ภาวะที่เหมาะสมในการตรึงไลเพสจาก *Candida rugosa* บน AMBERLITE XAD761 เพื่อการผลิตไบโอดีเซล

นางสาว สุธาสินี ภูธา

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OPTIMAL IMMOBILIZATION CONDITION OF LIPASE FROM *Candida rugosa* ON AMBERLITE XAD761 FOR BIODIESEL PRODUCTION

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A Thesis Submitted in Partial Fulfillment of Requirements
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Field of Study	Biotechnology
Thesis Advisor	Associate Professor Tikamporn Yongvanich

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

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สุธาสินี ภูธา : ภาวะที่เหมาะสมในการตรึงไลเพสจาก *Candida rugosa* บน AMBERLITE XAD 761 เพื่อการผลิตไบโอดีเซล (OPTIMAL IMMOBILIZATION CONDITION OF LIPASE FROM *Candida rugosa* ON AMBERLITE XAD761 FOR BIODIESEL PRODUCTION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ทิฆัมพร ยงวณิชย์, 75 หน้า

ปัจจุบันการผลิตไบโอดีเซลด้วยปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นที่เร่งด้วยเอนไซม์ได้รับความสนใจมากขึ้น เพราะได้ผลิตภัณฑ์ที่บริสุทธิ์และแยกไบโอดีเซลออกจากกลีเซอรอลที่เป็นผลิตภัณฑ์ร่วมได้ง่าย ในอุตสาหกรรมนิยมใช้ไลเพสจากเชื้อ *Candida rugosa* (CRL) มากที่สุด แต่ราคาของเอนไซม์ยังคงเป็นอุปสรรค ดังนั้นเพื่อเป็นการลดต้นทุน ทางอ้อมจึงนำเอนไซม์มาตรึงรูป วิธีการที่สามารถใช้ตรึงรูปเอนไซม์มีหลายชนิดขึ้นกับลักษณะของเอนไซม์ ตัวค้ำจุนและสารตั้งต้น ในงานวิจัยนี้ใช้ CRL หยาบ 200 มิลลิกรัม ละลายในฟอสเฟตบัฟเฟอร์ความเข้มข้น 20 มิลลิโมลาร์ pH 7.5 ผสมกับตัวค้ำจุน Amberlite XAD761 1 กรัม ปั่นกวนที่ความเร็วรอบ 350 รอบต่อนาที เป็นเวลา 5 ชั่วโมง เพื่อให้ได้ประสิทธิภาพการตรึงที่สูงขึ้น จึงได้ทำการหาภาวะที่เหมาะสม พบว่าความเป็นกรดด่างเท่ากับ 6 ความแรงไอออน 20 มิลลิโมลาร์ ปริมาณเอนไซม์ 3 มิลลิกรัมต่อมิลลิลิตร ที่อุณหภูมิ 30 องศาเซลเซียสเป็นเวลา 3 ชั่วโมง ได้ค่าการทำงานของ CRL ตรึงรูป คือ 0.62 ไมโครโมลต่อนาทีต่อกรัมตัวค้ำจุน เมื่อได้ภาวะที่เหมาะสมในการตรึงรูป CRL แล้วนำไปเร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชันเพื่อการผลิตไบโอดีเซลโดยใช้น้ำมันปาล์มเป็นสารตั้งต้น ทำ ปฏิกิริยาที่ 40 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง จากการวิเคราะห์ไบโอดีเซลที่ได้ด้วยเครื่องโครมาโทกราฟีของเหลวสมรรถนะสูง พบว่ามีประมาณ 35 เปอร์เซ็นต์ หลังจากนั้นหาภาวะที่เหมาะสมสำหรับปฏิกิริยาทรานส์เอสเทอริฟิเคชันที่เร่งด้วยเอนไซม์ตรึงรูปเพื่อผลิตไบโอดีเซล พบว่าได้แก่ การเติมเมทานอลแบบ 7 ขั้น อัตราส่วนของเมทานอลต่อน้ำมัน 4 ต่อ 1 ปริมาณเอนไซม์ 30 เปอร์เซ็นต์ของน้ำหนักน้ำมัน ระยะเวลาในการทำปฏิกิริยา 24 ชั่วโมง ที่อุณหภูมิ 40 องศาเซลเซียส โดยไม่ต้องเติมน้ำ สามารถผลิตไบโอดีเซลได้เพิ่มขึ้นเป็น 72 เปอร์เซ็นต์ ในปฏิกิริยาทรานส์เอสเทอริฟิเคชัน เอนไซม์ตรึงรูปมีความเสถียรในการนำมาใช้ซ้ำได้อีก 2 ครั้งและยังคงให้ไบโอดีเซลได้ 30 เปอร์เซ็นต์ จากผลการทดลองทั้งหมดชี้ให้เห็นว่าเอนไซม์ไลเพสจาก *Candida rugosa* ที่ตรึงบน Amberlite XAD761 สามารถนำมาใช้ผลิตไบโอดีเซลได้

