oil and 20% by weight of the immobilized lipase and later added with 3:1 mole ratio of methanol using 3-steps addition mode of methanol at 0, 3 and 6 hours. The reactions were magnetically stirred in a water bath for 24 hours at 40°C. After completion of reaction, samples were taken from the reaction and analyzed by HPLC as described in section 3.4.11.2.

3.4.8 Optimization of the transesterification catalyzed by immobilized *Candida rugosa* lipase

After the optimal conditons for immobilization of enzyme were obtained, the optimized conditions for transesterification were determined as follows: addition mode of methanol, oil:methanol ratio, amount of enzyme, water content and reaction time and temperature.

3.4.8.1 Effect of addition mode of methanol

Starting mixture of 1 g palm oil and methanol was prepared in 20 ml screw-cap bottles containing 0.3 g immobilized lipase. Then, 3 moles of methanol (71.1 µl) were added using 1, 2, 3, 4, 5, 6 and 7 steps. The reactions were magnetically stirred in a water bath for 24 hours at 40°C.

3.4.8.2 Effect of oil: methanol molar ratio

When the proper addition mode of methanol from the method described above was obtained, the effect of oil:methanol molar ratio on biodiesel production from palm oil was studied at different ratios of 1:3, 1:4, 1:5, 1:6, 1:7, 1:8 and 1:9. In this study, 20% (w/w of oil) of the immobilized

lipase and ⬛.5 g palm oil were mixed and different ratios methanol were added into the reaction using the obtained optimal addition mode of methanol from section 3.4.8.1. Experiments were carried out for the reaction periods of 24 hours and stirred in a water bath at 40°C.

3.4.8.3 Effect of Enzyme loading

When the proper addition mode of methanol, oil:methanol molar ratio from the method described above were obtained, the effect of enzyme loading of 10%, 20% and 30% (w/w of oil) on biodiesel production from palm oil was studied. This experiment was studied by adding 0.5 g of oil with each amount of the immobilized lipase for transesterification. Then, methanol was added into the reaction using the addition mode of methanol, and ratio of oil and methanol obtained from section 3.4.8.1 and 3.4.8.2, respectively. The reactions were magnetically stirred in a water bath for 24 hours at 40°C.

3.4.8.4 Effect of water content

When the proper addition mode of methanol, oil:methanol molar ratio and enzyme loading from the method described above were obtained , the effect of water content on lipase activity in transesterification reaction was examined at 0, 0.5, 1.0, 2.0, 5.0, 7.0 and 10.0 % (v/v) of the oil. The reaction mixtures were magnetically stirred in a water bath for 24 hours at 40°C.

3.4.8.5 Effect of reaction time and temperature

After the reaction mixtures were mixed using the optimized conditions from section 3.4.8.1-3.4.8.4, the reaction was incubated at different temperature 30, 40, 50, and 60 °C and stirred in a water bath for 48 hours to obtain the optimal time and temperature for the transesterification. Then, 100 μl of samples were taken from the reaction mixtures at 3, 6, 9, 12, 24, 36 and 48 hours.

3.4.9 Comparative studies of transesterification catalyzed by immobilized *Candida rugosa* lipase with Novozyme® 435 and Lipozyme® RM IM

After the optimal conditions for transesterification catalyzed by immobilized *Candida rugosa* lipase were obtained from section 3.4.8.1-3.4.8.5., lipase activities of immobilized *Candida rugosa* were tested. The selected non-edible and waste plant oils from section 3.4.6 were used as substrates for transesterification catalyzed by the optimized immobilized *Candida rugosa* lipase compared with Novozyme® 435 and Lipozyme® RM IM. Transesterification catalyzed by immobilized *Candida rugosa* lipase was carried out as follows; 0.5 g of oils were added with 30% (w/w of oil) of the immobilized *Candida rugosa* lipase with 1:3 mole ratio of methanol. The addition of methanol was performed at 0, 2, 4, 6, 8 and 10 hours and continuously stirred at 40°C for 12 hours. Transesterification catalyzed by commercial immobilized lipases were carried out as follows; one gram of oil was added with 20% (w/w of oil) of the Novozyme® 435 and Lipozyme® RM IM and later mixed with 1:3 mole ratio of methanol. The addition of methanol was performed at 0, 8 and 16 hours and continuously stirred with magnetic stirrer at 55°C for 24 hours. Samples were taken from the reaction mixture and later analyzed for the products by HPLC as described in 3.4.11.2.

3.4.10 Stability of immobilized lipase

3.4.10.1 Thermal stability

The thermal stability of immobilized enzyme was tested by incubating 2 mg of immobilized lipase at various temperatures from 30 to 80 °C in the temperature controlled heating block for 15 min. Then, the residual activities were determined to obtain proper temperature for the half life of the immobilized lipase. Then, the thermal stability of immobilized enzyme was tested by incubating 2 mg of immobilized lipase at that proper temperature as described above until none of the remained activities was detected. Then, the residual activities were determined as the percentage yield of activity compared to the activity at the optimum conditions and the half life time ($t_{1/2}$)

were calcu ated as shown in Appendix D. The results were expressed as the percentage of relative of the residual activity and half life time.

3.4.10.2 Repeated use of the immobilized *Candida rugosa* lipase

3.4.10.2.1 Repeated use on transesterification

The reusability of immobilized enzyme was tested by triplicate trials of reactions under optimum conditions. The optimum conditions determined in the present study were 2 g of palm oil, immobilized lipase content of 30% (w/w of oil), 1:3 oil:methanol ratio and water content of 5% (v/w of oil) and magnetically stirred in a water bath for 12 hours. In the present study, the immobilized lipase was rinsed with water, t-butanol and hexane after each batch reaction to remove glycerol and oil. The immobilized lipase was dried in desiccator at room temperature. The dried immobilized lipase was used in the next batch reaction composed of new substrates. The residual activity determined after complete reaction was expressed as relative conversion. The conversion achieved in the first batch was set to 100.

3.4.10.2.2 Repeated use on hydrolysis

The reusability of immobilized enzyme was tested by triplicate trials of reactions under optimum conditions. The optimum conditions in the present study were 3 g of palm oil, immobilized lipase content of 20% (w/w of oil) ,water content of 100% (v/w of oil) and magnetically stirred in a water bath at 40°C for 9 hours. In the present study, the immobilized lipase was rinsed with water after each batch reaction to remove glycerol and oil. The immobilized lipase was dried in desiccator at room temperature. The dried immobilized lipase was used in the next batch reaction with new substrates. The residual activity determined after complete reaction was expressed as relative conversion to the first batch set at 100.

3.4.11 Analysis of the fatty acid methyl ester (Biodiesel)

The biodiesel content in the reaction mixture was analyzed using a gas chromatography and high performance liquid chromatography.

3.4.11.1 Analysis of the fatty acid methyl ester by GC
3.4.11.1.1 Preparation of sample

Samples were taken from the reaction mixture at specified time and centrifuged at 12,000 rpm 30 min to obtain the upper layer. The 1 μl upper layer, 489 μl chloroform and internal standard 10 μl were precisely weighed into 1.5 ml vial.

3.4.11.1.2 Preparation of GC

FAME or biodiesel analysis was performed using gas chromatography (GC) (Shimadzu, GC 2010 series, Japan). Sample analysis was carried out on a DB-WAX fused silica capillary column (30m x 0.53mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). Acquisition and processing of data were obtained using the GC-solution software version 2.30.00 SU6 (Shimadzu, Japan).

Sample (1μl) was injected into GC column by an auto-sampler injector. The temperature program was set as follows: an isothermal period of 1 min at 70°C, then, the GC oven was heated at 20°C/min to 180°C, then at 3°C/min to 220°C and hold for 15 min. The temperature of injector and FID detector were set up for 250°C and 300°C, respectively. The fatty acids of plants seed oil were identified in Appendix E.

3.4.11.2 Analysis of the fatty acid methyl ester by HPLC
3.4.11.2.1 Preparation of sample

Samples were taken from the reaction mixture at specified time and centrifuged at 12,000 rpm 30 min to obtain the upper layer. The 10 μl upper layer, 490 μl chloroform and internal standard 10 μl were precisely weighed into 1.5 ml vial.

3.4.11.2.2 Preparation of HPLC

Reaction products were analyzed by normal phase HPLC to separate and quantify the FAME, free fatty acid and acylglycerols. The LC-20A HPLC apparatus (Shimadzu Corp., Kyoto) was equipped with Apollo Silica 5U column (250 m x 4.6 mm x 5 μm) from Alltech (Deerfield, IL) and ELSD-LT Evaporative Light Scattering Detector (Shimadzu Corp., Kyoto). Two mobile phases were employed: phase A consisted of hexane, 2-propanol, ethyl acetate and formic acid (80:10:10:0.1 v/v) and phase B consisted of hexane and formic acid (100:0.05 v/v). The flow rate was 1.5 ml/min and the injection volume was 20 μl. The protocol employed for the mobile phase involved a linear elution gradient of 1% (v/v) phase A increasing to 98% (v/v) in 20 min. The final mixture (A:B, 98:2 v/v) was employed for 3 min. Next, the system was restored to its initial condition by passing the A:B, 1:99 (v/v) mixture through the column for 15 min.

3.4.11.3 Calculation of biodiesel

Biodiesel yield was calculated as the percentage of the actual amount of methyl ester detected in the reaction process divided by the theoretical quantity of methyl ester. Calculation of the biodiesel yield was described in Appendix F.

CHAPTER IV

RESULTS

4.1 Support selection

Candida rugosa lipases were immobilized on 7 types of commercial hydrophobic supports namely Amberlite XAD 2, Amberlite XAD 4, Amberlite XAD 7, Amberlite XAD 16, Amberlite XAD 761, Sepabeads EC-BU and Sepabeads EC-OD. Lipases were adsorbed on each type of support under the same conditions and the activity was measured by the method described in 3.4.1 and 3.4.5.

Supports were prepared by suspending 1 g of support powder in 3 ml methanol. The suspension was kept stirred at 350 rpm at room temperature. After 30 min, methanol was removed from the reaction and supports were washed with 20 mM phosphate buffer pH 7.5 and kept stirred at 350 rpm at room temperature for 30 min 3 times. After that, the supports were separated into 2 groups. The first group was pretreated by drying at $45^{\circ}C$ and the other was immediately used for immobilization. Then, crude Candida rugosa lipase was dissolved in 20 mM phosphate buffer solution, pH 7.5 and the solution was centrifuged to remove insoluble components. The supernatant was then brought in contact with 1 g of support and magnetically stirred at 350 rpm for 6 hours at room temperature. After incubation, the solution was removed from immobilized enzyme and washed with buffer, Then, the immobilized lipases were dried at room temperature in desiccator and the enzyme was finally assayed for activities as described in section 3.4.5
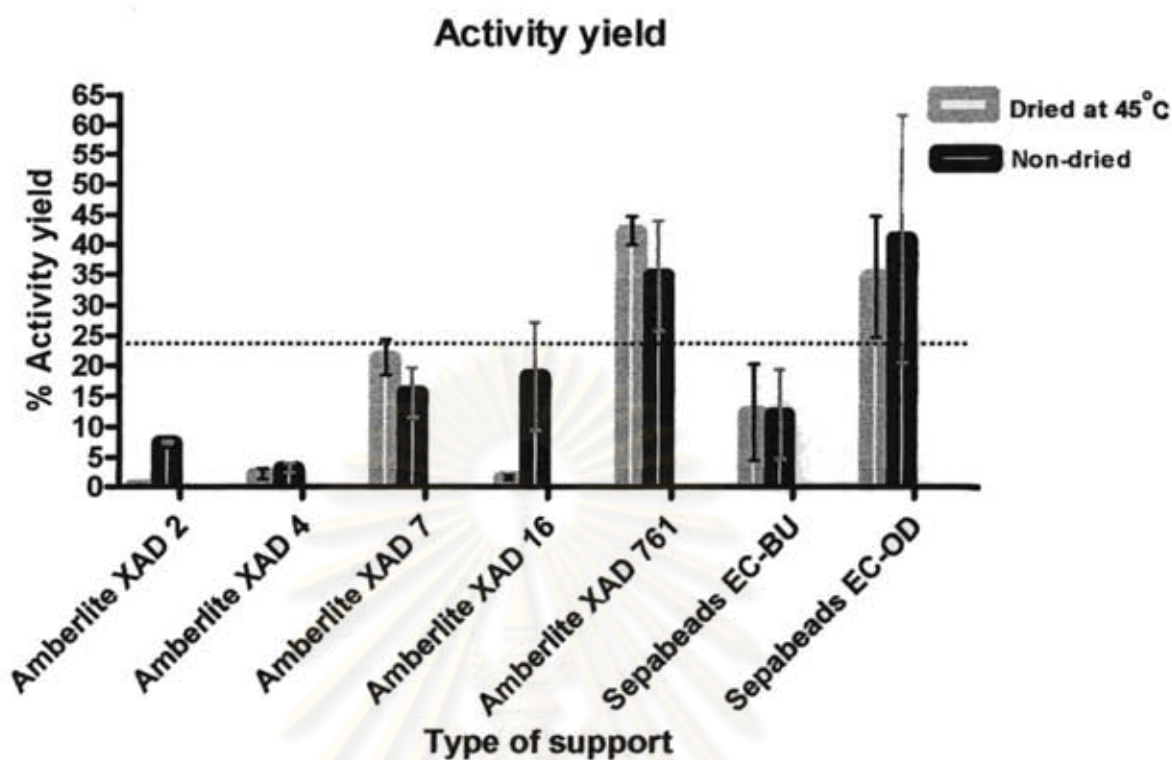
Activity yield

Figure 4-1. Yield of activity of the immobilization of lipase on hydrophobic supports. The reactions were carried out in a mixture of 3 mg/ml lipase solution prepared by dissolving crude lipase in 20 mM phosphate buffer solution pH7.5 and added with 1 g of support and magnetically stirred for 6 hr at room temperature (25°C).

Figure 4-1 showed the activity yields of the lipase from 7 types of hydrophobic supports. From the graph, it was found that even the same support gave different results if they were prepared from different methods. When the dried supports were used, the result showed that Amberlite XAD 7, Amberlite XAD 761 and Sepabeads EC-BU gave higher activity yield than the wet supports. When the wet supports were used, the resulted showed that Amberlite XAD 2, Amberlite XAD 4, Amberlite XAD 16 and Sepabeads EC-OD gave higher activity yield than the dried supports. So, from these results, more than 20% activity yield were obtained when dried Amberlite XAD 7 and Amberlite XAD 761 as well as wet Amberlite XAD 16 and Sepabeads EC-OD were selected for subsequent experiment.

Next, the immobilized enzymes with these supports were used to catalyze transesterification for the selection of the appropriate support. The conditions were 20 % (w/w of oil) immobilized lipase, the ratio of oil to methanol was one to three and three addition steps of methanol. The reactions were carried out at 40 °C for 24 hours with continuous stirring by magnetic stirrer. The results were illustrated in Figure 4- 2 and the production of biodiesel obtained was expressed as percent conversion. It could be seen that Sepabeads EC-OD gave the highest % conversion approximately, 34%. Therefore, wet Sepabeads EC-OD was selected as the optimal support for the subsequent immobilization.
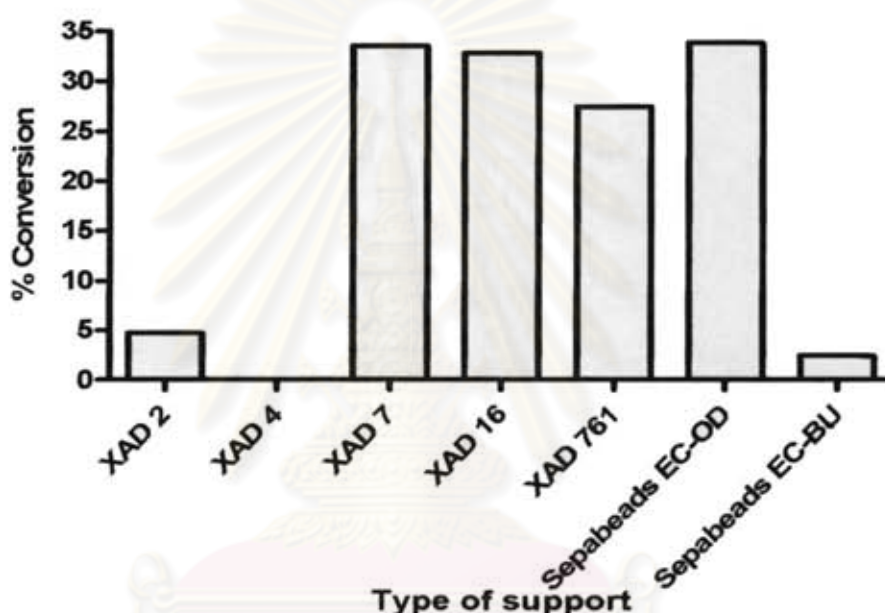
Figure 4-2. Support screening on transesterification of palm oil, a loading of 0.20 g immobilized lipase, 1 g oil, ratio of oil to methanol was one to three and added into reaction using 3 steps and continuously stirring for 24 hr at 40°C

4.2 Optimization of immobilization of lipase

The effect of pH, ionic strength, protein loading, immobilization time, temperature and adjuvants for the immobilization of *Candida rugosa* lipase onto Sepabeads EC-OD were investigated. The result for each factor was illustrated in Figure 4-3 to Figure 4-9.
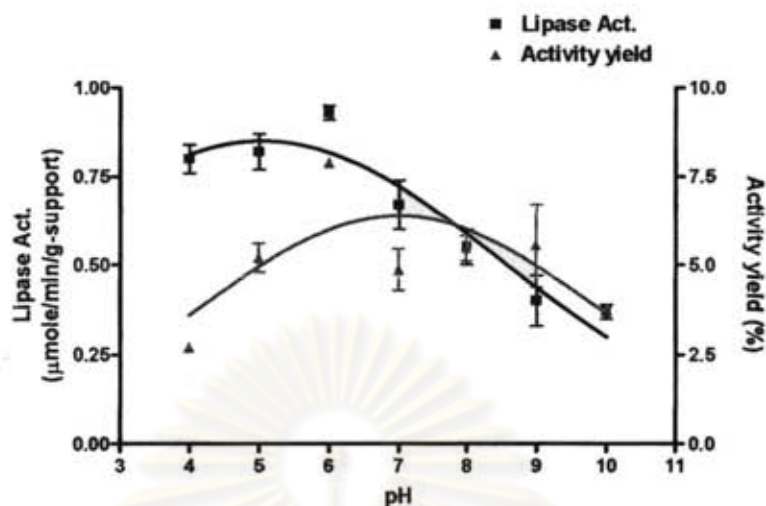
4.2.1 Effect of pH on immobilization

Figure 4-3. The effect of pH on the lipase activity and activity yield (%) of lipase immobilization. The enzyme solution was prepared by dissolving crude *Candida rugosa* lipase in 3 ml 20 mM buffer solution at various pH. Then, 2.5 ml lipase solution (3mg/ml) was added to 1 g of Sepabeads EC-OD and magnetically stirred for 5 hours at room temperature (25°C). Activities shown on the y-axis are the means ± SD of three individual experiments.

The activities of lipase and the activity yields at various reaction pH were studied and the results were shown in Figure 4-3. It can be seen that maximal activity was obtained when pH of the system reached pH 6 and started to decrease by nearly 3 folds when the pH subsequently rose to 10 since denaturation tend to increase under high pH values. The highest activity of lipase (0.93 (±0.02) umol/min/g-support) and activity yield (7.5 (±0.06) percent) were obtained at pH 6 in phosphate buffer (20 mM). As can be seen from the figure, the activity of lipase and activity yield increased from 0.80 (±0.04) to 0.93 (±0.02) umol/min/g-support and 2.71 (±0.14) to 7.5 (±0.06) percent when pH equaled to 4 and 6 respectively. From pH 6 to pH 10, the activity of lipase and activity yield dramatically decreased from 0.93 (±0.02) to 0.37 (±0.02) umol/min/g-support and 7.5 (±0.06) to 3.81 (±0.01)

percent respectively. The results indicated that immobilized lipase from *Candida rugosa* appeared more stable in acidic environments. The optimum pH for the maximum of activity of lipase was therefore fixed as 6.

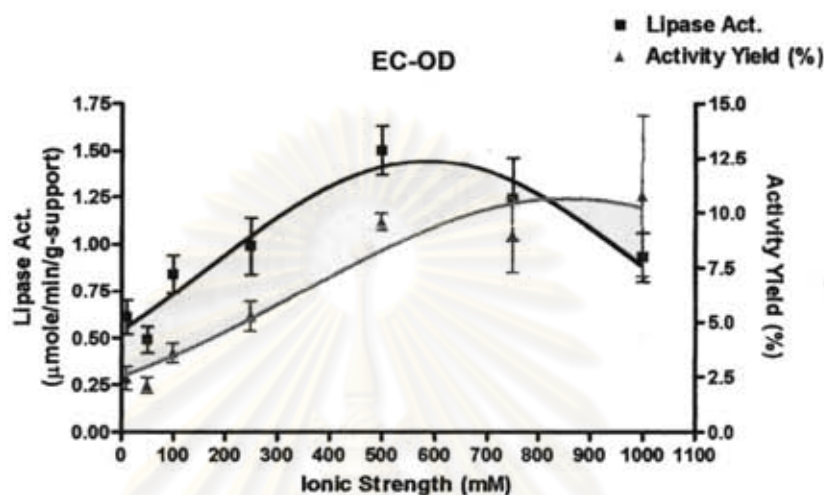### 4.2.2 Effect of ionic strength on immobilization

Figure 4-4. The effect of ionic strength on the lipase activity and activity yield (%) of lipase immobilization. 3 mg/ml lipase solution prepared by dissolving crude lipase in buffer solution pH6 at various concentrations was added to 1 g of Sepabeads EC-OD and magnetically stirred for 5 hr at room temperature (25°C). Activities shown on the y-axis are the means ± SD of three individual experiments.

When the optimal pH for immobilization was obtained at 6, the phosphate buffer, pH 6 at various concentrations from 10 mM to 1 M were therefore prepared to study the effect of ionic strength on activity of immobilized *Candida rugosa* lipase. The effect of the ionic strength of enzyme solution on the adsorbed amount of protein was investigated and these results were shown in Figure 4-4. It can be seen that the activity of lipase increased from 0.61 (±0.09) to 1.50 (±0.13) umol/min/g-support with increasing ionic strength from 10 mM to 500 mM and percentage of activity yield increased from 2.46 (±0.53) to 9.62 (±0.38). On the other hand, when the ionic strength was elevated from 500 mM to 1 M, the activity of

lipase was decreased from 1.50 (±0.13) umol/min/g-support to 0.93 (±0.13) umol/min/g-support and percentage of activity yield was rather stabilized at approximately 10 percents. Therefore, the optimal concentration (500mM) of phosphate buffer pH 6 was used as immobilization of *Candida rugosa* lipase to study the effect of protein loading on activity of immobilized lipase.