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

ปีการศึกษา.....2553.....ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

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SUTHASINEE PUTHA: OPTIMAL IMMOBILIZATION CONDITION OF LIPASE FROM *Candida rugosa* ON AMBERLITE XAD761 FOR BIODIESEL PRODUCTON.

ADVISOR: ASSOC. PROF. TIKAMPORN YONGVANICH, 75 pp.

Recently, lipase catalyzed transesterification for the production of biodiesel has attracted more attention as it produces higher purity product and enables easy separation from the byproduct, glycerol. *Candida rugosa* lipase (CRL) is the enzyme mostly used for industries but the cost remains a barrier which can be indirectly solved through the immobilization . There are many available methods for immobilization depending on the nature of enzymes , supports and substrates. In this research, CRL was immobilized on hydrophobic support namely, Amberlite XAD761 by adsorption. 200 mg of crude CRL in 20 mM phosphate, pH 7.5 were mixed with 1 g of Amberlite XAD761 and stirred at 350 rpm for 5 hours. In order to obtain the high efficiency of the immobilization, various optimal conditions were determined. The results were as follows; pH at 6, ionic strength of 20 mM with 3 mg/ml of enzyme loading at 30^{o}C for 3 hours. The activity of the immobilized CRL was 0.62 μmole/min/g-support. When this optimized CRL was used to catalyze the transesterification for the production of biodiesel using palm oil as the substrate at 40^{o}C for 24 hours, the obtained conversion from the HPLC analysis was found to be approximately 35%. Then the optimal conditions for transesterification of the obtained immobilized enzymes for the production of biodiesel were investigated. The results were as follows: 7 steps of addition mode of methanol, 4 to 1 molar ratio of methanol to palm oil, 30% of oil by weight enzyme loading, reaction time of 24 hours at 40^{o}C. The production of biodiesel obtained was increased to approximately 72%. The immobilized lipase was stable and retained 30% relative transesterification activity after two cycles. Overall results indicated that biodiesel could be produced by the *Candida rugosa* lipase immobilized on Amberlite XAD761.