4.2.3 Effect of protein loading on immobilization

(a)
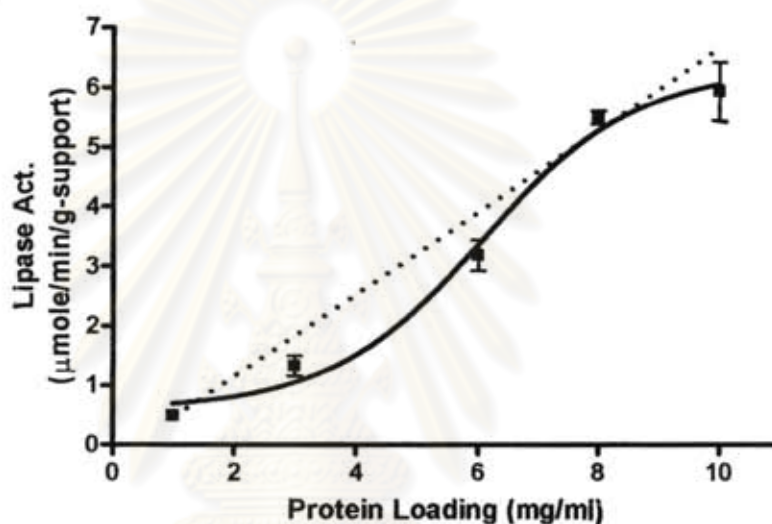
(b)
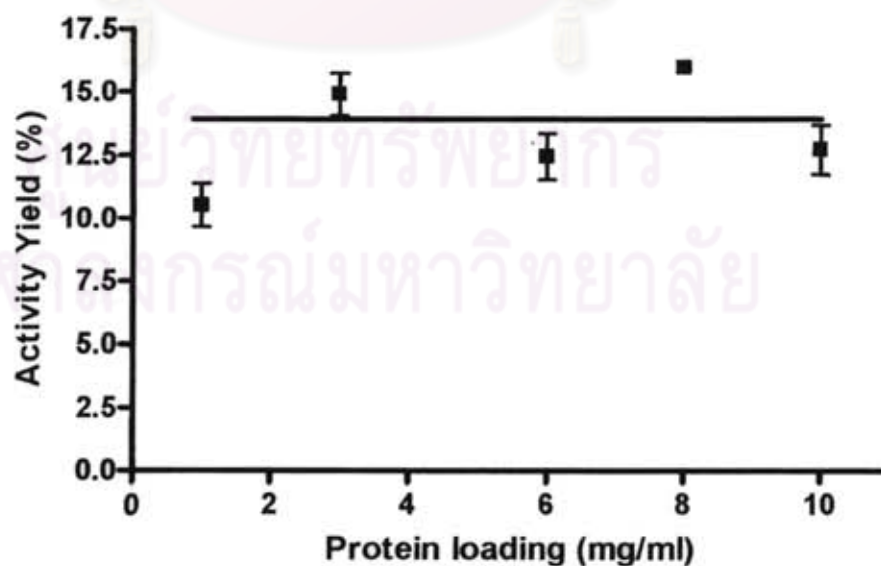
Figure 4-5. The effect of protein loading on (a) lipase activity and (b) % activity yield of lipase immobilization. Crude lipase was dissolved in 500 mM phosphate buffer, pH 6.0. The various quantities of enzyme were added to 1 g of Sepabeads EC-OD and magnetically stirred for 5 hr at room temperature ($25°C$). Activities shown on the y-axis are the means ± SD of three individual experiments.

When the suitable pH and ionic strength (500 mM phosphate buffer, pH 6.0) were obtained, the effect of protein loading was later studied. In this study, different quantities of enzyme were immobilized on the support from 1 to 10 mg/ml and the results were shown in Figure 4-5. It can be seen that the activity significantly increased from 0.49 (±0.01) to 3.18 (±0.26) umol/min/g-support when the protein loading was increased from 1 to 6 mg/ml and the maximal activity (5.93 (±0.48) umol/min/g-support) was obtained at approximately 10 mg/ml of protein (20 folds higher compared to 1 mg/ml of enzyme). However, when the activity of enzyme reached 8 mg/ml, the activity was not much different from 10 mg/ml of the enzyme. Therefore, in order to reduce the cost for further applications, 8 mg/ml was finally used as optimal amount of protein loading. Hence, the optimal condition was selected as described in section 4.2.1-4.2.3 to study the effect of time of immobilization.

4.2.4 Effect of immobilization time
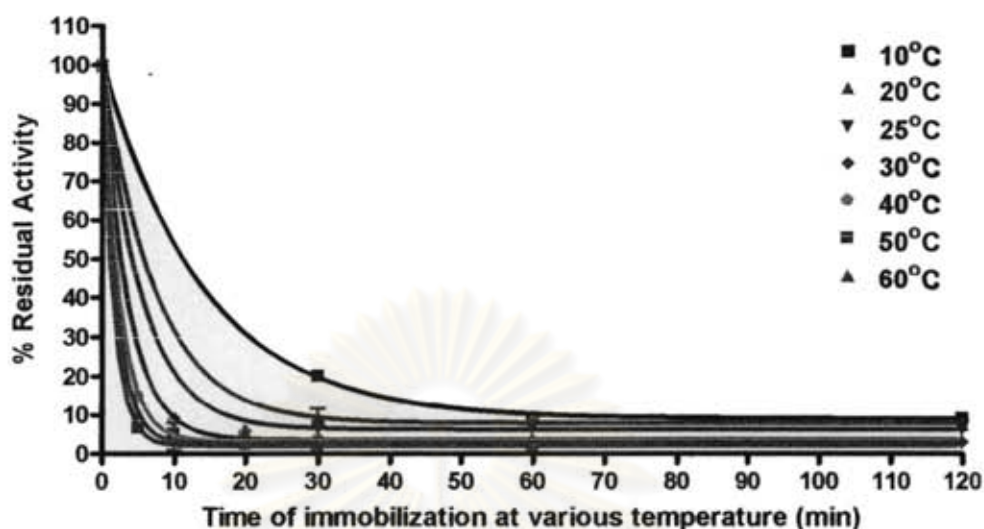
Figure 4-6. The effect of immobilization time on the residual activity of lipase. 8 mg/ml of crude *Candida rugosa* lipase solution were incubated at various temperatures. Residual activities on the y-axis are the means ± SD of three individual experiments.

The effect of time of immobilization on activity of immobilized *Candida rugosa* lipase was studied by using optimal conditions from the results described above. The residual activity of lipase solution was checked for each time of immobilization at various temperatures as described in 3.4.5.1. The relationship of the residual activity with immobilization time at various temperatures was shown in Figure 4-6. The results were expressed as the percentage of the residual activity at room temperature (25°C). When lipase was incubated at 10°C for 180 min, the residual activity of lipase appeared unchanged at 10.12 (±0.03) %. Furthermore, when incubation of lipase solution at 20°C and room temperature (around 25°C), the results showed that the residual activity leveled off in both temperatures at 6.50 (±0.11) and 7.67 (±0.16) % respectively until 120 min. At 30, 40, 50 and 60°C, the residual activity of lipase solution gave the similar pattern that they significantly

decreased from initial time of the incubation to around 20-30 minutes and stayed unchanged to 60 minutes. Therefore, the optimal time for immobilization was 3 hr, 2hr, 1hr and 20- 30 min at 10, 20, 25 (RT) and 30-60 $^{\circ}$C respectively and subsequently selected for the next experiment namely, optimal condition for the temperature.

4.2.5 Effect of temperature on immobilization
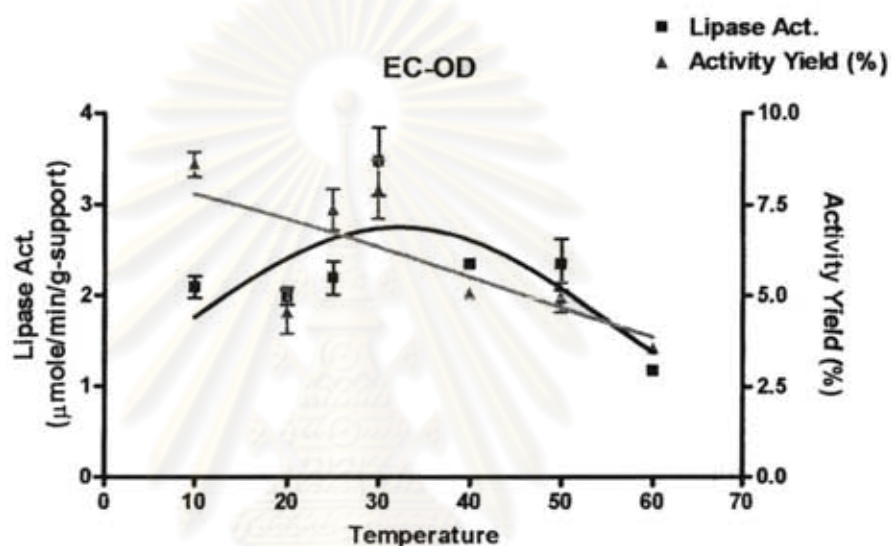
Figure 4-7. The effect of temperature on the lipase activity and % activity yield of lipase immobilization. The immobilization was performed in 500 mM phosphate buffer pH 6.0 and 8 mg/ml lipase solution at specific time for each temperature. Activities shown on the y-axis are the means ± SD of three individual experiments.

Lipase was immobilized on Sepabeads EC-OD at different temperatures from 10 to 60°C.The immobilization reaction was carried out under the optimal conditions obtained from all 4 previous sections. In this experiment, the immobilization of Candida rugosa lipase was determined by checking the activity of immobilized lipase. From Figure 4-7, it was shown that when 10°C was used for immobilization, the activity of lipase and activity yield were 2.09 (±0.12) umol/min/g-support and 6.62 (±0.34) respectively. They considerably increased to 3.47 (±0.37)

umol/min/g-support and 7.93 % (±0.79) respectively, when temperature rose to 30°C. However, when temperature was increased to 40, 50 and 60 °C, the lipase activity and activity yield decreased dramatically from 2.34 (±0.01) to 1.17 (±0.04) umol/min/g-support and 5.07 (±0.15) to 3.57 (±0.01) percent, respectively. From the above 4 optimal conditions, the activities of the immobilized lipase was increased approximately 3 folds higher from 0.93 (±0.02) to 3.47 (±0.37) umol/min/g-support. From these results, 30 °C was the optimal temperature for *Candida rugosa* lipase immobilization. Therefore, the optimal conditions from described 4.2.1 to 4.2.5 were subsequently selected for the next experiment namely, adjuvant.

4.2.6 Effect of adjuvant on immobilization

Once the optimal conditions of 500 mM phosphate buffer, pH 6, 8 mg/ml of *Candida rugosa* lipase solution for immobilization at 30 °C for 30 min were obtained, the effect of adjuvant on the activity of immobilized *Candida rugosa* lipase was examined by using two types of adjuvants as described in section 3.4.3.5.

4.2.6.1 Effect of concentration of adjuvant

Table 4-1. The concentrations of various adjuvants with the highest activities of *Candida rugosa* lipase solution.

Type of adjuvants	Concentration of adjuvants (%v/v)
Methanol	5
Ethanol	5
Isopropanol	20
Butanol	5
t-butanol	5
SDS	0.75
Tween- 80	2.50
Ethylene glycol	2.50
Triton X-100	1

From Table 4-1, the effect of concentration of adjuvant on activity of *Candida rugosa* lipase solution was studied by using optimal conditions from the results described above. The activities of lipase solution were examined by using two types of adjuvants with various different concentrations as described section 3.4.3.5.1. The results were expressed as the percentage of the activity of untreated lipase solution. Then, the various concentrations of each adjuvants were selected from the highest activity of lipase. Table 4-1 showed the highest activity of each type of adjuvant. Therefore, the concentration of each adjuvant was used for the immobilization of lipase to further select the type of adjuvant.

4.2.6.2 Effect of the type of adjuvant

(a)
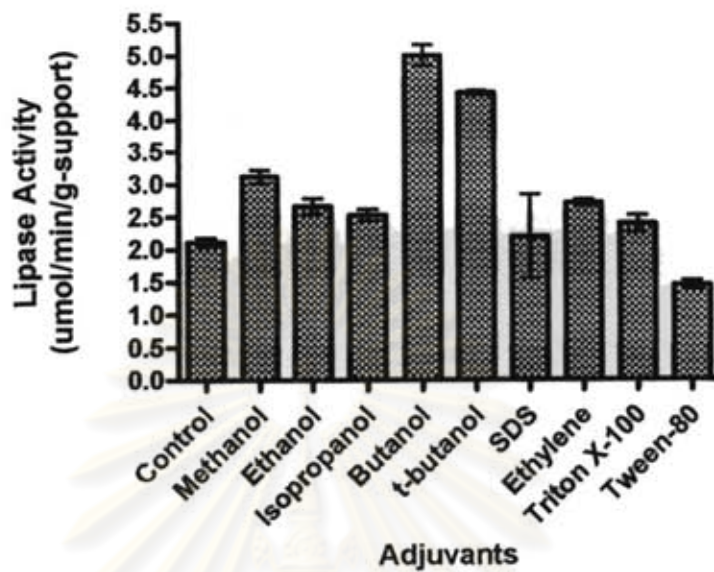
(b)
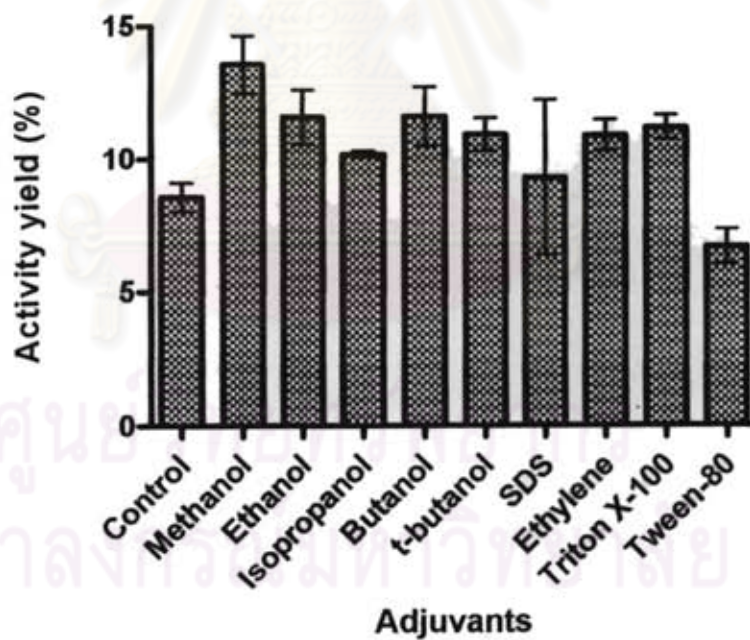
Figure 4-8. The effect of adjuvant types on the lipase activity (a) and %

activity yield (b) of lipase immobilization. The suitable concentration of each

adjuvant was added in to the enzyme solution (8 mg/ml) for 2 min before contact with support. The reaction was magnetically stirred for 30 min at 30°C.

When the proper concentration of each adjuvant from the method described above was obtained, the effect of the type of adjuvant on the immobilization of *Candida rugosa* lipase was then determined as described in 3.4.5.1. The relationship between the lipase activity and the % activity yield with type of adjuvant were shown in Figure 4-8a and Figure 4-8b.

From Figure 4-8a, the result showed that the highest lipase activity (4.99 (±0.16) umol/min/g-support) was achieved when butanol was used as adjuvant. However, from Figure 4-8b, the result showed that the highest % activity yield (13.55 % (±0.16)) was obtained when methanol was used. Since both results were inconsistent, the immobilized lipases were therefore tested again for transesterification to determine the selection of the optimal type of adjuvant by the method described in section 3.4.7
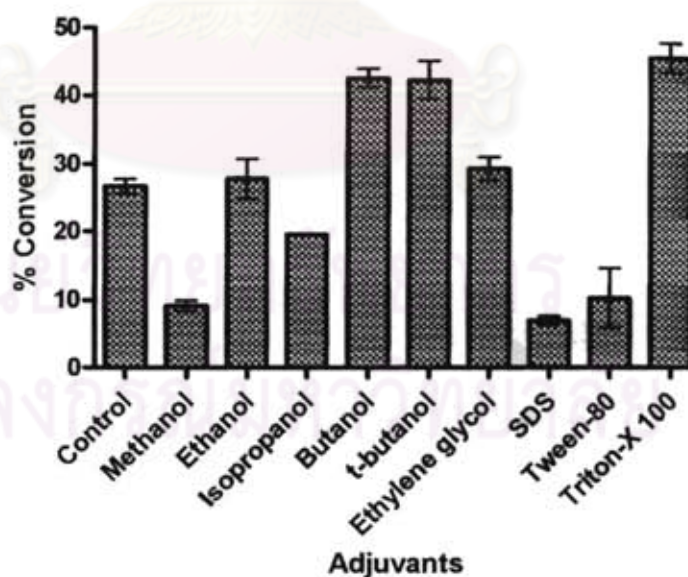
Figure **4-9**. The effect of immobilized lipase with added various adjuvants on transesterification reaction. The reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio, 3 step addition mode of methanol and 20% (w/w of oil) immobilized lipase magnetically stirred in a water bath for 24 hr at 40°C. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

From Figure4-9, the results showed that %conversion was highest when butanol, t-butanol and triton-X 100 were used as adjuvants. So, t-butanol was selected as the optimal adjuvant for the *Candida rugosa* lipase immobilization and subsequently selected for the next experiment.

From overall results, the optimal conditions of lipase immobilization were obtained and summarized in Table 4-2. Under these conditions, the lipase activity of the immobilized lipase was 4.41 umol/min/g-support, specific activity was 0.15 umol/min/mg-protein, activity yield was 10.89 percent and with a protein loading of 83.81 percent.

Table **4-2**. Optimal conditions of lipase immobilization

pH	6
Ionic strength (mM)	500
Protein loading (mg/ml)	8
Time (min)	30
Temperature (°C)	30
Type of adjuvant	t-butanol

4.3 Transesterification and hydrolysis catalyzed by immobilized *Candida rugosa* lipase
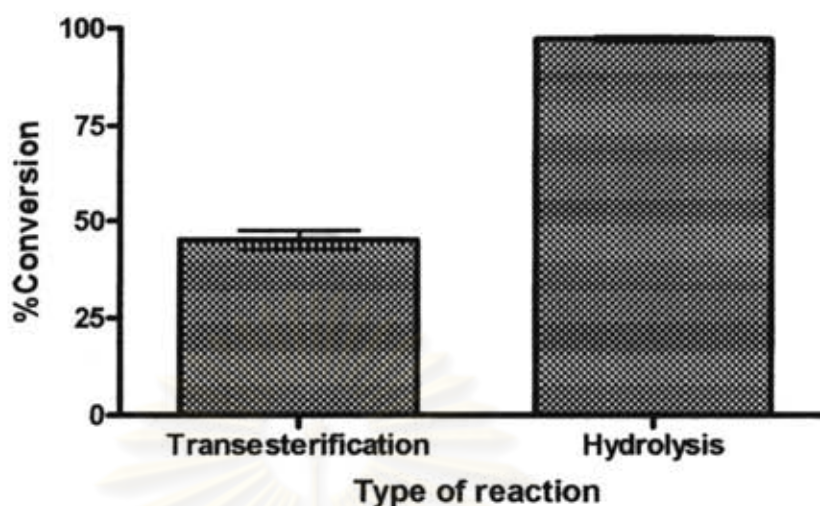
Figure **4-10**. Transesterification and hydrolysis catalyzed by immobilized lipase. The transesterification reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio, 3 step addition mode of methanol and 20% (w/w of oil) immobilized lipase and magnetically stirred for 24 hr at 40°C. The hydrolysis reaction was carried out in a mixture of 20 % (w/w of oil) enzyme and 100 % (w/w of oil) water were magnetically stirred at 40 °C for 9 hours at 40°C.Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

After the optimal conditions for immobilization of *Candida rugosa* lipase from section 4.2.1-4.2.6 were obtained, the activity of immobilized lipase was investigated on transesterification and hydrolysis. The transesterification reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio, 3 step addition mode of methanol and 20% (w/w of oil) immobilized lipase magnetically stirred in a water bath for 24 hr at 40°C. The hydrolysis reactions were carried out in a mixture of 20 % (w/w of oil) enzyme and 100 % (w/w of oil) water and mixed well at 40 °C for 9 hours by magnetic stirrer in a water bath. From Figure 4-10, it was shown that 45.23 % (±2.43) biodiesel and 97.21% (±0.69) free fatty acid were obtained from transesterification and hydrolysis, respectively.

4.4 Screening of raw materials for feedstock

Biodiesel can be produced from any vegetable oil such as rapeseed, soybean, cottonseed, physic nut, rubber seed and others. Oils are primarily composed of triacylglycerols (TAGs), a form of lipid comprised of three fatty acid molecules attached to a glycerol backbone.

4.4.1 Extraction of seed oil

Table 4-3. Oil content of non-edible and waste plant seeds

Common name	Scientific Name	% oil content
Papaya	Carica papaya Linn.	25.00
Physic nut	Jatropha curcas L.	43.75
Pomelo	Citrus maxima (Burm.) Merr.	42.78
Pumpkin	Cucurbita moschata Duchesne	38.12
Rambutan	Nephelium lappaceum L.	41.25
Rubber	Dipterocarpus alatus Roxb.ex G.Don	38.77
Tangerine	Citrus reticulate Blan co	29.10
White silk cotton	Ceiba pentandra (L.)Gaertn.	29.21
Wild almond	Irvingia malayana Oliv. ex A.W. Benn.	72.06

The oil of non-edible and waste plant seeds was extracted using soxhlet method. 15 g of plant seeds were packed in a thimble and the oil was extracted with 250 ml n-hexane for 6 hr. The extracted oil was then measured to calculate the content of oil in the plant seeds shown in Table 4-3. The %oil content from non-

edible and waste plant seed oils were in the range of 25 in papaya seeds to approximately 72 dry weight in wild almond seeds.

4.4.2 Fatty acid composition analysis

Seed oil from nine types of non-edible and waste plants commonly found in Thailand with oil contents ≥ 30% (dry weight) were analyzed for their fatty acid composition using chemical transesterification. One gram of oil sample was added with 1:3 molar ratio of methanol. The reaction was catalyzed with 10% by oil weight of NaOH at 55°C. Then, the fatty acid composition of obtained methyl esters was quantitated by GC.

The composition for various types of waste and non-edible oils is shown in Table 4-4. From this table, it was observed that the major oil composition in both non-edible and waste plant oils is generally similar. The major fatty acids content in both non-edible and waste plant oils are oleic, linoleic and palmitic acid, while the latter include lauric, myristic, palmitoleic, stearic, linolenic, arachidic and behenic acid. Noticeably, the major content in wild almond were found to be saturated fatty acids, lauric and myristic acids. White silk cotton and pomelo had high content of linolenic acid with ≥ 40% while physic nut, papaya, rambutan, and pumpkin contained similar high content of oleic acid. The obtained fatty acid composition of each non-edible and waste plant oils was then later used to calculate their saponification number (SN), iodine value (IV), cetane number (CN) and viscosity (η).

4.4.3 Characterization of oil

The quality of oil is expressed in terms of the physicochemical properties such as saponification value, iodine value, cetane number and viscosity (η) by equation shown in Appendix E. These values established their suitability for used as

substrates as shown in Table 4-4. From the results, it was shown that the calculated saponification number (SN), iodine value (IV) ranged from 195 to 204 and 3 to 147, respectively. Cetane number (CN) and viscosity (η) values among the species varied from 40 to 66 and 2.29 to 3.95, respectively. The values obtained were used to predict the quality of oil for use as biodiesel. It was found that biodiesel obtained from 6 plant species such as white silk cotton, physic nut, pomelo, papaya, rambutan and pumpkin met the major specification of biodiesel standards of USA, and European Standard Organization. All of the non-edible and waste plant oils were then used as substrates for transesterification catalyzed by immobilized *Candida rugosa* lipase, Novozyme® 435 and Lipozyme® RM IM in comparison.

Table 4-4. Fatty acid composition, Saponification number (SN), Iodine value (IV), Cetane number (CN) and viscosity (η) of fatty acid methyl ester of non-edible and waste plant oils

No.	Common Name	Scientific Name	SN	IV	CN	η	Fatty Acid Composition (%)
1	Wild almond	Irvingia malayana Oliv. ex A.W. Benn	204	3.60	66.13	2.29	12:0(55.55), 14:0(38.19), 16:0(2.55), 16:1(0.44), 18:0(0.22), 18:1(2.63), 18:2(0.44)
2	White Silk Cotton	Ceiba pentandra (L.) Gaertn.	204	104.70	49.52	3.41	12:0(0.06), 14:0(0.16), 16:0(24.19), 16:1(0.21), 18:0(4.15), 18:1(26.30), 18:2(42.03), 18:3(1.73), 20:0(0.39), 22:0(0.69)
3	Rubber	Dipterocarpus alatus Roxb. ex G.Don	202	146.90	40.29	3.24	12:0(0.30), 14:0(0.42), 16:0(9.87), 16:1(0.22), 18:0(5.28), 18:1(21.76), 18:2(45.82), 18:3(16.12), 20:0(0.14), 22:0(0.09)
4	Physic Nut	Jatropha curcas L.	202	108.40	48.91	3.46	12:0(0.14), 14:0(0.17), 16:0(14.82), 16:1(0.81), 18:0(4.15), 18:1(40.98), 18:2(38.61), 18:3(0.27), 20:0(0.06)
5	Pomelo	Citrus maxima (Burm.) Merr.	203	106.20	49.29	3.40	12:0(0.01), 14:0(0.14), 16:0(25.24), 16:1(0.17), 18:0(4.25), 18:1(24.53), 18:2(43.32), 18:3(2.00), 20:0(0.27), 22:0(0.11)
6	Papaya	Carica papaya Linn.	202	75.60	56.27	3.69	12:0(0.26), 14:0(0.46), 16:0(17.12), 16:1(0.45), 18:0(2.98), 18:1(72.91), 18:2(4.83), 18:3(0.29), 20:0(0.67), 22:0(0.07)
7	Rambutan	Nephelium lappaceum L.	195	58.13	61.17	3.95	12:0(0.08), 14:0(0.11), 16:0(8.77), 16:1(0.96), 18:0(7.25), 18:1(55.25), 18:2(3.72), 18:3(0.26), 20:0(22.05), 22:0(1.34)
8	Pumpkin	Cucurbita moschata Duchesne	202	95.19	51.87	3.52	12:0(0.01), 14:0(0.18), 16:0(20.53), 16:1(0.07), 18:0(6.51), 18:1(38.68), 18:2(32.96), 18:3(0.22), 20:0(0.28), 22:0(0.60)
9	Tangerine	Citrus reticulate Blan co	203	118.40	46.48	3.34	12:0(0.01), 14:0(0.03), 16:0(21.67), 16:1(0.39), 18:0(4.03), 18:1(20.99), 18:2(48.00), 18:3(4.45), 20:0(0.24), 22:0(0.05)

4.5 Optimization of the transesterification reaction

This work purported to study the ability of the immobilized *Candida rugosa* lipase preparation to catalyze the synthesis of fatty acid methyl ester (biodisel). The following six main variables: addition mode of methanol, oil:methanol molar ratio, amount of lipase, water content, time and temperature were investigated.

4.5.1 Effect of addition mode of methanol on transesterification

Figure 4-11. The effect of addition mode on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio with 20% (w/w of oil) immobilized lipase and continuously stirred at 40°C for 24 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

The effect of addition mode of methanol from once to seven steps on transesterification was studied using the conditions as follows; 0.5 g of oil, 20% (w/w of oil) immobilized lipase and 1:3 mole ratio of oil:methanol. From Figure 4-11, it was found that when three mole of methanol were added at once and two steps, 3.16 % (±2.46) and 4.12 % (±3.95) biodiesel were obtained, respectively. The yield suddenly rose from 4.12 % (±3.95) to 31.88% (±3.48) when the adding step of methanol increased from two to three steps and increased further from

31.88% (±3.48) to 53.90 % (±0.86) from three to six steps and declined lightly to 50% at 7 steps. The optimal addition mode of methanol for the maximum yield of biodiesel was therefore fixed at 6 steps and subsequently selected for the next experiment namely: optimal condition for the oil to methanol molar ratio.

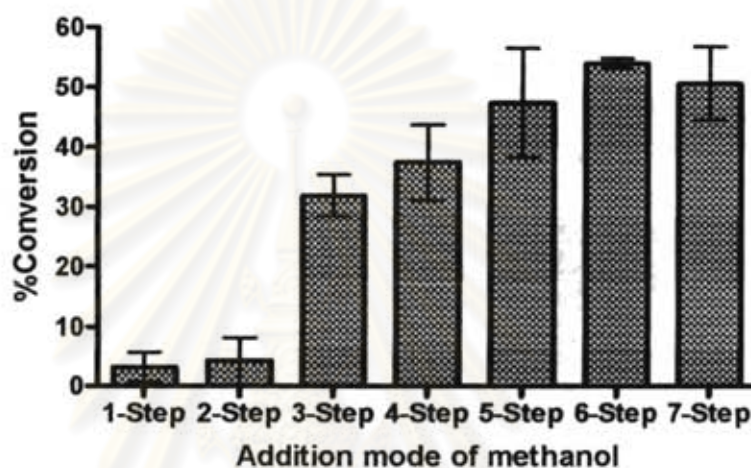### 4.5.2 Effect of oil to methanol molar ratio on transesterification
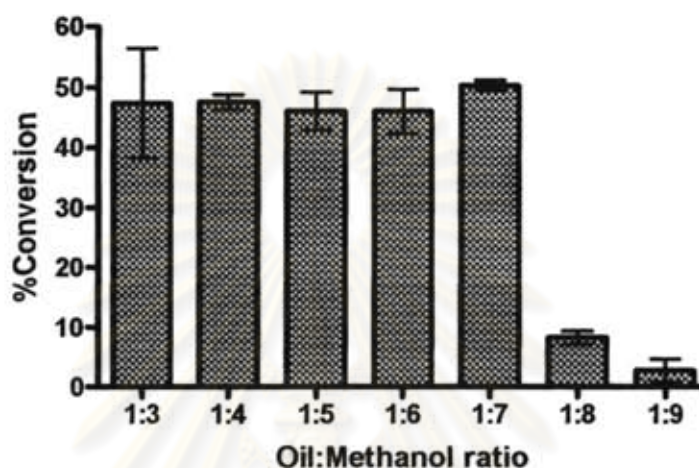
Figure 4-12. The effect of oil to methanol ratio on percentage of conversion to biodiesel. The reactions were carried out with 0.5 g of palm oil, 20% (w/w of oil) immobilized lipase and 6 steps addition mode of methanol and continuously stirred at 40°C for 24 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

The effect of oil to methanol molar ratio on the conversion was performed at 40 °C, immobilized lipase concentration of 20 % (w/w of oil), and varying the oil to methanol molar ratio at the values of 1:3, 1:4, 1:5, 1:6, 1:7,1:8 and 1:9. Figure 4-12 showed the fatty acid methyl esters conversion obtained. As can be seen from this figure, the highest conversion (50.24%) was achieved at the molar ratio of 1 to 7. From the figure, the percent conversion from 1:3 to 1:7 was approximately 48% which was not much different. However, when the ratio was increased to 1:8, the yield of biodiesel was dramatically decreased nearly 6 folds to approximately 8%.

Moreover, when the ratio was increased to 1:9, the yield of biodiesel was continuously decreased for 24 folds to 2%. Therefore, in order to reduce the cost for further applications, 1 to 3 ratio was subsequently used as optimal oil to methanol ratio from this study. As a result, 6 addition mode of methanol and 1 to 3 molar ratio of oil to methanol were used as optimal conditions for transesterification to further study the effect of amount of enzyme on conversion.

4.5.3 Effect of amount of enzyme on transesterification
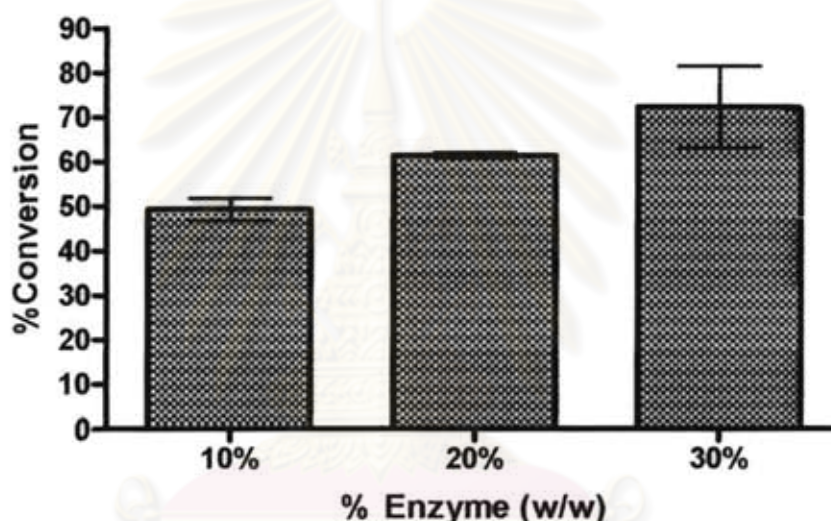
Figure 4-13. The effect of amount of enzyme on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio, 6 step addition mode of methanol and magnetically stirred in a water bath at 40°C for 24 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

Since the optimal mode of methanol addition and the ratio of oil to methanol were 6 steps and 1 to 3 respectively, the effect of immobilized lipase concentration on conversion was performed with enzyme concentrations of 10%,

20% and 30% w/w of oil at 40 ˚C. Figure 4-13 illustrated the conversion of biodiesel when various amounts of immobilized lipase were used. From this Figure, it can be observed that the maximal yield of FAME at 72.3% was achieved from 30% immobilized lipase. When 10 and 20 % immobilized enzyme were used, the % conversion of fatty acid methyl ester increased from 50 - 60%. Therefore, the optimal conditions were as follows: 6 step of addition mode of methanol, 1 to 3 oil to methanol ratio and 30% amount of enzyme were subsequently selected for the next optimal condition namely; water content.

4.5.4 Effect of water content on transesterification
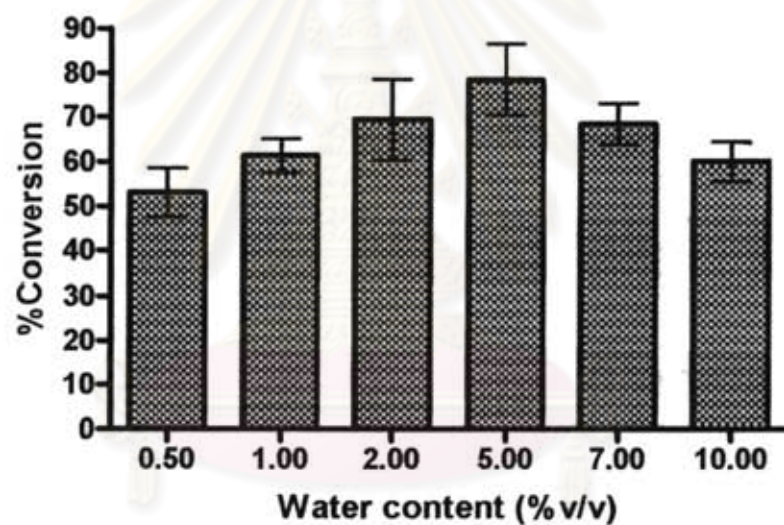
Figure 4-14. The effect of water content on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g of palm oil, 30% (w/w of oil) immobilized lipase, 1:3 oil:methanol ratio, 6 steps addition mode of methanol and continuously stirred at 40˚C for 24 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

The reaction was carried out by adding water ranging from 0.5% to 10% (%v/w) of the oil with 30% (w/w of oil) immobilized *Candida rugosa* lipase. The

reactions were carried out in mixtures of 0.5 g palm oil, 0.15 g of immobilized lipase, 71 µl of methanol (added at six steps, each 11.9 µl) and magnetically stirred in a water bath for 24 hr at 40°C. The results were shown in Figure 4-14.

As indicated in Figure 4-14, the fatty acid methyl ester content gradually rose from 52- 78% as water content increased from 0.5% to 5.0% (v/w) of oil, and then declined from 78% to 60% as water content rose from 5% to 10%. It can be seen that the FAME content reached its maximum at water content of 5% (v/w of oil) which was about 78.50% conversion higher than that in absence of water. From previous optimal conditions, the % conversion was increased from 50% to 80%, approximately 30 % higher.

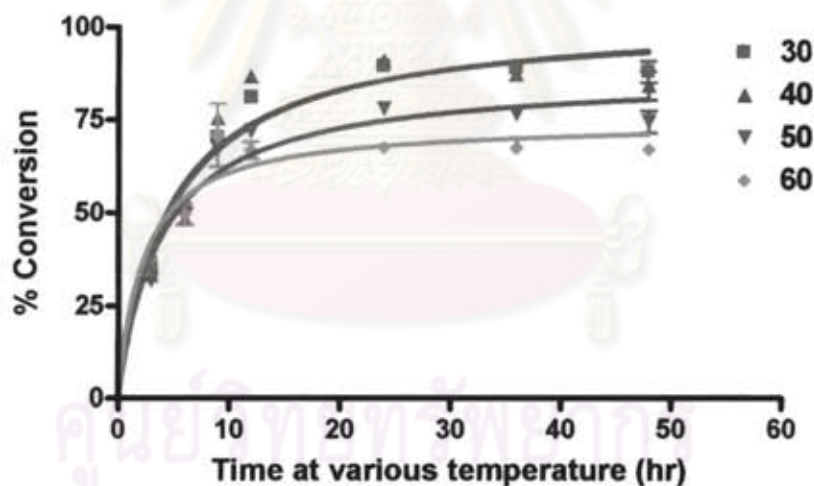### 4.5.5 Effect of time and temperature on transesterification

Figure 4-15. The effect of time and temperature on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 2 g of palm oil, 30% (w/w of oil) immobilized lipase, 1:3 oil:methanol ratio, 6 steps addition mode of methanol, 5% (v/w of oil) water and magnetically stirred in a water bath at each

temperature for 48 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

To study the effect of reaction temperature on fatty acid methyl esters formation, the transesterification reaction was carried out under the optimal conditions obtained from all 4 previous sections. The experiments were conducted at temperature ranging from 30 to 60 $^{\circ}$C shown in Figure 4-15.

Experimental results showed that the transesterification reaction could proceed within the temperature range studied but the reaction time to complete the reaction varied significantly with reaction temperature. It can be seen that highest yield of 91% of biodiesel was obtained at 40 $^{\circ}$C for the period of 24 hours whereas 87% conversion at 12 hours. The results in Figure 4-15 showed that the percentage yield of biodiesel suddenly rose when the reaction time was increased from 0 to 12 hours. After 12 hours, it was seen that increase in the reaction time did not have the effect on the production of biodiesel. When the reaction temperature increased to 50 $^{\circ}$C and 60°C, it was found that the product yield started to decrease since the enzyme lost its activity dramatically. From previous optimal conditions, the % conversion was increased from 50 to 90%.

4.6 Comparative studies of transesterification catalyzed by immobilized *Candida rugosa* lipase with Novozyme$^{®}$ 435 and Lipozyme$^{®}$ RM IM

The immobilized *Candida rugosa* lipase was used to catalyze the transesterification at the optimal conditions using six non-edible and waste oils compared with 2 commercial lipases. Transesterifications were carried out as described in section 3.4.9. The conversion of fatty acid methyl esters from these non-edible and waste plant oils were illustrated in Figure 4-16. From the results, it was shown that the production of biodiesel obtained from catalysis of three types of immobilized lipases in 6 types of plant seeds were approximately at 70-80 %. However, rambutan oil gave 90% of biodiesel

when catalyzed by both commercial lipases but only 60% was obtained by the immobilized *Candida rugosa* lipase. On the other hand, 80% of biodiesel from papaya oil was obtained when catalyzed by the immobilized *Candida rugosa* lipase when only 60% was obtained from Lipozyme® RM
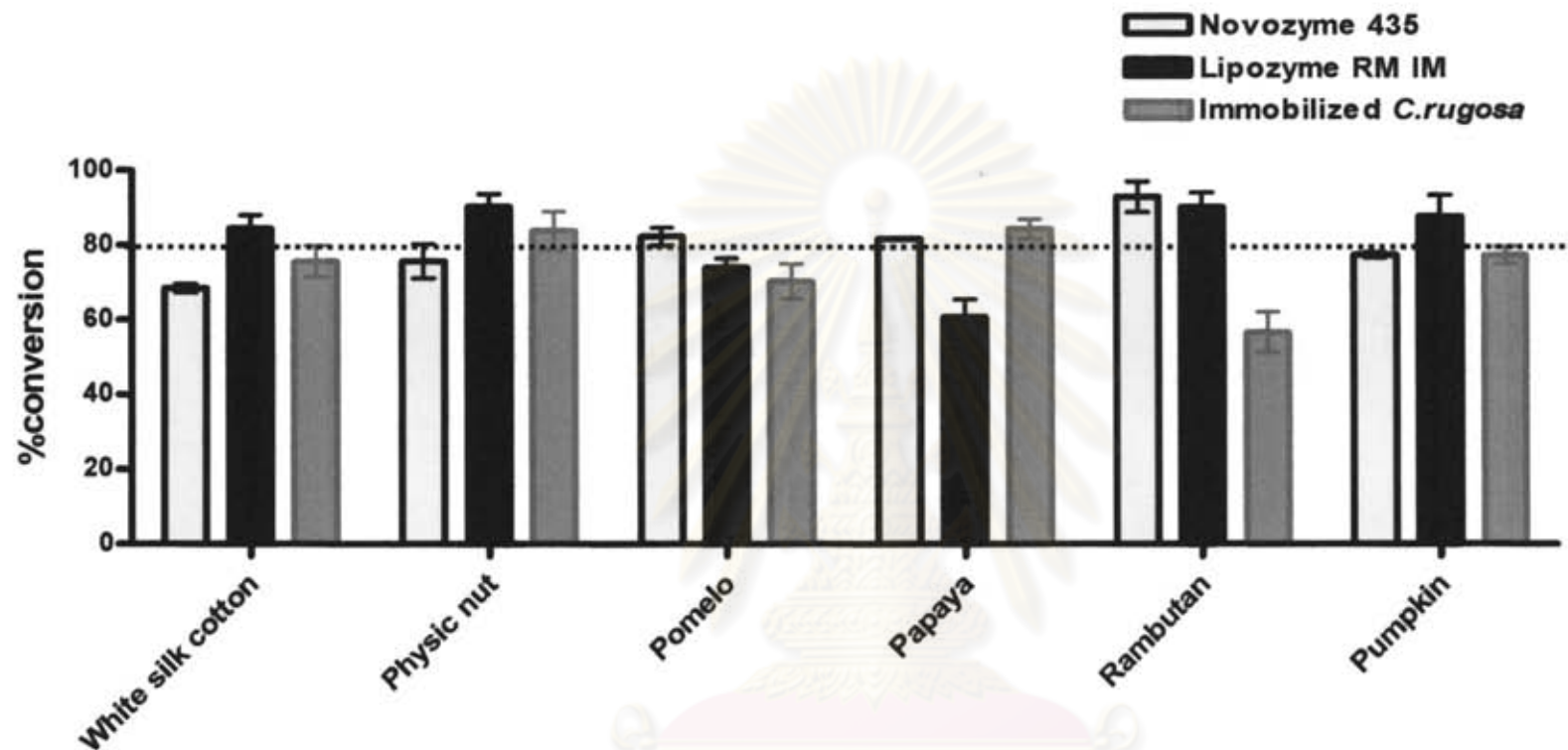
Figure 4-16. The percentage conversion of biodiesel from non-edible and waste oils catalyzed by 30% (w/w of oil) immobilized *Candida rugosa* lipase; grey column and later mixed with 1:3 mole ratio of methanol. The addition of methanol was performed using 6 steps at 0, 2, 4, 6, 6 and 10 hours by stirring the mixtures with magnetic stirrer for 12 hours at 40°C. The percentage conversion of biodiesel form non-edible and waste oils catalyzed by 20% (w/w of oil) Novozyme® 435; white column and Lipozyme® RM IM; black column and later mixed with 1:3 mole ratio of methanol. The addition of methanol was performed using 3 steps at 0, 8 and 16 hours by stirring the mixtures with magnetic stirrer for 24 hours at 55°C. Samples were taken from the reaction mixture and later analyzed for the products by high-performance liquid chromatography. The experiment was performed in triplicates, and the data are mean ± S.D.