Field of Study: ……Biotechnology…….. Student's Signature……

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CHAPTER I

Introduction

1.1 Statement of purpose

Due to the increasing energy demand and pollution problems caused by the use of fossil fuels, it has become necessary to develop alternative fuels as well as renewable sources of energy. Biodiesel is a diesel fuel substitute produced from renewable triglyceride sources such as vegetable oils, animal fats, and recycled cooking oils. Chemically, it is defined as the monoalkyl esters of long-chain fatty acids derived from lipid/triglyceride sources. Biodiesel has become more attractive because of its environmental benefits. The chemical processes for production of biodiesel are well known. It is most commonly produced by reacting lipids (triglycerides) with a primary alcohol (e.g., methanol) and a base (sodium hydroxide). The reaction, known as transesterification, results in the production of biodiesel and glycerin. However, new biochemical routes to biodiesel production, based on the use of enzymes, have become very interesting. Lipases, known as glycerol ester hydrolases (EC. 3.1.1.3), are widely spread in plants, animals and microorganisms. These enzymes can catalyze a wide range of reactions such as hydrolysis, esterification, interesterification, transesterification, etc (Deng *et al*., 2005). Utilization of lipase as a catalyst for biodiesel production is a clean technology due to its non-toxic and environmental friendly nature and requires mild operating conditions compared with chemical method. Nevertheless, the production cost of lipase catalyst is significantly greater than that of an alkaline. Consequently, further industrial applications of lipase are limited. However, by an appropriate choice of the immobilization process, operational costs for lipase industrial processes can be reduced by the selection of an appropriate immobilization method (Hung *et al*., 2003; Wang *et al*., 2006; Chang *et al*., 2007). Immobilizations of lipases have been achieved by adsorption onto support matrices such as particles, fibers, by entrapping them in gel matrices and by covalent attachment. The immobilization of lipase onto a porous support by adsorption has proved to be one of the useful techniques for improving enzymatic activity. It is believed that the immobilized lipase facilitates mass transfer by spreading the enzyme on a large surface area and by preventing the enzyme

particles from aggregation (Ghamgui *et al*., 2004). From the previous research by Miss Chutima Kaewpiboon from Biofuels by Biocatalysts reseach unit, the production of biodiesel obtained from 3 types of lipase producing microorganisms namely: bacteria (*Staphylococcus warneri*), filamentous fungus (*Fusarium solani*) and yeast (*Candida rugosa*) were compared. It was found that yeast; *Candida rugosa* showed higher activity than the bacterial and fungal lipases. Therefore, *Candida rugosa* lipase was selected for this study. In addition, studies by Miss Kingkaew Piriyakananon in which lipases from *Candida rugosa* were immobilized on 7 types of hydrophobic supports in comparison, showed that immobilized lipase on Sepabeads EC-OD and Amberlite XAD 761 gave higher specific activities than Amberlite XAD2, Amberlite XAD4, Amberlite XAD7, Amberlite XAD16 and Sepabeads EC-BU (Winayanuwattikun *et al.,* 2011). Since the immobilization efficiency of the enzyme depends on various factors, this study concentrated on the optimal immobilization conditions of lipase from *Candida rugosa* on Amberlite XAD 761 for the transesterication of palm oil with methanol for the production of biodiesel.

1.2 Objective of this research

The aim of this study was to investigate the optimal conditions for immobilization of lipase from *Candida rugosa* on Amberlite XAD 761 for biodiesel production.

1.3 Scopes of the investigation

1.3.1 To determine the optimal immobilization conditions of lipase

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

1.3.3 To determine the stability of immobilized lipase

1.4 Expected results

This research should provide the optimal immobilization conditions of lipase from *Candida rugosa* on Amberlite XAD 761 for biodiesel production so that these immobilized enzymes can be further applied for industrial applications.

1.5 Thesis organization

This thesis comprises five chapters as follows: Chapter 1 is the introduction. Chapter 2 gives the theoretical background and literature reviews. In Chapter 3, materials and methods are provided. The results can be found in Chapter 4. Chapter 5 is the discussion and conclusion.

CHAPTER II
THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Biodiesel

Biodiesel is a clean burning alternative fuel, produced from domestically grown, renewable resources. Biodiesel contains no petroleum products, but can be blended at any concentration with diesel from fossil sources to create a biodiesel blend. In addition, it is simple to use, biodegradable, non-toxic, and basically free of sulphur compounds and aromatics and can be used in compression-ignition (diesel) engines with little or no modification.