4.7 Stability of immobilized *andida rugosa* lipase

4.7.1 Thermal stability
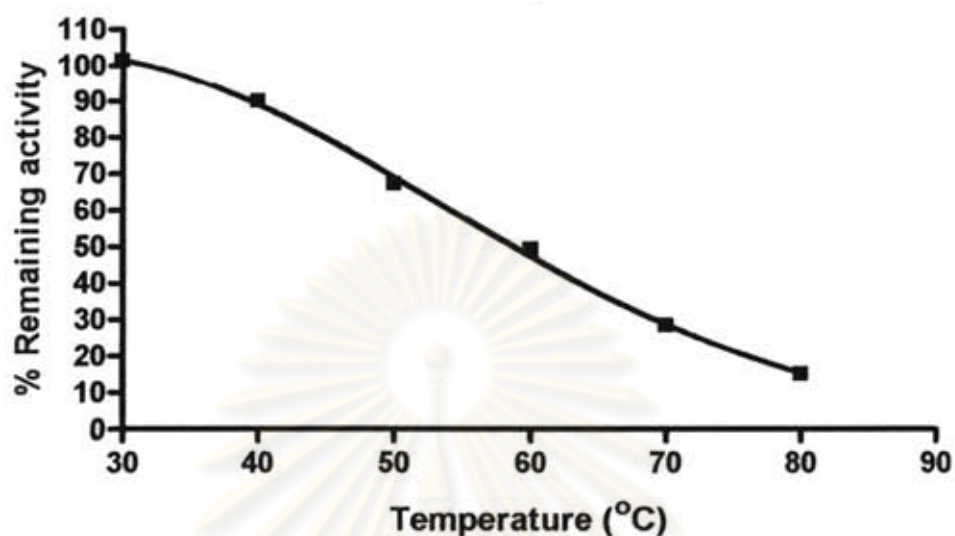
Figure 4-17. Effect of temperature on thermostability of immobilized *Candida rugosa* lipase. 2 mg of immobilized lipase were incubated at various temperatures from 30 to 80 °C in the temperature controlled heating block for 15 min. The results were average values of triplicate experiments.

500 M phosphate buffer, pH 6, 8 mg/ml of lipase solution, 30 min time of immobilization at 30 °C with t-butanol as adjuvant were selected as the optimal conditions for immobilization of *Candida rugosa* lipase to study the thermostability on residual activities of immobilized lipase using the method described in 3.4.10.1. The optimal temperature for thermostability study was screened by incubating 2 mg of immobilized lipase at various temperatures from 30 to 80 °C in the temperature controlled heating block for 15 min. Then, the residual activities were determined as percentage yield of activity at different temperatures compared to the activity at the optimal conditions. The results were shown in Figure 4-17.

Since the optimal temperature for the immobilization was found to be 30 °C, therefore, the selected temperature for this experiment was initially started at 30°C. From Figure 4-17, the results showed that the percentage of remaining activity significantly decreased from 100% to 15% when the temperature was increased from 30°C to 80°C and at 60°C, 50% of the activity was retained. Hence, the optimal temperature to study the thermal stability of immobilized *Candida rugosa* lipase was selected at this temperature. Then, the thermal stability of immobilized lipase was carried out by incubating 2 mg of immobilized lipase at 60°C. Later, the samples were periodically taken and the residual activities were determined as the percentage yield of activity compared to the activity at the optimum conditions. Then, the half life time ($t_{1/2}$) were calculated as shown in Appendix D. The results were expressed as the percentage of relative of the residual activity and half life time as shown in Figure 4-18. It was shown that, half-lives of the immobilized *Candida rugosa* lipase, Novozyme 435 and Lipozyme RM IM at 60°C was 14.35, 316.67 and 59.45 min respectively.
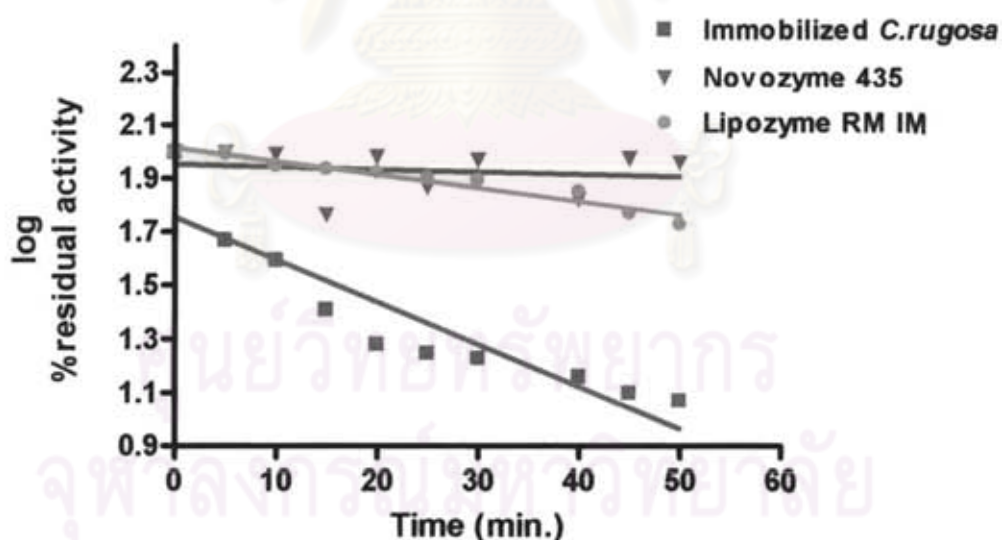
Figure 4-18. Half-life time ($t_{1/2}$) of immobilized *Candida rugosa* lipase, Novozyme 435 and Lipozyme RM IM. 2 mg of immobilized enzyme was incubated at 60 °C. The results were average values of triplicate experiments.

4.7.2 Repeated use of the immobilized *Candida rugosa* lipase

4.7.2.1 Repeated use on transesterification

Figure 4-19. Operational stability of immobilized *Candida rugosa* lipase catalysis for transesterification. The reactions were carried out in a mixture of 2 g of palm oil, 1:3 oil:methanol ratio, six steps addition mode of methanol, 20% (w/w of oil) immobilized lipase, 5% (v/w of oil) water and continuously stirred at 40°C for 12 hr. The lipase was transferred into the same system for a new cycle after completion of the former reaction in 12 hours. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

The immobilized *Candida rugosa* lipases were used consecutively in a series for biodiesel production. The optimal conditions applied in this reusability study were 2 g of palm oil, 30% (w/w of oil) of immobilized lipase, 1:3 oil:methanol ratio and water content of 5% (v/w of oil) and magnetically stirred in a water bath at 12 hr. Under these conditions, approximately 90% of FAME content were obtained. The immobilized lipase was rinsed with water, t-butanol and hexane after each batch reaction to select the optimal washing solution and to remove glycerol and

oil in carriers. The dried immobilized lipase was dried in the desiccators and later used in the next batch reaction composed of new substrates. The results of each batch for the production of FAME content were graphically shown in Figure 4-19. The % conversion in every condition decreased in the second and third cycles of use with the first cycle set at 100%. After 3 uses, it can be seen that immobilized lipases washed with water could retain approximately 45% of its initial activity after 4 more cycles. The immobilized lipases washed with hexane could only retain about 15% of its initial activity after 3 more cycles. On the other hand, when the enzymes were washed with t-butanol, 15% of its initial activity was retained while the enzyme with no washing lost all of its activity after 5 and 6 cycles.

4.7.2.2 Repeated use on hydrolysis

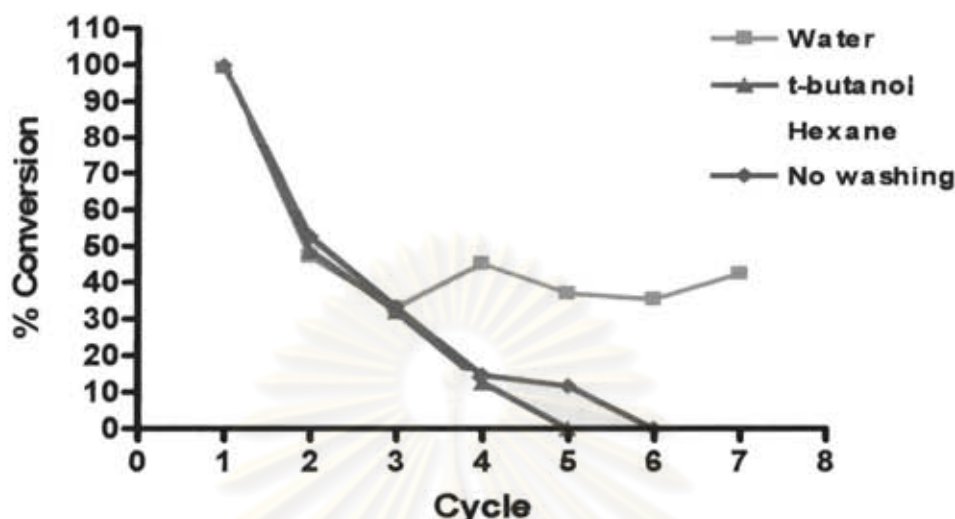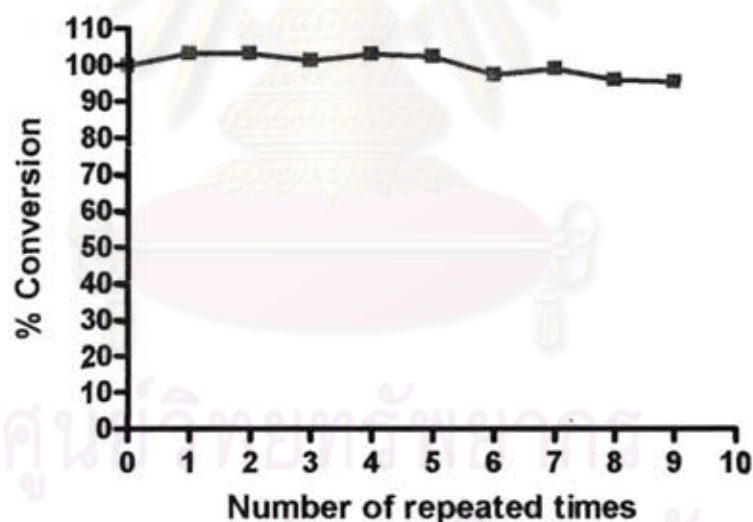Figure 4-20. Operational stability of immobilized *Candida rugosa* lipase catalysis for hydrolysis. The reactions were carried out in a mixture of 3 g of palm oil, 20% (w/w of oil) immobilized lipase, 100% (v/w of oil) water and continuously stirred at 40°C for 9 hours. The lipase was transferred

into the same system for a new cycle after completion of the former reaction in 9 hours. Percent conversion shown on the y-axis is the mean ± SD of three individual experiments.

The immobilized *Candida rugosa* lipases were used consecutively in a series for hydrolysis. The optimal conditions applied in this reusability study were 3 g of palm oil, immobilized lipase content of 20% (w/w of oil) and water content of 100% (v/w of oil) and magnetically stirred in a water bath at 40°C for 9 hours. Under these conditions, approximately 97% of FFA content were obtained. The immobilized lipase was rinsed with water after each batch reaction and to remove glycerol and oil in carriers. The dried immobilized lipase was dried in the desiccators and later used in the next batch reaction composed of new substrates. The results of each batch for the production of FFA content were graphically shown in Figure 4-20. From the results, it was shown that no evident decrease of the lipase activity was observed during the first cycle. Additionally, immobilized lipase could be used at least 10 repeated times without important loss of activity.

CHAPTER V

DISCUSSION

5.1 Selection of support

Lipases may exist in two different structural forms, the closed one where a polypeptide chain (lid or flat) isolates the active center from the medium, and the open form where this lid moves and the active center is exposed. This equilibrium is shifted towards the open form in the presence of hydrophobic surfaces, where the lipase becomes adsorbed by the large hydrophobic pocket around their active center and the internal face of the lid. Moreover, lipases may become adsorbed to other hydrophobic surfaces following a similar mechanism (Scheme1): droplets of oils, hydrophobic proteins, or on the surface of hydrophobic supports. The immobilization of lipases by their interfacial activation on hydrophobic supports may be suitable and simple method (Cabrera et al., 2009).
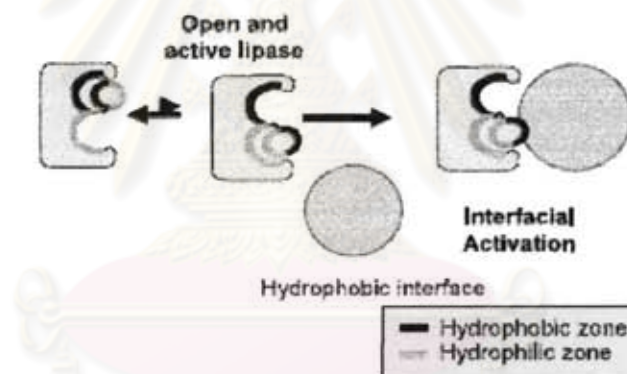
Scheme1. Mechanism of lipase in aqueous medium (Palomo et al., 2002)

Obviously, the characteristics of immobilized enzyme preparations are governed by the properties of both the enzyme and the carrier material. Numerous supports for the immobilization of lipases have been used. There are varieties of support materials such as chitosan (Feresti et al., 2007, Ye et al., 2007 and Amorim et al., 2003), silica (Blanco et al., 2004 and Blanco et al., 2007) and $CaCO_3$ (Ghamgui et al., 2004 and Rosu et al., 1998).

In this study, *Candida rugosa* lipases were immobilized on 7 types of commercial hydrophobic supports namely Amberlite XAD 2, Amberlite XAD 4, Amberlite XAD 7, Amberlite XAD 16, Amberlite XAD 761, Sepabeads EC-BU and Sepabeads EC-OD. Comparative studies indicated that dramatic differences exist in the activity of lipases supported on different materials. Figure 4-1 showed lipase activity of the 7 types of hydrophobic supports. From the graph, more than 20% activity yield were obtained when dried Amberlite XAD 7 and Amberlite XAD 761 as well as wet Amberlite XAD 16 and Sepabeads EC-OD were used. The choice of supports is often limited by some other factors related to their structure, such as the specific surface area, pore shape and particle size (Lei *et al.*, 2004, Panzavolta *et al.*, 2005, Blanco *et al.*, 2007 and Ghiaci *et al.*, 2009). Supports used in this work with pore diameter of around 10-60 nm, allow only the immobilization of small enzymes within the pores. In contrast, the larger enzymes can only be adsorbed on the external surfaces of the particles. Bosley and Clayton studied the adsorption of lipase from *Mucor miehei* on controlled pore glass of eight different pore sizes. They concluded that the larger the pore diameter, the faster the adsorption rate (Bosley and Clayton, 1994). For instance, the internal surface may not be fully used to adsorb enzyme molecules, even when pore sizes are wide enough (Blanco *et al.*, 2007). Another key factor is the specific surface area. There is probably significant contribution of the micropore regions in the immobilization of the enzyme (Table A-1) and the external surface area of the support is also accessible to the enzyme molecules. The support can affect the partitioning of substrates, products, and water in the reaction mixture, and thereby, can influence the catalytic properties of the enzyme (Palomo *et al.*, 2002).

The immobilized enzymes with these supports were used to catalyze transesterification for the selection of the appropriate support. The conditions were 20 % (w/w of oil) immobilized lipase, the ratio of oil to methanol was one to three and three addition steps of methanol. The reactions were carried out at 40 °C for 24 hours with continuous stirring by magnetic stirrer. The results were illustrated in Figure 4- 2 and the production of biodiesel obtained was expressed as percent conversion. It could be seen

that Sepabeads EC-OD gave the highest conversion approximately, 34%. Therefore, Sepabeads EC-OD was selected as the optimal support for the subsequent immobilization.

Sepabeads EC-OD which was selected as the carrier materials for optimal immobilization of *Candida rugosa* lipase are highly porous methacrylic polymer matrix spherical beads, with a high hydrophobicity for enzyme immobilization, low swelling tendency in high molar solutions. The support is resistant in common solvents and shows high resistance to microbial attack. The immobilization by physical adsorption on hydrophobic support, Sepabeads EC-OD is suitable to stabilize this enzyme. This result is consistent with other results concerning the immobilization of lipases on this kind of support. From the literature, immobilized lipase QL from *Alcaligenes* sp. by adsorption on octadecyl-sepabeads was studied. This immobilization technique improved the enzyme properties. The immobilized preparation exhibits a 135% of catalytic activity for hydrolysis of ρ-nitrophenyl propionate as compared to the soluble enzyme. The thermal stability of the immobilized enzyme is highly improved, a half-life time of 12 hr when incubated at $80^{\circ}C$ and the optimal temperature was increased from $50^{\circ}C$ (soluble enzyme) up to $70^{\circ}C$ (immobilized enzyme) (Wilson *et al.*, 2006). Moreover, using this kind of carrier presents an additional advantage which is the possibility of reuse of the support due to the reversible adsorption of the enzyme on the support (Palomo *et al.*, 2003).

5.2 Optimization of immobilization of lipase

When the Sepabeads EC-OD were selected, they were then prepared for the studies of the optimized immobilization of *Candida rugosa* lipase. Previous studies have shown that many factors can affect the activity recovery and reusability of enzymes in immobilization process. Some of the most important factors are the properties of the enzyme molecule, concentration of enzyme, temperature, ionic strength, pH, water and solutes present. Hence, the effect of immobilization parameters, i.e. pH, ionic strength, protein loading, immobilization time, immobilization temperature and adjuvant were investigated.

5.2.1 Effect of pH on immobilization

The optimum pH for lipase activity varies with the enzyme species. From the results, the effect of changing pH and the percent lipase activity and activity yield of immobilized lipase were shown in Figure 4-3. It could be seen that the shape of the graph is bell-shaped curve which the maximum activity of immobilized lipase was obtained at pH 6.0. The activity of lipase increased with the increment of pH values. This result suggested that electrostatic forces are important for the adsorption; changes in pH over the isoelectric point of the protein will have a large impact on the protein binding constant. The protonation and deprotonation of the charged functional groups were dependent upon the pH of the solution (Lei et al., 2009). Since isoelectric point of lipase from Candida rugosa is 4.6, overall net charge is close to 0. The lipase can be easily adsorbed to the nonionic or hydrophobic support by hydrophobic interaction. Moreover Candida rugosa is quite stable in acidic environments and the optimum pH values of Candida rugosa are between 6.0 and 7.0 which are correlated to the best pH during the enzyme immobilization process. It has been reported that the optimum of immobilized Candida rugosa lipase is slightly higher and lower than the free enzyme (Pereira et al., 2001, Blanco et al., 2004 and Yeşloğlu Y, 2005). However, the lipase activity started to decrease when the pH subsequently rose to more than 10. At more acidic or alkali pH, the denaturation of lipase tends to be increasing, like other proteins, which was in most cases greatly depended on the pH of the solution.

5.2.2 Effect of ionic strength on immobilization

The effect of ionic strength on immobilization was investigated and the results were shown in Figure 4-4. It could be seen that both lipase activity and activity yield of immobilized lipase were highest in 500 mM phosphate buffer pH 6.0. The activity of lipase and percentage of activity yield increased dependent on

increasing ionic strength from 10 mM to 500 mM. Conventionally, adsorption of proteins by hydrophobic interaction is stronger when the ionic strength is increased (Bastida *et al.*, 1998). Since at pH 6, lipase will exhibit net negative charge, therefore, the increase of ionic strength will gradually decrease the charges on enzyme molecules until the net charge reached near zero resulting in more pronounced hydrophobic interaction between enzyme and the support. In contrast, when the ionic strength was elevated from 500 mM to 1M, the activity of lipase and percentage of activity yield were decreased indicating that the higher ionic strength initiates more hydrophobic environment around the active sites. Accordingly, the numbers of active enzyme was further reduced.

5.2.3 Effect of protein loading on immobilization

The amount of enzyme loaded on the surface has a large effect on the performance of biocatalytic surface. In this study, different amount of enzymes were immobilized on the supports, by varying the protein loading (mg/ml) of enzyme solution from 1 to 10 mg/ml. The effect of protein loading on activities of lipase was shown in Figure 4-5. It could be seen that the activity of lipase significantly increased when the protein loading was increased. The highest activity of lipase was achieved when protein loading at 10 mg/ml was used. It is considered that the higher lipase loading makes the lipase form an intermolecular steric hindrance, which restrains the diffusion of the substrate and product. The similar phenomena were also observed in the previous studies. It was found that the activity of 12 g immobilized lipase/g ceramic from *Penicillium expansum* reached the maximum (Huang and Cheng, 2008). It is reasonable to conclude that the binding site on the surface areas of the support are limited (Jiang *et al.*, 2008 and Chang *et al.*, 2008) and the enzyme molecules need enough space for catalyzing the reaction of the substrate (Lei *et al.*, 2009). As could be seen from the figure, the activity of enzyme at 8 mg/ml was not much different from the activity obtained from 10mg/ml of the

enzyme. Therefore, in order to reduce the cost for further applications, 8 mg/ml was finally used as optimal amount of protein loading from this study.

5.2.4 Effect of immobilization times

The residual activity of lipase solution was checked for each time of immobilization at various temperatures as described in 3.4.3.4. The relationship of the residual activity with immobilization time at various temperatures was shown in Figure 4-6. The amount of soluble protein was rapidly decreased with the increment of the immobilization time. When lipase was incubated at $10°C$ for 180 min, the residual activity of lipase appeared unchanged. Furthermore, when lipase solutions were incubated at $20°C$ and room temperature (around $25°C$), the results showed that the residual activity leveled off until 120 min in both temperatures. At 30, 40, 50 and $60°C$, the residual activity of lipase solution gave the similar pattern that they significantly decreased from initial time of the incubation to around 20-30 minutes and stayed unchanged to 60 minutes. An extra incubation time of the immobilization leads to a plateau value. As long as the immobilization process continues, the lipase activity will decline. The protein conformation of lipase might be denatured leading to the limitation of immobilization processes from the effect of longer coupling reaction time. This result was similar to the immobilization studied by Chang et al. The immobilization time of Candida rugosa lipase on poly (alpha-glutamic acid) was varied from 1 to 18 hours. They found that the optimal immobilization time was less significantly effective after more than 6 hours (Chang et al., 2008). Similarly, Lei et al. studied the immobilization time of porcine pancreas lipase for 2 to 10 hours. The result indicated that the highest activity was obtained under immobilization time of 8 hours. After 8 hours of immobilization, the activity of immobilized enzyme was decreased (Lei et al., 2009). Therefore, the optimal time for immobilization was 3hr, 2hr, 1hr and 20-30 min at 10, 20, 25 (RT) and 30-60 $°C$

respectively and subsequently selected for the next experiment namely, optimal condition for the temperature.

5.2.5 Effect of temperature on immobilization

Lipase was immobilized on Sepabeads EC-OD at different temperatures; 10, 20, 25, 30, 40, 50 and 60 °C. The results in Figure 4-7 showed that the activity of lipase was initially increased under the temperatures from 10 to 30 °C and the highest activity and activity yield were obtained when the temperature of immobilization reached 30 °C. As shown in Figure 4-7, this result is consistent with the optimal temperature of the free Candida rugosa lipases obtained from other previous studies (Hung et al., 2003). Hung et al. reported that the optimum temperature of the lipase was not altered by immobilization. The activity of both free and immobilized lipases of Candida rugosa on chitosan were highest at 30°C. In contrast to Hung et al , Wilson et al. reported that the optimum temperatures of immobilized lipase QL from Alcaligenes sp. on octadecyl-sepabeads were altered from 50 to 70°C (Wilson et al., 2006). The alteration of optimum temperature of immobilized enzyme might be depending on type of enzymes and nature of supports. On the other hand, when the temperatures were elevated from 40 to 60 °C, the activities were reduced. This can be simply explained that the excessive temperature provided to the immobilization system would inhibit the activity of the free lipase, because lipase might be inactivated by thermal denaturation. From the optimal immobilization time obtained in section 4.2.4, the activity of immobilized lipase was found to be suitable at 30 °C. Therefore, the optimal immobilization time was 30 minutes at 30 °C and subsequently selected for the next experiment.