2.1.1 Biodiesel production

Considerable efforts have been made to develop vegetable oil derivatives that approximate the properties and performance of hydrocarbons-based diesel fuels. The problem with substituting triglycerides for diesel fuel is mostly associated with high viscosity, low volatility and polyunsaturated characters. These can be changed in at least four ways: direct use and blending, pyrolysis, microemulsion and transesterification.

2.1.1.1 Direct use and blending

The direct usage of vegetable oils as biodiesel is possible by blending it with conventional diesel fuels in a suitable ratio and these ester blends are stable for short term usages. But direct use and blending is not satisfactory and impractical for both direct and indirect diesel engines. The high viscosity, acid composition, free fatty acid content, as well as gum formation due to oxidation and polymerization during storage and combustion, carbon deposits and lubricating oil thickening are obvious problems (Ma and Hanna, 1999).

2.1.1.2 Pyrolysis

Pyrolysis can be defined as the conversion of one substance into another by means of heat in the absence of air (or oxygen) or by heat in the presence of a catalyst which results in cleavage of bonds and formation of a variety of small molecules. The pyrolysis of vegetable oil to produce biofuels has been studied and found to produce alkanes, alkenes, alkadienes, aromatics and carboxylic acids in various proportions (Alencar *et al.,* 1983; Peterson, 1986; Ma and Hanna, 1999;). The equipment for thermal cracking and pyrolysis is expensive for modest biodiesel production particularly in developing countries. Furthermore, the removal of oxygen during the thermal processing also removes any environmental benefits of using an oxygenated fuel (Ma and Hanna, 1999). Another disadvantage of pyrolysis is the need to separate distillation equipment for separation of the various fractions. Also, the product obtained is similar to gasoline containing sulphur which makes it less ecofriendly (Ranganathan *et al.,* 2008).

2.1.1.3 Microemulsion

The problem of the high viscosity of vegetable oils can be solved by microemulsion with solvents such as methanol, ethanol and 1-butanol (Agarwal, 2007). A microemulsion is defined as a colloidal equilibrium dispersion of optically isotropic fluid microstructures with dimensions generally in the 1 - 150 nm range formed spontaneously from two normally immiscible liquids and one or more ionic or non-ionic amphiphiles (Ma and Hanna, 1999). The components of a biodiesel microemulsion include diesel fuel, vegetable oil, alcohol, and surfactant and cetane improver in suitable proportions. Alcohols such as methanol and ethanol are used as viscosity lowering additives, higher alcohols are used as surfactants and alkyl nitrates are used as cetane improvers. Microemulsion can improve spray properties by explosive vaporisation of the low boiling constituents in the micelles. Microemulsion results in reduction in viscosity, increase in cetane number and good spray characters in the biodiesel. According to Srivastava and Prasad (2000), short term performance of microemulsion of

aqueous ethanol in soybean oil is nearly as good as that of No. 2 diesel despite the lower cetane number and energy content. However, continuous use of microemulsified diesel in engines causes problems like injector needle sticking, carbon deposit formation and incomplete combustion.

2.1.1.4 Transesterification

In the transesterification of different types of oils, triacylglycerol reacts with an alcohol, generally methanol or ethanol, to produce esters and glycerin. The overall process is normally a sequence of three consecutive steps, which are reversible reactions. In the first step from triacylglycerol, diacylglycerol is obtained. From diacylglycerol, monoacylglycerol is produced and in the last step, from monoacylglycerol, glycerin is obtained. In all these reactions, esters are produced. Among the alcohols that can be used in the transesterification process such as methanol, ethanol, propanol, butanol and amyl alcohol, methanol and ethanol are used most frequently. Methanol is preferred for all the commercial development because of its low cost and its physical and chemical advantages (polar and shortest chain alcohol). Biodiesel is based on fatty acid methylester and all the commercial development is focused on the manufacture of these materials. Although ethylesters can be produced but there is no current commercial demand for them nor the wish to produce them. The problem with producing ethylesters is that one has to use ethanol as the alcohol and this then poses severe problems of restricting access to it as a potential potable material. The chemical and the biological industries do not like to use ethanol because of the severe restrictions that are then imposed upon them. The transesterification is accelerated in a number of ways such as using an alkali, acid or biocatalyst (Meher *et al.* 2006).

2.1.1.4.1 Acid catalyst

The production of the biodiesel requires triglycerides catalyzed

with alcohol and an acid. Sulphuric acid, sulfonic acids, and hydrochloric acids are the common acid catalysts but the most commonly used is sulphuric acid. Acid catalysts are used if the triglyceride has a higher free fatty acid content and more water. Although the yields could be high, the corrosiveness of acids may cause damage to the equipment and the reaction rate can be low, sometimes taking more than a day to finish.

2.1.1.4.2 Alkali catalyst

Alkali-catalyzed transesterification is much faster than acid-catalysis and is less corrosive to industrial equipment and therefore is the most often used commercially (Ma and Hanna, 1999; Agarwal, 2007; Marchetti *et al.,* 2007; Ranganathan *et al*., 2008). Sodium hydroxide or potassium hydroxide is used as basic catalyst with methanol or ethanol as well as the vegetable oil. Sodium hydroxide is cheaper and is widely used for large scale-processing. There are several disadvantages in using an alkaline catalysis process although it gives high conversion levels of triglycerides to their corresponding methyl esters in short reaction times. The process is energy intensive, recovery of glycerol is difficult, the alkaline catalyst has to be removed from the product, alkaline wastewater generated requires treatment and the level of free fatty acids and water greatly interfere with the reaction. The risk of free acid or water contamination results in soap formation causing the difficulty of the separation process (Fukuda *et al*., 2001; Barnwal and Sharma, 2005).

2.1.1.4.3 Enzymatic catalyst

Biocatalysts are becoming increasingly important in biodiesel preparation as it is believed that these catalysts will eventually have the ability to outperform chemical catalysts (Al-Zuhair *et al.,* 2007). Biocatalysts are naturally occurring lipases used to catalyze some reactions such as hydrolysis of glycerol, alcoholysis and acidolysis. In addition, it has been discovered that they can be used as catalysts for transesterification and esterification

reactions too. Biocompatibility, biodegradability and environmental acceptability of the biocatalysts are the desired properties in agricultural and medical applications. The extracellular and the intracellular lipases are also able to catalyze the transesterification of triacylglycerol effectively.

2.2 Lipase

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoglycerols and glycerol. Besides, they are also efficient in various reactions such as esterification, transesterification and aminolysis in organic solvents. Therefore, these enzymes are nowadays extensively studied for their potential industrial applications.

2.2.1 Sources of lipase

Lipases can be obtained from animals, plants and also many natural or genetically engineered microorganisms both in endogenous and exogenous forms. Among lipases of plant, animal and microbial origin, it is the microbial lipases that find immense application. This is because microbes can be easily cultivated and their lipases can catalyze a wide variety of hydrolytic and synthetic reactions. The mechanisms of lipase-catalyzed reactions resemble closely to the natural metabolic pathways; hence, lipase-based processes may be viewed as more environment-friendly than some bulk chemical synthesis. Owing to their chemical and stereoselectivity, lipases can produce high added value products. Because of low activation energies, lipase-mediated processes require mild temperature and pH, energy consumption is small and there is little damage to reactants and products. Also, they are stable in organic solvents and do not require cofactors.