5.2.6 Effect of adjuvant on immobilization

Once the optimal conditions of 500 mM phosphate buffer, pH 6, 8 mg/ml of *Candida rugosa* lipase solution for immobilization at 30 °C for 30 min were obtained, the effect of adjuvant on the activity of immobilized *Candida rugosa* lipase were examined by using two categories of adjuvants namely; alcohol and detergents.

5.2.6.1 Effect of concentration of adjuvant

The concentrations of each type of adjuvant necessary to obtain the desired effect of the highest concentration of each type of adjuvant with no damage of the enzyme activity had to be determined. From Table 4-1, it was shown that the concentrations of various adjuvants with the activities of *Candida rugosa* lipase solution. The highest concentration of each adjuvant with maximum of lipase activity was selected and later used for immobilization.

5.2.6.2 Effect of the type of adjuvant

The relationship between the lipase activity and adjuvant was shown in Figure 4-8A. The activity and the ability to be non-covalently immobilized on hydrophobic supports are known to be closely related to the hydrophobicity environment. Thus, the presence of hydrophobic adjuvant possibly contributes to a rearrangement of the tertiary structure involving a higher accessibility of some hydrophobic side chains of amino acids, which in such less polar medium become more to the surface of the protein. Only small-size of hydrophobic adjuvant necessary to obtain the desired effect of decreasing the hydrophobicity of the pore channels for the enzyme was adsorbed (Blanco *et al.*, 2007). Moreover, two forms of lipase show very different activity; closed form (inactive form) and open form (active form). If

the enzyme was able to fix with higher activity, the final immobilized preparation may be more active than the native one (Mateo et al., 2007). Hydrophobic and small substrate such as detergent and short chain alcohol have been described to promote the interfacial activation of the lipase yielding the stabilization of the open form of the lipase (Fernândez-Lorente et al., 2006). From these features, strategies to get immobilized lipases molecules with improved activity have been developed, trying to fix the open form of the lipase. This assumption has been supported by López-Serreno et al. that improvement of lipase activity was found when the enzyme was prepared in the presence of SDS (López-Serreno et al., 2002). Furthermore, the stabilization of the fully open forms of lipases adsorbed to supports is extremely essential especially using immobilized lipase as catalyst in biodiesel production. To this goal, immobilized lipase was incubated in the presence of adjuvants such as methanol, ethanol, iso-propanol, butanol, t-butanol, SDS, ethylene glycol, tween 80 and triton X-100. The result showed that highest lipase activity with p-NPP was obtained when t-butanol was used that the activity of lipase was increased approximately 45%. In addition, increase of the conversion of biodiesel synthesis was about 43%, 44% and 46% when the lipase was immobilized in the presence of butanol, t-butanol and triton X-100, respectively (Figure 4-8B).

5.3 Transesterification and hydrolysis catalyzed by immobilized Candida rugosa lipase

After the optimal conditions for immobilization of Candida rugosa lipase from section 4.2.1-4.2.6 were obtained, the activity of immobilized lipase was investigated on transesterification and hydrolysis. The transesterification and hydrolysis reactions were conducted as described in section 3.4.4. From Figure 4-10, it was shown that 45.23 % (±2.43) biodiesel and 97.21% (±0.69) free fatty acids were obtained from transesterification and hydrolysis, respectively. From the results, it could be seen that hydrolysis may be more

preferable than transesterifi ation for the catalytic properties of lipase. Obviously, lipase is generally categorized as hydrolases which catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids (Hung *et al.*, 2003). Consistent to this study, it was the hydrolytic activities of *Candida rugosa* which was higher than synthetic activity (Teng *et al.*, 2009).

5.4 Screening of raw materials for feedstock

5.4.1 Extraction of seed oil

The oil of non-edible and waste plant seeds was extracted by using soxhlet extraction method. 15 g of plant seeds were packed in a thimble and the oil was extracted with 250 ml *n*-hexane for 6 hr. The extracted oil was then measured to calculate the content of oil in the plant seeds shown in Table 4-3. The %oil content from non-edible and waste plant seed oils were in the range of 25 % to approximately 72 % dry weight seeds. The oil yield from the non-edible and waste plant oils itself is always the key factor to decide the suitability of a feedstock for biodiesel production. From Table 4-3, it can be seen that wild almond was the plant yielding the highest oil (72%). On the other hand, among the various non-edible oils shown in Table 4-3, physic nut was found to give the high yield (43%). However, the oil yield depends on many factors such as plantation and oil extraction techniques (Gui *et al.*, 2008).

5.4.2 Fatty acid composition analysis

Another important criterion to determine the suitability of oil as a raw material for the production of biodiesel is the composition of the oil itself. The composition of oil will subsequently determine the properties of the biodiesel obtained. The effect of oil composition on the properties of the biodiesel produced will be discussed in the

subsequent section. The composition for various types of non-edible and waste oils was shown in Table 4-4. From this table, the major oil compositions in both non-edible and waste plant oils were tabulated. The major fatty acids content in both non-edible and waste oils are oleic, linoleic and palmitic acid, while the latter include lauric, myristic, palmitoleic, stearic, linolenic, arachidic and behenic acid. Furthermore, the fatty acids in the oils are further categorized into saturated and unsaturated fatty acids which are also an important for the storage stability of biodiesel. In this concept, the oxidative stability is obtained based on the relative rates of oxidation of these positions in unsaturated fatty acid as well as their amounts. Bouaid et al. reported that the oxidative stability of the oil may be more strongly influenced by the presence of small amounts of highly unsaturated fatty. Two important factors affecting the degradation of biodiesel were also observed in their study, which are water content and air exposure (Bouaid et al., 2007). However, the effect of the presence of unsaturated fatty acid on the storage stability of biodiesel can be avoided by taking proper precaution during the storage such as limiting contact to oxygen and exposure to light and moisture.

5.4.3 Characterization of oil

Apart from the oil yield of the raw materials, the properties of biodiesel vary accordingly to the fatty acid composition in the feedstock oil which is used to produce biodiesel. The properties include saponification number, iodine value, cetane number, and viscosity. Among all the properties listed in Table 4-4, it was shown that the calculated saponification number (SN), iodine value (IV) ranged from 195 to 204 and 3 to 147, respectively. Cetane number (CN) and viscosity (η) values among the species varied from 40 to 66 and 2.29 to 3.95, respectively.

CN is a significant expression of diesel fuel quality among a number of other measurements that determine overall diesel fuel quality. CN is actually a measure of a fuel's ignition delay, the time period between the start of injection and start of

combustion of the fuel. Fuels with higher CN, which have shorter ignition delays, provide more time for the fuel combustion process to be completed. Hence, higher speed diesels operate more effectively with higher CN fuels. This is one of the important parameters which are considered during the selection of FAMEs for use as biodiesel. The different countries or organizations have specified different minimal values. Biodiesel standards of Thailand (2007), USA (ASTM D6751-07a) and European Standards Organization (EN 14214:2003) have set value as 51, 47 and 51, respectively. Among the FAMEs of 9 species, 7 species (No.1–2, 4–8) have CN value higher than 51 ($\pm$2.5), which is the highest minimal value among the three biodiesel standards (Winayanuwattikun et al., 2008).

The degree of unsaturation, which is measured as IV, is an important considerable factor for the selection of FAMEs. The unsaturated fatty acid component in FAMEs is required as it restricts the FAMEs from solidification. However, the higher degree of unsaturated FAMEs is not suitable for biodiesel. The unsaturated molecules react with atmospheric oxygen and are converted to peroxide, crosslinking with the other unsaturated molecules. The material may be come polymerized into a plastic like body. It makes an internal combustion engine quickly gummed up with the polymerized FAMEs. To avoid this situation, biodiesel standards have set a maximum limit of IV in their specifications. All of 7 species, which qualify the specification of CN, also meet the specification of IV. All of them have IV less than 120, the lowest maximum limit among the three biodiesel standards (Winayanuwattikun et al., 2008).

Another important criterion for the selection of FAMEs is viscosity. The fuel viscosity significantly affects the atomization process that is the initial stage of combustion in a diesel engine. High viscosity interferes with injector operation, resulting in poorer atomization of the fuel spray, leading to the fuel injector operation problems such as injector coking, oil ring sticking and thickening and increased carbon deposits. The conversion of vegetable oil to their FAMEs results in a marked reduction in viscosity (Winayanuwattikun et al., 2008).

The values obtained were used to predict the quality of oil for use as biodiesel. It was found that biodiesel obtained from 6 plant species; white silk cotton, physic nut, pomelo, papaya, rambutan and pumpkin met the major specification of biodiesel standards of USA, and European Standard Organization.

All of the non-edible and waste plant oils were then used as substrates for transesterification catalyzed by immobilized *Candida rugosa* lipase, Novozyme[®] 435 and Lipozyme[®] RM IM in comparison.

5.5 Optimization of the transesterification reaction

In this study, the effects of various factors on the transesterification catalyzed by *Candida rogosa* lipase immobilized onto Sepabeads EC-OD were investigated. Biodiesel yield was increased significantly by each sequential variable, namely, addition mode of methanol, molar ratio of oil:methanol, enzyme loading, water content , reaction time and temperature.

5.5.1 Effect of addition mode on transesterification

Watanabe *et al.* reported that more than 1/2 molar equivalent of methanol is insoluble in vegetable oils and immobilized lipases are easily inactivated by contacting with insoluble methanol (Watanabe *et al.*, 2002). It is also known that lipase from *Candida antarctica* are deactivated when exposed to high concentration of methanol >0.5 M equivalent of MeOH for the stoichiometric amount (Shimada *et al.*, 2002). Hence, addition mode has been suggested as a means of circumventing the deactivation problem. Lu *et al.* studied the number of added times for methanol ranging from 1 to 6 and reported that the conversion was about 88% by more than three successive additions of methanol. So three-step methanolysis was sufficient to convert lard to FAME (Lu *et al.*, 2007). Furthermore, Nie *et al.* also studied the effect of methanol addition to the reaction. Methanol addition was performed from 1 to 10

times and they reported that when the methanol was stepwisely added more than three times, the conversion could be increased to 95% (Nie *et al.*, 2006). The effect of addition mode of methanol from one to seven steps on transesterification was studied. From Figure 7, very small conversion of 3.16 % (±2.46) and 4.12 % (±3.95) % were obtained when three moles of methanol were added at once and two steps respectively. The yield suddenly rose from 4.12 % (±3.95) to 31.88 % (±3.48) when the adding step of methanol increased from two to three steps and increased further from 31.88 % (±3.48) to 53.90 % (±0.86) from three to six steps. Therefore, six step addition mode of methanol was most effective for the production of biodiesel, approximately 50% of fatty acid methyl ester were obtained.

5.5.2 Effect of oil: methanol molar ratio on transesterification

The molar excess of alcohol over fatty acids contained in TAG always increases transesterification yield but it can also inactivate the enzyme. In particular, when the alcohol is insoluble in reaction mixture, it forms emulsion and the size of droplets depends on intensity of stirring (Antczak *et al.*, 2009). However, at least three molar equivalents of methanol are required for the complete conversion of the oil to FAME. Dizge and Keskinler studied the oil:methanol ratio from 1:1 to 1:10. They reported that the highest methyl ester yield could be obtained at the oil:methanol molar ratio of 1:6 and the higher methanol concentration (1:10 molar ratio) would decrease the methyl ester yield (Dizge and Keskinler., 2008). In addition, Liu *et al.* reported that the biodiesel yield was decreased when the oil:methanol was over 1:15. Additionally, when the amount of oil:methanol ratio was excessive, the glycerol separation becomes more difficult (Liu *et al.*, 2007). Therefore, the effect of oil: methanol molar ratio was studied at different ratios from 1:3 to 1:9 and the results were shown in Figure 8. The yield obtained for FAME was approximately 48% when the oil: methanol molar ratio increased from 1:3 to 1:7. However, when the ratio was increased to 1:8 and 1:9, the FAME yield was

decreased to 8.27 and 2.75 %, respectively. Therefore, in order to reduce the cost for further applications, 1 to 3 oil:methanol ratio was subsequently used as optimal oil:methanol ratio from this study.

5.5.3 Effect of enzyme loading on transesterification

The effect of immobilized lipase concentration on conversion was performed with enzyme concentrations of 10%, 20% and 30% w/w of oil. From Figure 9, it was shown that the FAME yield increased rapidly to 72% when the amount of lipase was increased up to 30% (w/w of oil). Obviously, more lipase showed abundant activated sites and sufficient mass contact, consequently the FAME yields were higher (Ghamgui et al., 2004, Rosa et al., 2008, Chen et al., 2009 and Dizge et al., 2009). In addition, the FAME content increased along with the increase in enzyme content because the more lipase available, the more substrate molecules can be adsorbed onto the active center of the lipase (Chen et al., 2009). Therefore, there exists an optimum enzyme loading leading to high conversion rate. The phenomenon has also been found by some other researchers. This result is in good agreement with results obtained by Lu et at. The synthesis of FAME from lard catalyzed by immobilized Candida sp. 99-125 was found to increase rapidly when the amount of lipase was increased to 20% (w/w) (Lu et at., 2007). Furthermore, Köse et al. studied the effect of immobilized Candida antarctica lipase quantity on alcoholysis of cotton seed. It was found that the FAME content was increased by increasing lipase quantity up to 30%. It was also seen that the highest FAME formation (83.6%) was observed with the reaction using 30% lipase based on oil weight (Köse et al., 2002). So from this study, the immobilized lipase at 30 % by weight of oil was selected for the optimal condition of transesterification.

5.5.4 Effect of wate content on tansesterification

Water plays an important role in enzyme structure and function (Ghamgui *et al.*, 2004). It is well known that lipase, as a form of protein, requires the presence of water to maintain its active three dimensional structures. The activity of the enzyme in non-aqueous media is affected by the water content. In this study, the effect of water content on the transesterification of palm oil is presented in Figure10. From the result, it was shown that the FAME content rose gradually from 53 to 78% as water content increased from 0.5% to 5% (v/w) of the oil. Similar results had been reported in the previous studies. Lu *et al.* studied the effect of water on methanolysis of glycerol trioleate catalyzed by immobilized lipase *Candida* sp. 99–125. It was found that the biodiesel yield was increased from 35 to 85 % when the amount of water increased from 0 to 20 wt% (Lu *et al.*, 2009). Moreover, the effect of water content on transesterification catalyzed by immobilized lipase from *Candida* sp. 99-125 was tested with increasing water content to 15% (w/w) of oil. The conversion obtained was 98% (Yang *et al.*, 2006). This effect could strongly confirm the hypothesis that the necessary quantities of water to stabilize the hydrophilic groups located on the surface of the enzyme molecule. This results in the changing of topology of active site and lid leading to an active conformation of lipase. Furthermore, activation of the enzyme involves in unmasking and restructuring of the active site through conformational changes of the lipase molecules, which also requires the presence of oil-water interface. Lipase activity generally depends on the available interface area. With the increasing of additional water, the amount of water available for oil to form oil-water droplets were increased, consequently increasing the available interfacial areas (Noureddini *et al*, 2005). The results presented in Figure 10 indicated that the FAME content reached its maximum with the water content of 5 wt% which was about 78% higher than that in absence of water. This supports the fact that amount of water is required to activate the enzyme. However, the amount of FAME was decreased when more than 5% (v/w of oil) of

water was added. In this case, the yield reduction might be caused by the reduced homogeneity of substrate mixtures owing to immiscibility between water and oil compounds (Lu *et al.*, 2007). This result suggests that excessive water content affects the mass transfer of the oil phase of the reaction product, and inhibits transesterification (Hama *et al*, 2006 and Chen *et al.*, 2009).

5.5.5 Effect of times and temperatures on transesterification

Temperature can influence the reaction rate and biodiesel yield. Figure 11 showed the transesterification activity of the immobilized enzyme with variations in temperature at specific times. The experiments were conducted at temperature ranging from 30 to 60 $^{\circ}$C illustrated in Figure. 11. The optimum temperature was 40°C after 12 hours and approximately 87.08 % of fatty acid methyl ester was obtained. This result is in agreement with results obtained by Nie *et al.* who conducted the methanolysis of salad oil using the immobilized *Candida* sp. 99-125 lipase from 27 $^{\circ}$C to 50°C. The highest yield (87%) was observed at 40 $^{\circ}$C when the reaction time was extended to 30 hours (Nie *et al.*, 2006). Furthermore, Lu *et al.* studied the transesterification of lard using the immobilized *Candida* sp. 99-125 lipase at various reaction temperatures from 40°C, 50°C and 60°C. It was found that the highest fatty acid methyl ester at 40°C was obtained and selected as the optimal reaction temperature. Devanesan *et al.* studied the effect of temperature on biodiesel production from *Jatropha* oil using immobilized cell of *P.fluorescens* at 30, 35, 40, 45 and 50°C. It was found that the maximum yield 70%of biodiesel at 40°C was obtained (Devanesan *et al.*, 2007). In addition, the effect of temperature on transesterification of canola oil catalyzed by immobilized *Thermomyces lanuginosus* lipase was studied and 40°C was found to be optimal temperature for biodiesel production (85.8%) (Dizge and Keskinler, 2008). From Figure 11, it was shown that when the reaction temperature increased to 50°C and 60°C, the product started to decrease, which is in agreement with the previous literature report (Dizge *et al.*,

2009 and Rashid *et al.*, 2008). A similar result was obtained by Yang *et al.* They studied the reaction temperature on transesterification of soybean oil catalyzed by *Candida* sp 99-125 and reported that the optimum temperature of the reaction was 40°C. Beyond 45°C, the relative conversion rate of biodiesel declined from 98% to 40% (Yang *et al.*, 2006). The advantage of higher temperature is a shorter reaction time. However, if the reaction temperature exceeds the boiling point of methanol (65°C), the methanol will vaporize from the reaction (Chen *et al.*, 2009) and high temperature certainly causes enzyme denaturation (Yang *et al.*, 2006, Devanesan *et al.*, 2007 and Dizge and Keskinler, 2009). Moreover, the results showed that when increasing the reaction time, the percentage yield of biodiesel was increased up to 12 hours. Thereafter, increase in the reaction time did not have the effect on the production of biodiesel. The phenomenon has also been observed by other researchers. Köse *et al.* studied the reaction time ranges from 2 to 24 hours on methanolysis of cotton seed oil. It was found that the methyl ester conversion was practically constant over reaction time ranges between 7 and 24 hours, indicating optimum reaction time could be 7 hours (Köse *et al.*, 2002).

5.6 Comparative studies of transesterification catalyzed by immobilized *Candida rugosa* lipase with Novozyme® 435 and Lipozyme® RM IM

It is well known that the ability of lipase is highly dependent on sources of lipase, substrates and reaction condition. Thus three different immobilized lipases and non-edible and waste oils were screened for FAME production. Transesterifications were carried out as described in section 3.4.7. The conversion of fatty acid methyl esters from these non-edible and waste plant oils were illustrated in Figure 4-16. From the results, it was shown that the efficiency of biodiesel production was different from the catalysis of Novozyme® 435, Lipozyme® RM IM and immobilized *Candida rugosa* depending on the types of feedstocks. The production of biodiesel obtained from catalysis of three types of immobilized lipases in 6 types of plant seeds were approximately at 70-80 %. However, rambutan oil gave 90% of

biodiesel when catalyzed b both commercial lipases but only 60% was obtained by the immobilized *Candida rugosa* lipase. On the other hand, 80% of biodiesel from papaya oil was obtained when catalyzed by the immobilized *Candida rugosa* lipase when only 60% was obtained from Lipozyme[®] RMIM.

Six out of nine plant oils being surveyed had suitable physical property as feedstock for biodiesel production catalyzed by lipase. Even though white silk cotton, pomelo and pumpkin seeds are agricultural waste but they could not be collected in large quantities. Hence, there is a limitation on the use of these oils for production of biodiesel. Under Thailand condition, physic nut, papaya and rambutan appear to be potential raw materials for the development of oils as diesel fuels. The cultivation and climatic conditions required for plantation of these four species are described here:

Physic nuts or *Jatropha curcas* produce non-edible oil in appreciable quantity and can be grown in large scale on non-cropped marginal lands and wastelands. It is well adapted in arid and semi-arid conditions and has low fertility and moisture demand. It can also grow on moderately sodic and saline, degraded and eroded soil. The ideal density of plants/hectare is 2500. It reaches maximum productivity by five years and can live up to 50 years. There are reports of oil yields of as high as 50% from the seed. Typically, the seed production would be 3.75 ton/ha, with oil yield of 30-35%, giving net oil yield of about 1.2 ton/ha. Although *Jatropha* oil seed is not yet cultivated on a large scale in Thailand, *Jatropha* oil is the major feedstock of the biodiesel program. The projection for plantation of this species was started in 2008 to supplement the utilized edible palm oil for production of biodiesel.

Papaya is a tropical or near tropical species, sensitive to frost and limited to the region between 32° north and 32° south of the equator. It needs plentiful rainfall or irrigation but must have good drainage. While doing best in light, porous soils rich in organic matter, the plant will grow in scarified limestone, marl, or various other soils if it is given adequate care. Optimum pH ranges from 5.5 to 6.7. Papaya plants bear well for 2 years and then productivity declines and commercial plantings are generally replaced after 3-4 years. The papaya black seeds contain 25-48% oil (w/w).

Rambutan is adapted to warm tropical climates and is sensitive to temperatures below 10 °C, and is grown commercially within 15° of the equator. Rambutan flourishes from sea-level to 1,600 or even 1,800 ft (500-600 m), in tropical, humid regions having well-distributed rainfall. The dry season should not last much over 3 months. The tree does best on deep, clay-loam or rich sandy loam rich in organic matter and thrive on hilly terrain as they require good drainage. Optimum pH ranges from 5.0 to 5.7. Rambutan trees may fruit after 2-3 years with optimum production occurring after 8-10 years. The seed kernel yield 37-43% oil (w/w). Both rambutan and papaya are food industrial waste products which can be collected in a large amount. Thus, they might be suitable for use as starting material for the production of biodiesel.

5.7 Stability of immobilized *Candida rugosa* lipase

5.7.1 Thermal stability

The resistance of immobilized lipase to temperature is an important potential advantage for practical applications of this enzyme. The residual activities of immobilized lipase on thermo stability were tested as described in 3.4.10.1. The optimal temperature for thermal stability was studied at various temperatures from 30 to 80 °C. Then, the residual activities were determined as percentage yield of activity at different temperatures compared to the activity at the optimal conditions. The results in Figure 4-17 revealed that the percentage of remaining activity significantly decreased from 100% to 15% when the temperature was increased from 30 °C to 80 °C. However, 50% of the activity was still retained at 60 °C. According to Hung *et al*, they reported that the inactivation of the enzyme occurred when they were treated at high temperature. Free lipase remained stable only up to 39 °C. At 60 °C, the residual activity of immobilized *Candida rugosa* lipase on chitosan was 23 % compared to 12 % for free lipase. Then, the thermal stability of immobilized lipase was carried out by incubating 2 mg of immobilized lipase at 60 °C. Later, the samples were periodically taken and the residual activities were

determined as the percentage yield of activity compared to the activity at the optimum conditions. Then, the half life time (t $_{1/2}$) were calculated as shown in Appendix D. The results were expressed as the percentage of relative of the residual activity and half life time as shown in Figure 4-18. It was shown that, half-lives of the immobilized *Candida rugosa* lipase at 60°C was 14.35 min. When an immobilization of *Candida rugosa* lipase by adsorption on bentonite was studied (Yeşiloğlu Y, 2005), the half –life of the immobilized enzyme was about 45 min, whereas for the soluble free lipase was 17 min at 50°C. Evidently, the immobilization has considerably increased the thermal stability of lipase. This result supported the fact that the strong hydrophobic interaction between the lipase and the hydrophobic carriers enhances the stability of the molecular conformation of the immobilized enzyme. This therefore will increase the thermal stability of the immobilized lipase (Huang and Cheng, 2008). The results indicated that immobilization helps preserve the enzyme structure from thermal inactivation.

5.7.2 Repeated use of the immobilized *Candida rugosa* lipase

The most important advantage of immobilization is the repetitive use of enzyme. The catalyst reusability was carried out to determine the stability of the immobilized lipase. The short operational life of the enzyme is normally caused by the negative effects of excessive methanol and by product glycerol. This phenomenon may be clarified by two explanations. Firstly, the glycerol adsorbed on the surface of immobilized lipase constrains the contact of substrate and enzyme molecules. This can be resolved by washing with a solvent such as acetone and t-butanol (Dizge *et al.*, 2008). Secondly, the lipase was inactivated or desorbed from carriers during repeated uses.

5.7.2.1 Repeated use on transesterification

It has been demonstrated that the cost of lipase accounts for a large part in the total cost of biodiesel production. One of the main advantages of an immobilized lipase is that it can be used repeatedly over an extended period of time (Ghamgui et al., 2004 and Lu et al., 2007). The byproduct; glycerol is the main problem of reusable of immobilized lipase in transesterification because it can deactivate enzymes, particularly in continuous and repeated-batch processes (Antczak et al., 2009). The glycerol molecules were adsorbed on the surface of these carriers thereby forming the hydrophilic coating which made enzyme molecules inaccessible to substrates. Addition of another hydrophilic substance like acetone to the reaction system partially removed glycerol from the lipase environment (Dossat et al., 1999). Du et al. also washed the immobilized lipase with iso-propanol for restoring its activity (Du et al., 2003). In addition, to increase the operational stability of the lipases, washing of used Candida antarctica immobilized lipase with t-butanol or 2-propanol can be an efficient method of regeneration of immobilized lipase (Chen and Wu, 2003). To investigate the stability of the immobilized lipase, the optimal conditions for transesterification were conducted to obtain the production of biodiesel and repeated every 12 hours. Under these conditions, approximately 90% of FAME content was obtained. After completion of the reaction for 12 hours of each cycle, the immobilized lipase was rinsed with water, t-butanol, hexane and acetone in comparison .The purpose was to select the best washing solution for the removal of glycerol and oil from the carriers. The immobilized lipase was dried in the desiccator and later used in the next batch reaction composed of new substrates. The results demonstrated that the % production of FAME in each batch was reduced. After three or four times of uses, immobilized lipase retained approximately 45% of its initial activity

when washed with water. On the other hand, enzyme lost all of its activity after 5 cycles with t-butanol, hexane and acetone as the washing solutions. From the results, water is selected as the best washing solution for cleaning the lipase immobilized on hydrophobic support. It appears that the immobilized lipase on Sepabeads EC-OD was inactivated by methanol or desorbed from carriers during repeated use. Therefore, the reusability of immobilized lipase in hydrolysis of palm oil was examined.

5.7.2.2 Repeated use on hydrolysis

Repeated use of the immobilized lipase in batch hydrolysis of palm oil was tested as described in section 3.4.10.2.2. Under these conditions, approximately 97% of FFA content was obtained. The immobilized lipase was rinsed with water after each batch reaction to remove glycerol and oil in carriers. From the results, it was shown that stability of the lipase activity was evidently observed as in the first cycle. Additionally, immobilized lipase could be used at least 10 repeated times without significant loss of activity. From this result, it strongly confirmed that immobilized lipase on Sepabeads EC-OD was inactivated by methanol. This problem can be reduced by the modification of the enzyme with chemical and genetic techniques for more tolerance ability in the organic solvents. Otherwise, the alternative method is to apply a novel technology for the immobilization of the enzyme.

CONCLUSION

In this research, lipase from *Candida rogusa* was successfully immobilized on selected Sepabeads EC-OD by adsorption. The optimal conditions for the immobilization obtained were as follows: pH 6, 500 mM ionic strength, 8 mg/ml enzyme loading at $30\,^{\circ}$C for 30 min and t-butanol as the adjuvant. When the immobilized lipase- catalyzed transesterification was carried out for the production of biodiesel, the maximal yield of 87% was finally achieved under the following optimal parameters: six step addition mode, 1to 3 molar ratio of oil: methanol, 30% (w/v) of oil enzyme loading at 40ºC for 12 hours. Potential feedstocks from non-edible and waste plant seed oils including physic nut, papaya and rambutan could be highly converted to biodiesel when the reactions were comparatively catalyzed by immobilized *Candida rugosa* lipase and commercial lipases; Novozyme 435 and Lipozyme RMIM. Finally, the enzyme could be reused for three cycles for the production of biodiesel whereas approximately 95% relative hydrolytic activities could be well maintained over ten repeated cycles. Therefore, it can be concluded that the immobilized *Candida rugosa* lipase on Sepabeads EC-OD can catalyze the transesterification for the production of biodiesel as efficiently as the commercial enzymes.

REFERENCES

Amorim, R.V.S., Melo, E.S., Carneiro-da-Cunha, M.G., Ledingham, W.M. and Campos-Takaki, G.M. 2003. Chitosan from *Syncephalastrum racemosum* used as film support for lipase immobilization. Bioresource Technology 89: 35-39.

Antczak, M.S., Kubiak, A., Antczak, T. and Bielecki, S. 2009. Enzymatic biodiesel synthesis-Key factor affecting efficiency of the process. Renewable Energy 34, 5: 1185-1194.

Antolin, G., Tinaut, F.V., Briceno, Y., Castano, V., Perez, C. and Ramirez, A.L. 2002. Optimization of biodiesel production by sunflower oil transesterification. Bioresource Technology 83:111–114.

Bako, K.B., Kova, F.C.S., Gubicza, L., Hansco, J.K., 2002. Enzymatic biodiesel production from sunflower oil by *Candida antarctica* lipase in a solvent free system. Biocatalyst Biotransform 20: 437–439.

Ban, K., Kaieda, M., Matsumoto, T., Kondo, A., Fukuda, H., 2001. Whole-cell biocatalyst for Biodiesel fuel production utilizing *Rhizopus oryzae* cells immobilized within biomass support particles. Biochemical Engineering 8: 39–43.

Barnwal, B.K. and Sharma, M.P., 2005. Prospects of biodiesel production from vegetable oils in India. Renewable Sustainable Energy Reversible 9: 363–378.

Bastida, A., Sabuquillo, P., Armisen, P., Fernandez-Lafuente, R., Huguet, J. and Guisan, J.M. 1998. A single step purification, immobilization and hyperactivation of lipase via interfacial adsorption on strongly hydrophobic supports. Biotechnology Bioengineering 58: 486-493.

Bayramoğlu, G. and Arıca, .Y. 2008. Preparation of poly (glycidylmethacrylate methylmethacrylate) magnetic beads: Application in lipase immobilization. Journal of Molecular Catalysis B: Enzymatic 55: 76–83.

Berchmans, H.J. and Hirata, S. 2008. Biodiesel production from crude Jatropha curcas L. seed oil with a high content of free fatty acids. Bioresource Technology 99: 1716–1721.

Blanco, R.M., Tereros, P., Fernândes-Pérez, M., Otero, C, and Diaz-Gonzâlez, G. 2004. Functionalization of mesoporous silica for lipase immobilization Characterization of the support and the catalysts. Journal of Molecular Catalysis B: Enzymatic 30: 83-93.

Blanco, R.M., Terreros, P., Muñoz, N. and Serra, E. 2007. Ethanol improves lipase immobilization on a hydrophobic support. Journal of Molecular Catalysis B: Enzymatic 47: 13-20.

Bosley, J.A., Clayton, J.C., 1994. Blueprint for a lipase support: use of hydrophobic controlled-pore glasses as model systems. Biotechnology Bioenergy 43: 934–938.

Bouaid, A., Martinez, M. and Aracil, J. 2007. Long storage stability of biodiesel from vegetable and used flying oils. Fuel 86: 2596-2602.

Cabrera, Z., Fernandez-Lorente, G., Fernandez-Lafuente, R., Palomo, J.M. and Guisan, J.M. 2009. Novozym 435 displays very different selectivity compared to lipase from Candida antarctica B adsorbed on other hydrophobic supports. Journal of Molecular Catalysis B: Enzymatic 57: 171-176.

Châabouni, M.K., Bouaziz, I., Boufi, S., Rego, A.M.B. and Gargouri, Y. 2008. Physical immobilization of *Rhizopus oryzae* lipase onto cellulose substrate: Activity and stability studies. Colloids and Surfaces B: Biointerfaces 66: 168–177.

Chang, S.F., Chang, S.W., Yen, T.H. and Shieh, C.J. 2007. Optimum immobilization of *Candida rugosa* lipase on Celite by RSM. Applied Clay Science 37: 67-73.

Chang, S.W., Shaw, J.F., Yang, K.H., Chang, S.F. and Shieh, C.J. 2008. Studies of optimum conditions for covalent immobilization of *Candida rugosa* lipase on poly (γ-glutamic acid) by RSM. Bioresource Technology 99: 2800-2805.

Chen, Y., Xiao, B., Chang, J., Fu, Y., Lv, P. and Wang, X. 2009. Synthesis of biodiesel from waste cooking oil using immobilized lipase in fixed bed reactor. Energy Conversion and Management 50: 668-673.

Cihangir, N. and Sarikaya, E. 2004. Investigation of lipase production by a new isolate of *Aspergillus* sp. World Journal of Microbiology & Biotechnology 20: 193–197.

Cruz, J.C., Pfromm, P.H. and Rezac, M.E. 2009. Immobilization of *Candida antarctica* Lipase B on fumed silica. Process Biochemistry 44: 62–69.

Dandavate, V. and Madamwar, D. 2007. Novel approach for the synthesis of ethyl isovalerate using surfactant coated *Candida rugosa* lipase immobilized in microemulsion based organogels. Enzyme and Microbial Technology 41: 265–270.

Darnoko, D. and Cheryan, M. 2000. Kinetics of palm oil transesterification in a batch reactor. JAOCS 77: 1263–1267.

Demirbas, A. 2003. Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey. Energy Conversion and Management 44: 2093–2109.

Demirbas A. 2008. Studies on cotton seed oil biodiesel prepared in non-catalytic SCF conditions. Bioresource Technology 99:1125–1130.

Demirbas, A. Progress and recent trends in biodiesel fuels. 2009. Energy Conversion and Management 50: 14–34.

Deng, H.T., Xu, Z.K., Dai, Z.W., Wu, J. and Seta, P. 2005. Immobilization of Candida rugosa lipase on polypropylene microfiltration membrane modified by glycopolymer: hydrolysis of olive oil in biphasic bioreactor. Enzyme and Microbial Technology 36: 996–1002.

Devanesan, M.G., Viruthagiri, T. and Sugumar, N. 2007. Transesterification of Jatropha oil using immobilized Pseudomonas fluorescens. Journal of Biotechnology 6 (21): 2497-2501.

Dizge, N. and Keskinler, B. 2008. Enzymatic production of biodiesel from canola oil using immobilized lipase. Biomass and Bioenergy 32: 1274 – 1278.

Dizge, N., Keskinler, B. and Tanriseven, A. 2008. Covalent attachment of microbial lipase onto microporous styrene–divinylbenzene copolymer by means of polyglutaraldehyde. Colloids and Surfaces B: Biointerfaces 66: 34-38.

Dizge, N., Aydiner, C., Imer, D.Y., Bayramoglu, M., Tanriseven, A. and Keskinler, B. 2009. Biodiesel production from sunflower, soybean, and waste cooking oils by transesterification using lipase immobilized onto a novel microporous polymer. Bioresource Technology 100: 1983–1991.

Dossat, V., Combes, D. and Marty, A. 1999. Continuous enzymatic transesterification of high oleic sunflower oil in packed bed reactor: influence of the glycerol production. Enzyme and Microbial Technology 25: 194-200.

Du W, Xu Y, Liu D. 2003. Lipase-catalysed transestrification of soya bean oil for biodiesel production during continuous batch operation. Biotechnology and Applied Biochemistry 38:103–106.

Du, W., Xu, Y., Liu, D., Zeng, J., 2004. Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors. Journal of Molecular Catalysis B: Enzymatic 30: 125–129.

Dwivedi, D., Agarwal, A.K. and Shama, M. 2006. Particular emission characterization of a biodiesel vs diesel-fuelled compression ignition transport engine: A comparative study. Atmospheric Environment 40: 5586-5595.

Fang, Y., Lu, Y., Lv, F., Bie, X., Zhao, H., Wang, Y. and Lu. Z. 2009. Improvement of alkaline lipase from Proteus vulgaris T6 by directed evolution. Enzyme and Microbial Technology 44: 84–88.

Feresti, M.L. and Ferreira, M.L. 2007. Chitosan- immobilized lipase for the catalysis of fatty acid esterifications. Enzyme and Microbial Technology 40: 769-777.

Fernandez-Lorente, G., Palomo, J.M., Mateo, C., Munilla, R., Ortiz, C., Cabrera, Z., Guisan, J.M. and Fernandez-Lafuente, R. 2006. Glutaraldehyde Cross-linking of Lipases Adsorption on Aminated Supports in the Presence of Detergents Leads to Improved Performanc. Biomacromolecules: 2610-2615.

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

Gao, Y.Y., Chen, W.W., Lei, H., Liu, Y., Lin, X. and Ruan, R. 2009. Optimization of transesterification conditions for the production of fatty acid methyl ester (FAME) from Chinese tallow kernel oil with surfactant-coated lipase. Biomass and bioenergy 33: 277–282.

Ghadge, S.V. and Raheman, H. 2005. Biodiesel production from mahua (Madhuca indica) oil having high free fatty acids. Biomass and Bioenergy 28:601–605.

Ghamgui, H., Châabounia, M.K. and Gargouri, Y. 2004. 1-Bytyl oleate synthesis by immobilized lipase from *Rhizopus oryzae*: a comparative study between *n*-hexane and solvent-free system. Enzyme and Microbial Technology 35: 355-363.

Ghiaci, M., Aghaei, H., Soleimanian, S. and Sedaghata, M. E. 2009. Enzyme immobilization. Part 1. Modified bentonite as a new and efficient support for immobilization of *Candida rugosa* lipase. Applied Clay Science 43: 289-295.

Gitlesen, T., Bauer, M. and Adlercreutz, P. 1997. Adsorption of lipase on polypropylene powder. Biochimica et Biophysica Acta 1345: 188-196.

Guan, G., Kusakabe, K., Sa urai, N. and Moriyama, K. 2009. Transesterification of vegetable oil to biodiesel fuel using acid catalysts in the presence of dimethyl ether. Fuel 88: 81-86

Guerreiro, L., Castanheiro, J.E., Fonseca, I.M., Martin-Aranda, R.M., Ramos, A.M. and Vital, J. 2006. Transesterification of soybean oil over sulfonic acid functionalized polymeric membranes. Catalysis Today 118:166–171.

Gui, M.M., Lee, K.T. and Bhatia, S. 2008. Feasibility of edible oil vs. non-edible oil vs. waste edible oil as biodiesel feedstock. Energy 33: 1646– 1653.

Halim, S.F.A. and Kamaruddin, A.H. 2008. Catalytic studies of lipase on FAME production from waste cooking palm oil in a tert-butanol system. Process Biochemistry 43: 1436–1439.

Hama, S., Yamaji, H., Fukumizu, T., Numata, T., Tamalampudi, S., Kondo, A., Nodac, H., Fukuda, H., 2007. Biodiesel fuel production in a packed-bed reactor using lipase-producing Rhizopus oryzae cells immobilized within biomass support particles. Biochemical Engineering 34: 273–278.

Hasan, F., Shah, A.A. and Hameed, A. 2006. Industrial applications of microbial lipases. Enzyme and Microbial Technology 39: 235–251.

Huang, L., and Cheng, Z.M. 2008. Immobilization of lipase on chemically modified bimodal ceramic foams for olive oil hydrolysis. Chemical Engineering Journal 144: 103-109.

Hung, T.C., Giridhar, R., Chiou, S.H. and Wu, W.T. 2003. Binary immobilization of Candida rugoza lipase on chitosan. Journal of Molecular Catalysis B: Enzymatic 26: 69-78.

Ibrahim, C.O. 2008. Development of applications of industrial enzymes from Malaysian indigenous microbial sources. Bioresource Technology 99: 4572–4582.

Iso, M., Chem, B., Eguchi, M., Kudo, T., Shrestha, S., 2001. Production of Biodiesel from triglycerides and ethanol using immobilized enzyme. Journal of Molecular Catalysis B: Enzymatic 16: 53–58.

Jeong, G.T., Yang, H.S. and Park, D.H. 2009. Optimization of transesterification of animal fat ester using response surface methodology. Bioresource Technology 100: 25–30.

Jiang, Y., Guo, C., Xia, H., Mahmood, I., Liu, C. and Liu, H. 2008. Magnetic nanoparticles supported ionic liquids for lipase immobilization: Enzyme activity in catalyzing esterification. Journal of Molecular Catalysis B: Enzymatic. Available online 11 December 2008

Kamini, N.R. and Iefuji, H. 2001. Lipase catalyzed methanolysis of vegetable oils in aqueous medium by *Cryptococcus* spp. S-2. Process Biochemistry 37:405–410.

Karadzic, I., Masui, A., Zivkovic, L.I. and Fujiwara, N. 2006. Purification and Characterization of an Alkaline Lipase from *Pseudomonas aeruginosa* Isolated from Putrid Mineral Cutting Oil as Component of Metalworking Fluid. Journal of bioscience and bioengineering 102,2: 82–89.

Komers, K., Stloukal, R., Machek, J. and Skopal, F. 2001. Biodiesel from rapeseed oil, methanol and KOH 3: Analysis of composition of actual reaction mixture. Europe Journal Lipid Science Technology 103: 347–363.

Köse, Ö., Tüter, M. and Aks y, H.A. 2002. Immobilized *Candida antarctica* lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. Bioresource Technology 83: 125-129.

Lei, J., Fan, J., Yu, C., Zhang, L., Jiang, S., Tu, B. and Zhao, D. 2004. Immobilization of enzyme in mesoporous materials: controlling the entrance to nanospace. Microporous and Mesoporous Materials 73: 121-128.

Lei, L., Bai, Y., Li, Y., Yi, L., Yang, Y. and Xi, C. 2009. Study on immobilization of lipase onto magnetic microspheres with epoxy groups. Journal of Magnetism and Magnetic Materials 321: 252-258.

Leung, D.Y.C. and Guo, Y. 2006. Transesterification of neat and used frying oil: Optimization for biodiesel production. Fuel Process Technology 87: 883–890.

Li, L., Du, W., Liu, D., Wang, L., Li, Z., 2006. Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. Journal of Molecular Catalysis B: Enzymatic 43: 58–62.

Liu, X., He, H., Wang, Y. and Shenlin Zhu, S. 2007. Transesterification of soybean oil to biodiesel using SrO as a solid base catalyst. Catalysis Communications 8: 1107–1111.

López-Serrano, P., Cao, L., Van Rantwijk, F. and Shedon, R.A. (2002) Cross-linked enzyme aggregates with enhanced activity: application to lipase. Biotechnology letter 24: 1379-1383.

Lu, J., Nie, K., Xie, F., Wang F. and Tan, T. 2007. Enzymatic synthesis of fatty acid methyl esters from lard with immobilized *Candida* sp. 99-125. Process biochemistry 42: 1367-1370.

Lu, J., Nie, K., Wang, F. and Tan, T. 2008. 2008. Immobilized lipase *Candida sp.* 99-125 catalyzed methanolysis of glycerol trioleate: Solvent effect. Bioresource Technology 99: 6070–6074.

Lu, J., Chen, Y., Wang, F. and Tan, T. 2009. Effect of water on methanolysis of glycerol trioleate catalyzed by immobilized lipase *Candida* sp. 99–125 in organic solvent system. Journal of Molecular Catalysis B: Enzymatic 56: 122–125.

Ma, F., Hanna, M.A., 1999. Biodiesel Production: a review. Bioresource Technology 70: 1–15.

Mamoru, I., Chen, B., Eguchi, M., Kudo, T. and Shrestha, S. 2001. Production of biodiesel fule from triglycerides and alcohol using immobilized lipase. Journal of Molecular Catalysis B: Enzymatic 16: 53-58.

María, P.D. and Gago, J.O.S. 1999. Causes of Unreproducibility of *C. rugosa* Lipase-catalyzed Reactions in Slightly Hydrated Organic Media. Tetrahedron 55: 8555-8566.

Martín, E.H. and Otero, C. 2008. Different enzyme requirements for the synthesis of biodiesel: Novozym® 435 and Lipozyme® TL IM. Bioresource Technology 99: 277-286.

Masse, L., Kennedy, K. J. and Chou, S. 2001. Testing of alkaline and enzymatic hydrolysis pretreatments for fat particles in slaughterhouse wastewater. Bioresource Technology 77, 2: 145-155.

Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M. and Fernandez-Lafuente, R. 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme and Microbial Technology 40: 1451-1463.

.

Modi, M.K., Reddy, J.R.C., Rao, B.V.S.K., Prasad, R.B.N., 2007. Lipase mediated conversion of vegetable oils into biodiesel using ethyl acetate. Bioresource Technology 98: 1260–1264.

Narayan, V., Diwekar, U.M. and Hoza, M. 1996. Synthesizing optimal waste blends. Computers & Chemical Engineering 20: S1443-S1448.

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

Noureddini, H., Gao, X., Phikana, R.S., 2005. Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from Soybean oil. Bioresource Technology 96: 767–777.