2.2.2 Lipase producing microorganisms

Lipases can be found widely in nature, but microbial lipases are commercially significant because of low production cost, greater stability and wider availability than plant and

animal lipases. They may originate from fungi, yeasts or bacteria and most of them are formed extracellularly. This availability has created an enormous spin-off with respect to the enantioselective hydrolysis and formation of carboxyl esters. The enormous biotechnological potential of microbial lipases are related to their exquisite chemoselectivity, regioselectivity and stereoselectivity. They are readily available in large quantities because many of them can be produced in high yields from microorganisms. The crystal structures of many lipases have been studied, considerably facilitating the design of rational engineering strategies. Finally, lipases do not usually require cofactors nor do they catalyze side reactions. More than 50% of reported producing yeasts produce lipases in the forms of various isozymes. These lipase isozymes are in turn produced by various lipase encoding genes. Among many lipase producing yeasts, *Candida rugosa* is the most frequently used as the commercial source of lipase .Though several microbial lipases are produced commercially, the high cost of lipase seems to be a major factor in its successful application. This may be overcome by immobilization from the reusability of the immobilized enzyme.

2.2.3 Properties of lipase

Lipases are amongst the most important biocatalysts that carry out novel reactions in aqueous and non – aqueous media. They show wide variety of enantioselective transformations. The ease in handling them, their broad substrate tolerance, high stability under varied temperature and solvents, high enantioselectivity and easy availability account for their widespread popularity (Vakhlu and Kour, 2006). Lipases can hydrolyse fats into fatty acids and glycerol at the water-lipid interface and can reverse the reaction in non-aqueous media. This is responsible for both hydrolysis and synthesis of reactants and products which is under the control of the water activity of the enzyme. The enzyme acts on the substrate in a specific or non-specific manner resulting in either the complete hydrolysis of triglycerides into free fatty acid and glycerol or, along with triglycerides, diglycerides, monoglycerides, fatty acids and glycerol are also formed. Strong interactions with hydrophobic substrates at an interface are

probably caused by hydrophobic patches on the other lipase surface. Such patches may also be responsible for self association behaviour shown by the enzymes in aqueous solutions.

Under certain experimental conditions such as in the absence of water in a system, lipases are capable of reversing the reaction that leads to esterification and interesterification.

The activity of lipase is pH dependent. Some lipases have shown considerable stability over a wide range of pH values. The ideal lipase would be the one which is active over the full pH range of 0-14; such a lipase may be available in nature. Nevertheless, the lipases studied have usually shown profound stability at neutral pH or near the neutral pH range of 6.0 to 7.5, with considerable stability at acidic pH down to 4.0 and to alkaline pH up to 8.0.

Extracellular lipases produced by *Aspergillus niger*, *Chromobacterium viscosum,* and *Rhizopus* spp. are particularly active at acidic pH. An alkaline lipase active at pH 11.0 has been isolated from *Pseudomonas nitroreducens.*

The pancreatic lipases lose activity on storage at temperatures above 40^{o}C but some microbial lipases are more resistant to heat inactivation. The enzymes produced by *Aspergillus niger*, *Rhizopus japonicas* and *Chromobacterium viscosum* are stable in solution at 50^{o}C and the thermotolerant fungus *Humicola lanuginosa* excretes a lipase which is stable at 60^{o}C. A strain of *Pseudomonas nitroreducens* produces a lipase which is stable at 70^{o}C.

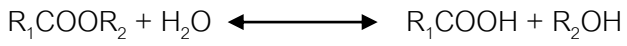
The temperature stability profiles determined by half-life values show maximum stability at lower temperature. Thus, *Calvatia gigantea* lipase had values of half-life of 35.7, 46.4 and 22.9 minutes at 45^{o}C, 50^{o}C and 55^{o}C. The results are similar for the lipases obtained from *Rhizopus japonicus.* Regarding the maximum lipase activities versus temperature, the highest activities for *Calvatia gigantea* lipase and many others are about 30-35^{o}C. All these lipase are however, derived from mesophilic sources. Thermophilic bacterial lipases obtained from organisms at Icelandic hot springs show higher lipase activity at 40-60^{o}C. The characteristics of lipases obtained from different sources thus need careful study at molecular level to enable insights into the protein structure and sequence for enabling the designing of thermostable lipases (Ghosh *et al.,* 1996).