Oliveira, L.S., Leite, P.M., Souza, L.B., Mello, V.M., Silva, E.C., Rubim, J.C., Meneghetti, S.M.P. and Suarez, P.A.Z. 2009. Characteristics and composition of *Jatropha gossypiifolia* and *Jatropha curcas* L. oils and application for biodiesel production. Biomass and bioenergy 33: 449 – 453.

Öztürk, N., Akgöl, S., Arısoy, M. and Denizli, A. 2007. Reversible adsorption of lipase on novel hydrophobic nanospheres. Separation and Purification Technology 58: 83–90.

Palomo, J.M., Muñoz, G., Lo ente, G.F., Cesar Mateo, Lafuente, R.F. and Guisán, J.M. 2002. Interfacial adsorption of lipases on very hydrophobic support (octadecyl– Sepabeads): immobilization, hyperactivation and stabilization of the open form of lipases. Journal of Molecular Catalysis B: Enzymatic 19–20: 279–286.

Palomo, J.M., Mateo, C., Fernández-Lorente, G., Solares, L.F., Diaz, M., Sánchez, V.M., Bayod, M., Gotor, V., Guisana, J.M. and Fernandez-Lafuente. R. 2003. Resolution of (±)-5-substituted-6-(5-chloropyridin-2-yl)-7-oxo-5,6-dihydropyrrolo[3,4b]pyrazine derivatives-precursors of (S)-(+)-Zopiclone, catalyzed by immobilized *Candida antarctica B* lipase in aqueous media. Tetrahedron: Asymmetry 14: 429–438.

Panzavolta, F., Soro, S., Amato, R.D., Palocci, C., Cernia, E. and Russo, M.V. 2005. Acetylenic polymers as new immobilization matrices for lipolytic enzymes. Journal of Molecular Catalysis B: Enzymatic 32: 67-76.

Patil, P.D. and Deng, S. 2009. Optimization of biodiesel production from edible and non-edible vegetable oils. Fuel 88: 1302-1306.

Pereira, E.B., Castro, H.F., Moraes, F.F. and Zanin, G.M. 2001. Kinetic studies of lipase from *Candida rugosa*:: a comparative study between free and immobilized enzyme onto porous chitosan beads. Applied Biochemical Biotechnology 91: 739-752.

Petkar, M., Lali, A., Caimi, P. and Daminati, M. 2006. Immobilization of lipases for non-aqueous synthesis. Journal of Molecular Catalysis B: Enzymatic 39: 83-90.

Qian, J., Wang, F., Liu, S. and Yun, Z. 2008. *In situ* alkaline transesterification of cottonseed oil for production of biodiesel and nontoxic cottonseed meal. Bioresource Technology 99: 9009–9012.

Rahman, M.B.A., Zaidan, U. ., Basri, M., Hussein, M.Z., Rahman, R.N.Z.R.A., and Salleh, A.B. 2005. Enzymatic synthesis of methyl adipate ester using lipase from *Candida rugosa* immobilised on Mg, Zn and Ni of layered double hydroxides (LDHs). Journal of Molecular Catalysis B: Enzymatic 50: 33–39.

Rajendran, A., Palanisamy, A. and Thangavelu, V. 2008. Evaluation of Medium Components by Plackett-Burman Statistical Design for Lipase Production by *Candida rugosa* and Kinetic Modeling. Chinese Journal of Biotechnology 24, 3: 436-444.

Ramadhas, A.S., Jayaraj, S. and Muraleedharan, C. 2004. Use of vegetable oils as I.C. engine fuels-A review. Renewable Energy 29: 727–742.

Ramadhas, A.S., Muraleedharan, C. and Jayaraj, S. 2005. Performance and emission evaluation of a diesel engine fueled with methyl esters of rubber seed oil. Renewable Energy 30:1789–1800.

Ranganathan, S.V., Narasimhan, S.L. and Muthukumar, K. 2008. An overview of enzymatic production of biodiesel. Bioresource Technology 99: 3975–3981.

Rashid, U., Anwar, F., Moser, B.R. and Ashraf, S. 2008. Production of sunflower oil methyl esters by optimized alkali-catalyzed methanolysis. Biomass and Bioenergy 32:1202-1205.

Rosa, C.D., Morandim, M.B., Ninow, J.L., Oliveira, D., Treichel, H. and Oliveira, J .V. 2008. Lipase-catalyzed production of fatty acid ethyl esters from soybean oil in compressed propane. Journal of Supercritical Fluids 47: 49-53

Rosu, R., Iwasaki, Y., Shimiz , N., Doisaki, N. and Yamane, T. 1998. Intensification of lipase performance in a transesterification reaction by immobilization on CaCO$_3$ powder. Journal of Biotechnology 66: 51-59.

Royon, D., Daz, M., Ellenrieder, G., Locatelli, S., 2007. Enzymatic production of Biodiesel from cotton seed oil using t-butanol as a solvent. Bioresource Technology 98: 648–653.

Salis, A., Pinna, M., Monduzzi, M. and Solinas, V. 2008. Comparison among immobilised lipases on macroporous polypropylene toward biodiesel synthesis. Journal of Molecular Catalysis B: Enzymatic 54: 19–26.

Samukawa, T., Kaieda, M., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., Fukuda, H., 2000. Pretreatment of Immobilized *Candida antarctica* lipase for Biodiesel fuel production from plant oil. Journal Bioresource Bioengineering 90: 180–183.

Schinas, P., Karavalakis, G., Davaris, C., Anastopoulos, G., Karonis, D., Zannikos, F., Stournas, S. and Lois, E. 2009. Pumpkin (*Cucurbita pepo* L.) seed oil as an alternative feedstock for the production of biodiesel in Greece. Biomass and Bioenergy 33: 44–49.

Shao, P., Meng, X., He, J. and Sun, P. 2008. Analysis of immobilized *Candida rugosa* lipase catalyzed preparation of biodiesel from rapeseed soapstock. Food and Bioproducts Processing 86: 283-289.

Sharma, R., Chisti, Y. and Banerjee, U.C. 2001. Production, purification, characterization and applications of lipases. Biotechnology Advances 19: 627–662.

Sharma, Y.C. and Singh, B. 2008. Development of biodiesel from karanja, a tree found in rural India. Fuel 87: 1740-1742.

Shimada, Y., Watanabe, Y., Sugihara, A., Tominaga, Y., 2002. Enzymatic alcoholysis for biodiesel fuel production and application of the reaction oil processing. Journal of Molecular Catalysis B: Enzymatic 17: 133–142.

Takaç, S. and Bakkal, M. 2007. Impressive effect of immobilization conditions on the catalytic activity and enantioselectivity of Candida rugosa lipase toward S-Naproxen production. Process Biochemistry 42: 1021-1027.

Tamalampudi, S., Talukder, M.R., Hama, S., Numata, T., Kondo, A. and Fukuda, H. 2008. Enzymatic production of biodiesel from Jatropha oil: A comparative study of immobilized-whole cell and commercial lipases as a biocatalyst. Biochemical Engineering Journal 39:185-189.

Teng, Y., Xu, Y. and Wang, D. 2009. Production and regulation of different lipase activity from Rhizopus chinensis in submerged fermentation by lipaids. Journal of Molecular Catalysis B: Enzymatic 57: 292-298.

Tiwari, A.K., Kumar, A. and Raheman, H. 2007. Biodiesel production from jatropha oil (Jatropha curcas) with high free fatty acids: an optimized process. Biomass and Bioenergy 31: 569–75.

Vakhlu, J., Johri, S., Verma, V., Koul, S., Parshad, R., Taneja, S.C. and Qazi, G.N. 2005. Purification and properties of enantioselective ester hydrolase from a strain of Trichosporon species (DSMZ 11829). Enzyme and Microbial Technology 37: 330-339.

Villeneuve, P., Muderhwa, J. ., Graille, J. and Haas, M.J. 2000. Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. Journal of Molecular Catalysis B: Enzymatic 9:113–148.

Wang, L., Du, W., Liu, D., Li, L. and Dai, N. 2006. Lipase-catalyzed biodiesel production from soybean oil deodorizer distillate with absorbent present in *tert*-butanol system. Journal of Molecular Catalysis B: Enzymatic 43: 29–32.

Wang, Y., Ou, S., Liu, P. and Zhang, Z. 2007. Preparation of biodiesel from waste cooking oil via two-step catalyzed process. Energy Conservat Manage 48:184–188.

Watanabe, Y., Shimada, Y., Sugihara, A., Noda, H., Fukuda, H., Tominaga, Y., 2000. Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase. Journal of the American Oil Chemists' Society 77: 355–360.

Watanabe, Y., Shimada, Y., Sugihara, A. and Tominaga Y. 2002. Conversion of degummed soybean oil to biodiesel fuel with immobilized *Candida antarctica* lipase. Journal of Molecular Catalysis B: Enzymatic 17:151–155.

Wilson, L., Palomo, J.M., Fernández-Lorente, G., Illanes, A., Guisan, J.M. and Fernandez-Lafuente. R. 2006. Improvement of the functional properties of a thermostable lipase from *Alcaligenes* sp. via strong adsorption on hydrophobic supports. Enzyme and Microbial Technology 38: 975–980.

Winayanuwattikun, P., Kaewpiboon, C., Piriyakananon, K., Tantong, S., Thakernkarnkit, W., Chulalaksananukul, W. and Yongvanich, T. 2008. Potential plant oil feedstock for lipase-catalyzed biodiesel production in Thailand. Biomass and Bioenergy 32: 1279–1286.

Wu, J.C., Lee, S.S., Mahmood, M.M.B., Chow, Y., Talukder, M.M.R. and Choi, W.J. 2007. Enhanced activity and stability of immobilized lipases by treatment with polar solvent prior to lyophilization. Journal of Molecular Catalysis B: Enzymatic 45: 108-112.

Xu, Y., Du, W., Zeng, J., Liu, D., 2004. Conversion of soybean oil to biodiesel fuel using lipozyme TL IM in a solvent free medium. Biocatalyst Biotransform 22: 45–48.

Xu, J., Wang, Y., Hu, Y., Luo, G. and Dai, Y. 2006. *Candida rugosa* lipase immobilized by a specially designed microstructure in the PVA/PTFE composite membrane. Journal of Membrane Science 281: 410-416.

Yang, G., Tian-Wei, T., Kai-Li, N. and Fang, W. 2006. Immobilized of lipase on Macroporous Resin and Its Application in Synthesis of Biodiesel in Low Aqueous Media. Chinese Journal of Biotechnology 22(1): 114-118.

Ye, P., Xu, Z.K., Wu, J., Innocent, C. and Seta, P. 2006. Nanofibrous poly (acrylonitrile-*co*-maleci acid) membranes functionalized with gelatin and chitosan for lipase immobilization. *Biomaterials* 27: 4169- 4176.

Ye, P., Jiang, J. and Xu, Z.K. 2007. Adsorption and activity of lipase from *Candida rugosa* on the chitosan-modified poly (acrylonitrile-co-maleic acid) membrane surface. Colloids and Surfaces B: Biointerfaces 60: 62-67.

Yeşiloğlu, Y. 2005. Utilization of bentonite as a support material for immobilization of *Candida rugosa* lipase. Process biochemistry 40: 2155-2159.

Zeng, J., Du, W., Liu, X., Liu, D. and Dai, L. 2006. Study on the effect of cultivation parameters and pretreatment on *Rhizopus oryzae* cell-catalyzed transesterification of vegetable oils for biodiesel production. Journal of Molecular Catalysis B: Enzymatic 43: 15-18.

Zeng, L., Luo, K. and Gong, Y. 2006. Preparation and characterization of dendritic composite magnetic particles as a novel enzyme immobilization carrier. Journal of Molecular Catalysis B: Enzymatic 38: 24–30.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

APPENDIX A

Properties of enzyme carrier

Table A-1. Details of seven types of hydrophobic support for immobilization of *Candida rugosa* lipase

Type of support	Polymer base	Functional group	Spec. surface area (m^2/g)	Pore diameter	Particle size (mm)
Amberlite XAD 2	styrene DVB	-	300	90 Å	-
Amberlite XAD 4	Polystyrene DVB	-	750	100 Å	490-690
Amberlite XAD 7	Aliphatic ester	-	$\geq$ 380	450 Å	560-710
Amberlite XAD 16	Polystyrene DVB	-	$\geq$ 800	150 Å	560-710
Amberlite XAD 761	Formophenolic	Phenol	150-250	600 Å	560-760
Sepabeads EC-BU	Acrylic	Butyl	-	30-40 nm	150-300
Sepabeads EC-OD	Acrylic	Octadecyl	-	30-40 nm	150-300

APPENDIX B

Hydrolysis assays

Preparation of solutions for hydrolysis assays

1 Tris buffer solution (Tris HCl)

1 M Tris buffer, pH 8.0

Tris base	121	g
Distilled water	800	ml

Tris base was dissolved and pH was adjusted to 8 with HCl. Then, solution was adjusted to 1L with distilled water. Tris buffer solution was later steriled at 121 °C, pressure 15 psi for 15 min. The buffer solution was kept at 4 °C

50 mM Tris buffer, pH 8.0

1 M Tris buffer, pH 8.0	25	ml
Distilled water	475	ml

buffer solution was kept at 4 °C

2 p-nitrophenyl palmitate solution

p-nitrophenyl palmitate	50	mg
Absolute ethanol	10	ml

p-nitrophenyl palmitate was dissolved with absolute ethanol. Then, the solution was mixed well and kept in the brown bottle. (This solution was prepared before use)

APPENDIX C

Protein determination

1. Preparation of solutions for protein assays

The assay reagent is prepared by diluting 1 volume of the dye stock with 4 volumes of distilled H_2O. Then solution was filtered by filter paper, Whatman No. 1. The solution should appear brown, and have a pH of 1.1. It is stable for 4 weeks in a brown bottle at 4°C.

2. Standard curve of BSA

Protein standards should be prepared in the same buffer as the samples to be assayed. A convenient standard curve can be made using bovine serum albumin (BSA) with concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/ml. The method is as follows;

1. Prepare stock bovine serum albumin with concentration 20 mg/ml.

2. 20 mg/ml BSA was diluted with distilled water as 0.1-0.6 mg/ml (Table C-1)

Table C-1 Composition for standard BSA

BSA	Reagent volume (µl)	
(mg)	stock of BSA	dH_2O
0	-	1000
0.1	5	995
0.2	10	990
0.3	15	985
0.4	20	980
0.5	25	975
0.6	30	970

2. Pipet 5 µl of each standard from stock solution was into 96 wells microplate. Protein solutions are normally assayed in duplicate.

3. Add 300 µl f diluted dye reagent to each well and incubated at room temperature for 5 minutes.

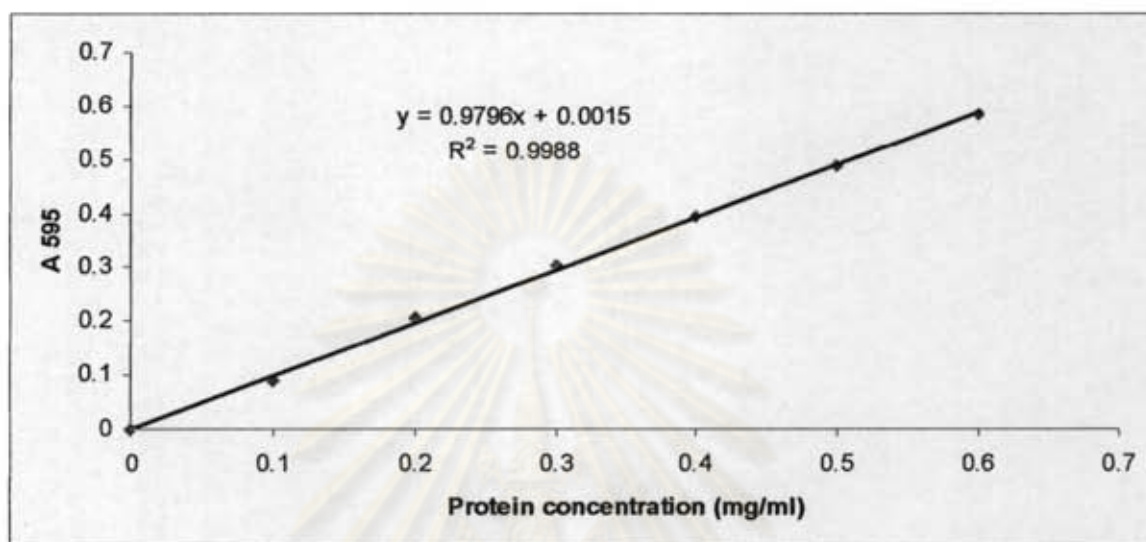4. The product was measured by an increase in the absorbance at 595 nm with micro plate reader.

Figure C-1. Calibration curve for protein determination by Bradford's method

3. Calculation of total protein

The absorbance value at 595 nm was calculated by:

$$Y = aX + b$$

Where

Value X axis = Standard protein concentration (mg/ml)

Value Y axis = Absorbance at 595 nm

The amount of bound protein on the support was calculated from the difference between the amount of protein introduced into the reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization. Amount of bound enzyme onto support (mg/g) was calculated from the following formula:

$$p \quad = \quad \frac{C_i V_i - (C_f V_f + C_w V_w)}{m_s}$$

p = Amount of bound enzyme onto support (mg/g)

C_i = Initial protein concentration (mg/ml)

C_f = Protein concentration of filtrate (mg/ml)

C_w = Protein concentration of washing solution (mg/ml)

V_i = Initial volume of enzyme solution (ml)

V_f = Volume of filtrate (ml)

V_w = Volume of washing solution (ml)

m_s = Weight of the support (g)

APPENDIX D

Calculation of the lipase activity

1. Calculation of enzyme immobilization

The efficiency of immobilization was evaluated in terms of lipase activity, specific activity, protein loading and activity yields as follows:

$$A_{410} = \varepsilon_{410}bc \qquad \text{Equation D 1.1}$$

Where

A_{410}	=	Absorbance at 410 nm
ε_{410}	=	Molar extinction coefficient of p-nitrophenol at 410 nm
	=	15,000 M^{-1} cm^{-1}
b	=	1 cm
c	=	Concentration of p-nitrophenyl palmitate

One unit (1 U) was defined as that amount of enzyme that liberated 1 µmol of pNPP per minute under the test conditions. Lipase activity was calculated from

$$\text{Lipase activity (U/g-support)} = \frac{\text{Activity of immobilized lipase}}{\text{Amount of immobilized lipase}}$$

$$\text{Specific activity (U/mg-protein)} = \frac{\text{Activity of immobilized lipase}}{\text{Amount of protein loading}}$$

$$\text{Protein loading yield (\%)} = \frac{\text{Amount of protein loading}}{\text{Amount of protein introduced}} \times 100$$

$$\text{Activity yield (\%)} = \frac{\text{Specific activity of immobilized lipase}}{\text{Specific activity of free lipase}} \times 100$$

(Chang *et al.*, 2007)

2. Calculation of thermal stability

Thermal stability was calculated according to equation (1) and (2)
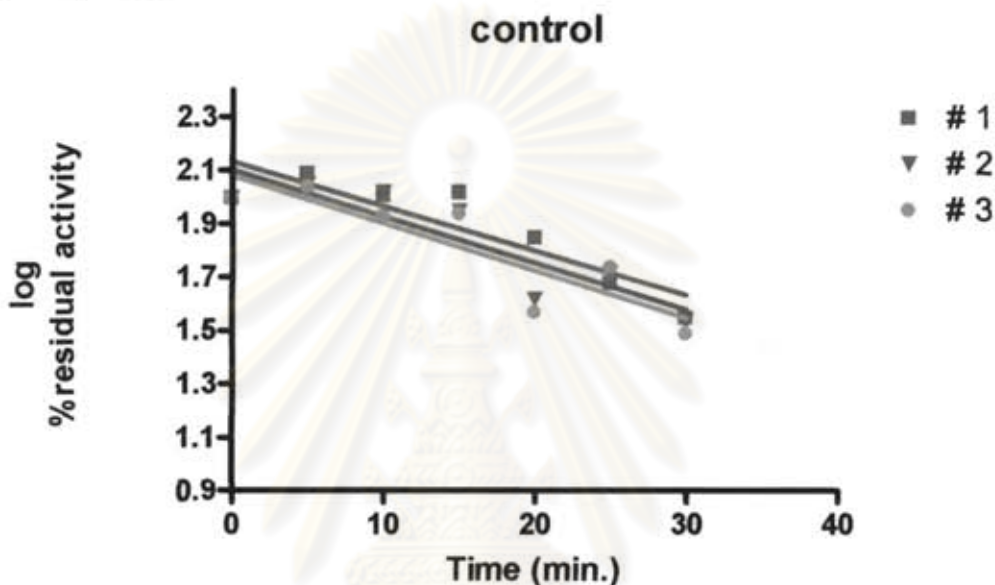(Santos *et al.*, 2008)

Figure D-1 Thermostability of untreated immobilized enzyme

$$A_{in} = A_{in0} \exp(-k_d t) \quad \dots\dots\dots\dots\dots\dots\dots(1)$$

$$t_{1/2} = \frac{\ln 2}{K_d} \quad \dots\dots\dots\dots\dots\dots\dots(2)$$

where

A_{in} = the hydrolytic activity at given time

A_{in0} = the initial hydrolytic activity at given time

K_d = thermal deactivation constant

t = the incubation time

Since,   slope $= \dfrac{K_d}{2.3}$ (From Fig D-1)

So, $t_{1/2} = \dfrac{0.693}{2.3 \times slope}$

untreated immobilized enzyme

APPENDIX E

Properties of plant oil

1. Calculation of oil content

In oil extraction, the thimbles were weighed before and after addition of the ground plant seed including the flask before and after extraction.

Calculation method was as follows;

$$\% \text{ Oil content} = \frac{(W_4 - W_3)}{(W_2 - W_1)} \times 100 \quad \text{w/w of seed}$$

W_1 = weight of thimble before paked (g)

W_2 = weight of thimble include plant seed (g)

W_3 = flask (500 ml) + boiling chip (g)

W_4 = flask (500 ml) + boiling chip (g) + oil

2. Calculation of each fatty acid composition in oil

Fatty acid composition of plants oil (Table E-1) was determined by GC analysis. The chromatogram of fatty acid methyl ester as shows in Figure E-1: The percentage of each fatty acid in oil was calculated as follows:

$$\text{Percentage of fatty acid (\%)} = \frac{\text{Peak area of fatty acid}}{\text{Total peak area of fatty acids}} \times 100$$

The example of fatty acid composition in palm oil

$$\text{Palmitic acid (\%)} = \frac{155596}{1075+3021+155596+3156+367686+39777+723745+40218+1692+1673} \times 100$$

$$= 11.63 \%$$

Figure E-1. Chromatogram of methyl ester from transesterification of the 1g of palm oil with 10% (w/w of oil) sodium hydroxide at 55oC, 350 rpm at 24 hr.
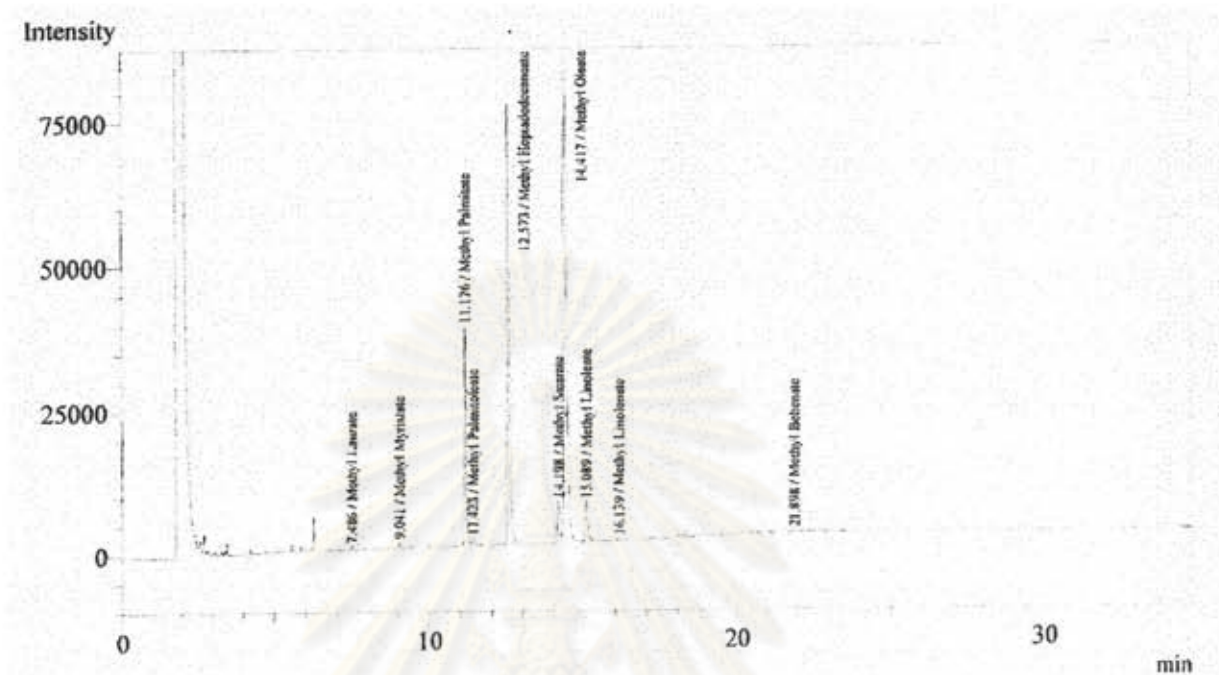

Table E-1. Retention time, peak area and peak height of standard fatty acid methyl ester, used in the calculation of fatty acid composition in oil.

Peak	Compound name	RT	Area	Height
1	Methyl Laurate	7.486	1075	477
2	Methyl Myristate	9.041	3021	962
3	Methyl Palmitate	11.176	155596	37879
4	Methyl Palmitoleate	11.423	3156	916
5	Methyl Standard	12.573	367686	76057
6	Methyl Stearate	14.198	39777	7310
7	Methyl Oleate	14.417	723745	127619
8	Methyl Linoleate	15.089	40218	7002
9	Methyl Linolenate	16.139	1692	289
10	Methyl Arachidate	21.898	1673	217

2. Molecular weight of palm il

Triglyceride (TAG) is the major composition in oil. Therefore, the molecular weight of triglyceride represents the molecular weight of oil. To calculate the molecular weight of TAG (i.e. molecular weight of oil), equation E-1 was use.
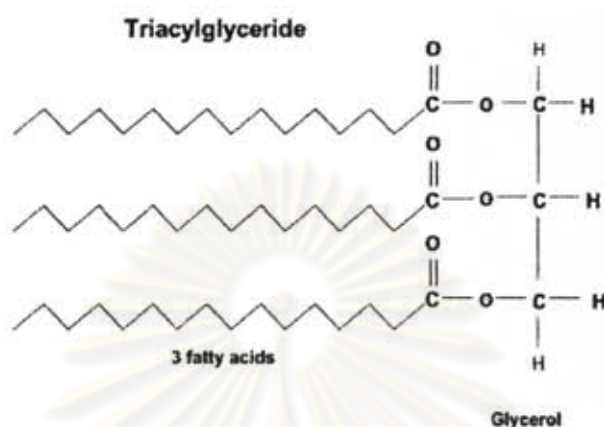
Triacylglyceride

Figure E-2 Molecular structure of triglyceride

$$MW_{TG} = 3R_{Aver} + 173 \qquad \text{equation E-1}$$

$$R_{Aver} = \frac{(\%FA_n \times MW_n)}{100}$$

Where,

MW_{TG} = Molecular weight of triglyceride = Molecular weight of palm oil

R_{Aver} = Mass of three fatty acid esterified with glycerol
(minus molecular weight of COOH)

$\%FA_n$ = Percentage of each fatty acid in oil

MW_n = Molecular weight of three fatty acid – COOH

= MW_{FA} – 45 (from main structure of triglyceride) (Fig E-2)

Table E-2 Fatty acid composition of palm oil

Common name	Abbreviation	% Fatty acid
Lauric acid ($C_{12}H_{24}O_2$)	12 : 0	0.59
Myristic acid ($C_{14}H_{28}O_2$)	14 : 0	0.96
Palmitic acid ($C_{16}H_{32}O_2$)	16 : 0	38.67
Palmitoleic acid ($C_{16}H_{30}O_2$)	16 : 1	0.11
Stearic acid ($C_{18}H_{36}O_2$)	18 : 0	3.32
Oleic acid ($C_{18}H_{34}O_2$)	18 : 1	45.45
Linoleic acid ($C_{18}H_{32}O_2$)	18 : 2	10.87
Linolenic acid ($C_{18}H_{30}O_2$)	18 : 3	0.20
Arachidic acid ($C_{20}H_{40}O_2$)	20 : 0	0.23
Behenic acid ($C_{22}H_{44}O_2$)	22 : 0	0.02

The example of triglyceride in used palm oil calculation

$$R_{Ave} = \left(\frac{0.59}{100}\times155\right)+\left(\frac{0.96}{100}\times183\right)+\left(\frac{38.67}{100}\times211\right)+\left(\frac{0.11}{100}\times209\right)+\left(\frac{3.32}{100}\times239\right)$$
$$+\left(\frac{45.45}{100}\times237\right)+\left(\frac{10.87}{100}\times235\right)+\left(\frac{0.20}{100}\times233\right)+\left(\frac{0.23}{100}\times267\right)+\left(\frac{0.02}{100}\times295\right)$$

$$= 0.915 + 1.757 + 81.594 + 0.221 + 7.935 + 107.717 + 25.545 + 0.466 + 0.614 + 0.059$$

$$= 226.823$$

$$MW_{TG} = (3 \times 226.823) + 173$$

$$= 853.469$$

Therefore molecular weight of palm oil is equal to 853.47

Table E-3. Molecular weight of non-edible and waste plant oil

Common name		MW
Papaya	*Carica papaya* Linn.	871.18
Physic nut	*Jatropha curcas* L.	870.41
Pomelo	*Citrus maxima* (Burm.) Merr.	860.27
Pumpkin	*Cucurbita moschata* Duchesne	867.38
Rambutan	*Nephelium lappaceum* L.	899.13
Rubber	*Dipterocarpus alatus* Roxb.ex G.Don	872.15
Tangerine	*Citrus reticulate Blan co*	901.66
White silk cotton	*Ceiba pentandra* (L.)Gaertn.	865.65
Wild almond	*Irvingia malayana* Oliv. ex A.W. Benn.	748.51

3. Calculation SN,IV, CN and η values

Calculation IV of plant seed oils were calculated from their fatty acid methyl ester compositions as Equation E-2, SN were calculated from the equation E-3 and CN of fatty acid methyl ester were calculated from Equation E-4. Also, the mass fraction was used in preference to the mole fraction to conform to the mass unit that is implicit in the units for viscosity used in this study. With these two modifications, Equation E-5 was used to predict the viscosity of biodiesel fuels based on their fatty acid composition

$$IV = \sum (254 \times D \times A_i) / MW_i \qquad \text{Equation E-2}$$
$$SN = \sum (560 \times A_i) / MW_i \qquad \text{Equation E-3}$$
$$CN = 46.3 + 5458 / SN - 0.225 \times IV \qquad \text{Equation E-4}$$
$$\ln \eta_m = \sum Z_i \ln \eta_i \qquad \text{Equation E-5}$$

Where,

A_i = Percentage of each component in the chromatogram

MW_i = Molecular mass
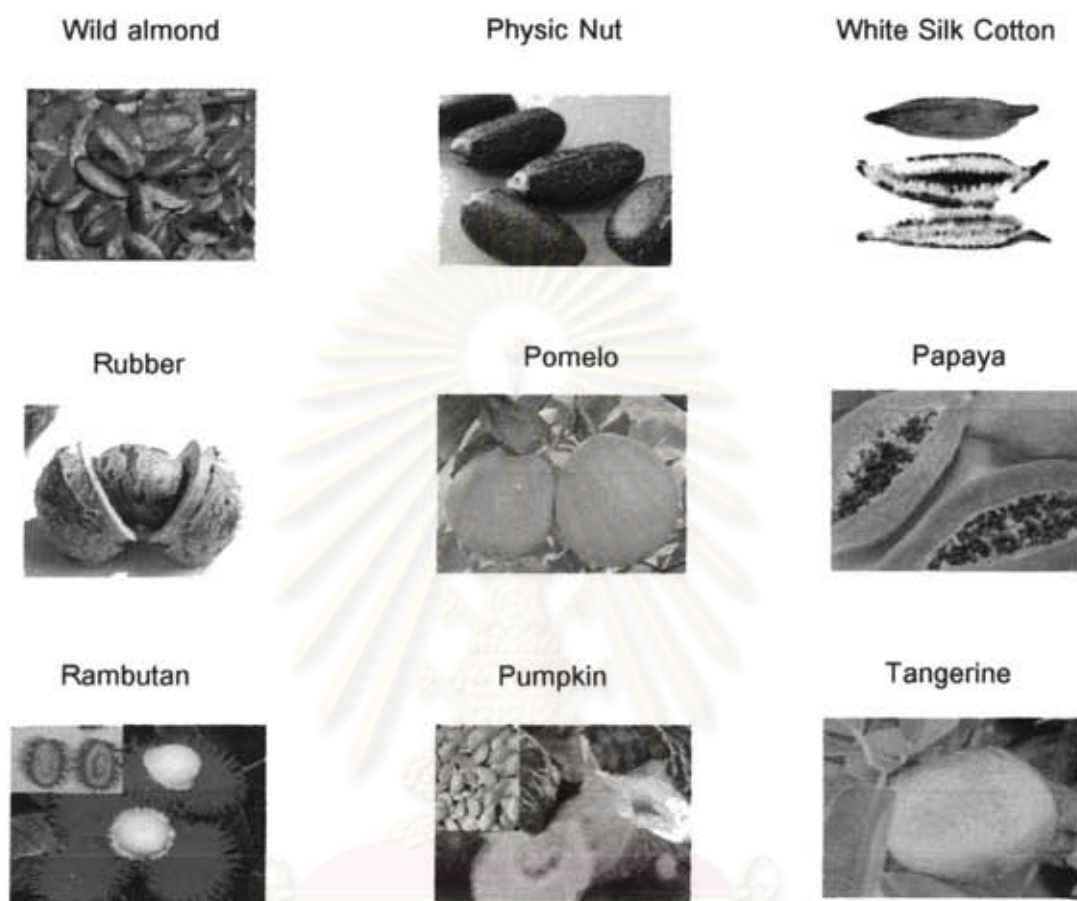
D   = Number of double bonds

Z_i = Mass fraction

Wild almond

Physic Nut

White Silk Cotton

Rubber

Pomelo

Papaya

Rambutan

Pumpkin

Tangerine

Figure E-3. Non-edible and waste plant oil

APPENDIX F

Calculation of transesterification reaction

1. Volume of methanol

The stoichiometry of this reaction requires 3 mol methanol per mol triglyceride to yield 3 mol fatty acid methyl ester or biodiesel and 1 mol glycerol. The biodiesel yield could be elevated by introducing an excess amount of methanol to shift the equilibrium to the right-hand side. So, the ratio of oil and methanol is 1:3 according to the equation in Fig D-3. The applied volume of methanol was determined by using the molecular weight of palm oil from section 4 equal to 853.47.
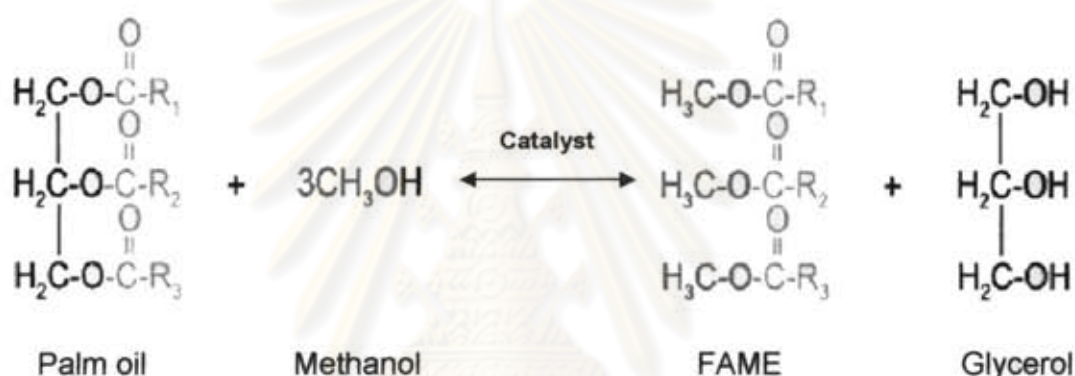

Figure F-1 Transesterification of palm oil and methanol

So, palm oil 1 g $= 1/853.47$

$\quad\quad = 1.171 \times 10^{-3}$ mole

Thus, the volume of methanol $= 3 \times 1.171 \times 10^{-3}$

$\quad\quad = 3.513 \times 10^{-3}$ mole

Since molecular weight of methanol is equal to 32

So, methanol 3.513×10^{-3} mole $= 3.513 \times 10^{-3} \times 32 = 0.1124$ g

From $\quad\quad\quad\quad\quad\quad D = \dfrac{M}{V}$

D = Density of methanol $\quad$ (0.792)

$$M = \text{Mass of methanol} \qquad (0.1124 \text{ g})$$

$$V = \text{Volume of methanol} \qquad (\text{ml})$$

$$V = \frac{0.1124}{0.792}$$

So, the volume of methanol is equal to 0.140 ml.

2. %conversion yield from HPLC analysis

All FAME were assumed by HPLC can be calculated as follows;

$$\% \text{ FAME} = \frac{\text{FAME}}{\{\text{FAME} + \text{FFA} + (\text{TAG} \times 3) + (1,3 \text{ DAG} \times 2) + (1,2 \text{ DAG} \times 2) + \text{MAG}\}} \times 100$$

FAME = Concentration of methyl ester

FFA = Concentration of free fatty acid

TAG = Concentration of triglyceride

DAG = Concentration of diglyceride
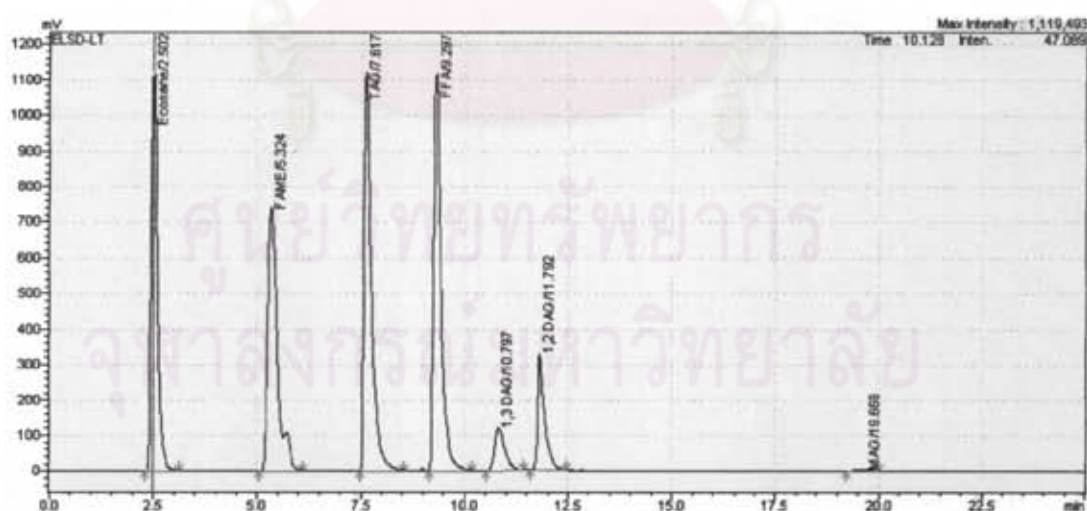
MAG = Concentration of monoglyceride

Figure F-2 Chromatogram of methyl ester from transesterification catalyzed by immobilized lipase and analyzed by high performance liquid chromatography

Where

Peak 1 = Eicosane

Peak 2 = Fatty acid methyl ester (FAME or Biodiesel)

Peak 3 = Triglyceride (TAG)

Peak 4 = Free fatty acid (FFA)

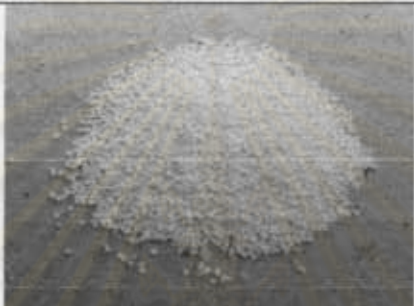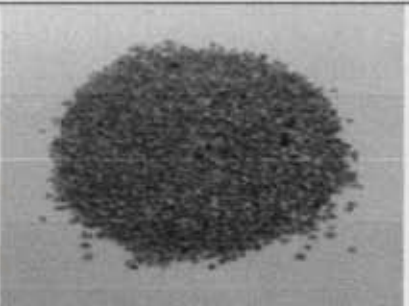Peak 5 = 1,3 Diglyceride (1,3 DAG)

Peak 6 = 1,2 Diglyceride (1,2 DAG)

Peak 7 = Monoglyceride (MAG)

APPENDIX G

Property of commercial lipase

Property	Novozyme® 435	Lipozyme® RM IM
Microorganism	*Candida antarctica* fraction B	*Rhizomucor miehei*
Type of support	Macroporous resin	Acrylic resin
Specificity	Non-specific	1,3-specific

References: Martin. E. H. and Otero. C., 2007

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

BIOGRAPHY

Miss Kingkaew Piriyakananon was born on November 27, 1983 in Bangkok, Thailand. She graduated with the Bachelor Degree of Science from the Department of General Science, Faculty of Science, Kasetsart University in 2005. Later in 2006, she furthered her Master's Degree of Science in Biotechnology from Program in Biotechnology, Department of Biochemistry, Faculty of Science, Chulalongkorn University, at which she finished with the "very good" merit from her defense examination on the 2nd of April of 2009. During her postgraduate studies, she had presented her work at both national and international levels. In addition, she received 2 awards from oral presentation competition and poster presentation. Most importantly, part of her thesis was already published in *Biomass and Bioenergy* in the year 2008. The details are as follows:

AWARDS

Oral presentation

Second prize for the thesis oral research presentation at the national competition for postgraduate students: The CGI Award for Young Scientists 2008. 21-22 November 2008. organized at Chulabhorn Research Institute & Chulabhorn Graduate Institute. Piriyakananon. K., Winayanuwattikun, P., Chulalaksananukul, W., and Yongvanich, T. 2008. Optimal immobilization condition of lipase from *Candida rugosa* for biodiesel production.

Poster presentations

1. **Commendable prize** for Poster Presentation at the Third Annual Symposium Protein Society of Thailand: Frontiers in Protein Research. 28-29 August 2008. Chulabhorn Research Institute Conference Center.

Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W., and Yongvanich, T. 2008.Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. Third Annual Symposium Protein Society of Thailand: Frontiers in Protein Research. Chulabhorn Research Institute, Bangkok, Thailand. 28-29 August, 2008.

2. **First prize** for Poster Presentation at the The Science Forum 2009. Symposium 1: Energy and Environmental Research For Sustainable Development Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W., and Yongvanich, T. 2008. Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. The Science Forum 2009. Faculty of Science, Chulalongkorn University, Bangkok, Thailand. 12 -13 March, 2009.

PUBLICATION

Winayanuwattikun, P., Kaewpiboon, C., Piriyakananon, K., Tantong, S. Thakernkarnkit, W., Chulalaksananukul, W. and Yongvanich, T. 2008.Potential Plant Oil Feedstock for Lipase-catalyzed Biodiesel Production in Thailand. 2008. *Biomass and Bioenergy* 32. 1279-1286.

PROCEEDINGS

Piriyakananon, K., Thakernkarnkit, W., Chulalaksananukul, W., Winayanuwattikun, P. and Yongvanich, T. 2008. Lipase catalyzed biodiesel production from non-edible and waste plant oils. The 7th International Symposium of High Temperature Air Combustion and Gasification (HiTACG 2008). Phuket Spa and Resort , Phuket , Thailand. 13-16 January, 2008

PRESENTATIONS

Poster presentations

1. Kaewpiboon, C., Piriyakananon, K., Chulalaksananukul, W. and Yongvanich, T. 2007. Comparative studies on biodiesel production from physic nut and coconut oils by different immobilized lipases. The 8th International Conference on Agricultural, Food and Biological Engineering & Post Harvest/Production Technology. Sofitel Raja Orchid Hotel, Khon Kaen, Thailand. 21 – 24 January, 2007.

2. Piriyakananon, K., Thakernkarnkit, W., Chulalaksananukul, W., Winayanuwattikun, P. and Yongvanich, T. 2008. Lipase catalyzed biodiesel production from non-edible and waste plant oils. The 7th International Symposium of High Temperature Air Combustion and Gasification (HiTACG 2008). Phuket Spa and Resort, Phuket, Thailand. 13-16 January, 2008.

3. Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W. and Yongvanich, T. 2008. Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. Third Annual Symposium Protein Society of Thailand: Frontiers in Protein Research. Chulabhorn Research Institute, Bangkok, Thailand. 28-29 August, 2008.

4. Winayanuwattikun, P., Piriyakananon, K., Chulalaksananukul, W. and Yongvanich, T. Lipase-mediated biodiesel production using various acyl acceptors. Third Annual Symposium Protein Society of Thailand: Frontiers in Protein Research. Chulabhorn Research Institute, Bangkok, Thailand. 28-29 August, 2008.

5. Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W. and Yongvanich, T. 2008. Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. 13th Biological Science Graduated Congress. National University of Singapore, Singapore. 15-17 December, 2008.

6. Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W. and Yongvanich, T. 2008. Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. The Science Forum 2009, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. 12 -13 March, 2009.