2.2.4 Enzymatic reaction of lipase

Lipases have been used as biocatalysts for a variety of reactions. Unlike other hydrophilic enzymes, lipases from different origins are uniquely stable in non-polar organic solvents and can accept a broad range of substrates of varying size and stereochemical complexities. Their flexible protein backbones, which assume a variety of conformations, give them the ability to carry out many profitable reactions such as hydrolysis, and also reverse of hydrolysis reaction; esterification, transesterification (acidolysis, interesterification, alcoholysis) and aminolysis. The equilibrium between the forward (hydrolysis) and the reverse (synthesis) reactions is controlled by the water activity of the reaction mixture.

2.2.4.1 Hydrolysis

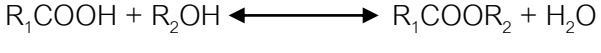
Lipases catalyze the cleavage of ester bonds of triacylglycerols with the concomitant consumption of water molecules, which is called as hydrolysis.



$$R_1COOR_2 + H_2O \longleftrightarrow R_1COOH + R_2OH$$

2.2.4.2 Esterification

Esterification reactions between alcohol and free fatty acids, that is the reverse of the hydrolysis, are catalyzed by lipases in water-poor organic solvents under conditions of low water activity or even solvent free systems.

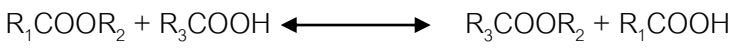


$$R_1COOH + R_2OH \longleftrightarrow R_1COOR_2 + H_2O$$

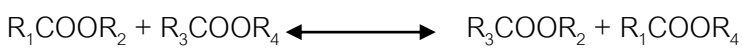
2.2.4.3 Transesterification

The term transesterification refers to the process of exchanging acyl radicals between an ester and an acid (acidolysis), an ester and another ester (interesterification), or an ester and an alcohol (alcoholysis).

2.2.4.3.1 Acidolysis



2.2.4.3.2 Interesterification



2.2.4.3.3 Alcoholysis

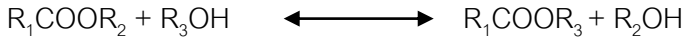


Figure 2-1 Reactions catalyzed by lipases.

2.2.5 Application of lipases

Although lipases can be produced easily on a large scale by growing microorganisms in a fermentor, yet their use is till recently confined largely to oleo-chemistry and dairy based industry. However, the last quarter of the 20th century has witnessed unprecedented use of lipases in biotechnology, manufacture of pharmaceuticals and pesticides, single cell protein production, biosensor preparation and in waste management etc. Lipases have become an integral part of the modern food industry and are used in the preparation of a variety of products including fruit juices, baked food, vegetable fermentation and dairy enrichment. They are also used in leather industry for processing hides and skins (bating) and for treatment of activated sludge and other aerobic waste products where they remove the thin layer of the fats and by so doing provide for oxygen transport. Lipases may also assist in the regular performance of anaerobic digesters. Nearly 1000 tonnes of lipase are used annually in detergent industry, primarily as lipid stain digesters. They also are used as flavour development agents in the preparation of cheese, butter and margarine.

2.3 Immobilization of lipase

Economical usage of lipases in industry requires enzyme immobilization, which enables enzyme reuse and facilitation of the continuous process. Immobilized enzymes are the confinement or localization of enzyme physically in a certain defined region of space with retention to their catalytic activities, and which can be used repeatedly and continuously.

2.3.1 Methods of enzyme immobilization

The selection of an immobilization technique is based on process specification for the catalyst, including such parameters as overall enzymatic activity, effectiveness of the lipase utilization, deactivation and regeneration characteristics, cost of immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized lipase. Chemical methods feature the formation of covalent bonds between the lipase and the modifier, while physical methods are characterized by weaker interactions of the enzyme with the support material, or mechanical containment of the lipase within the support. Methods for enzyme immobilization can be classified into three main categories:

- Cross linking
- Entrapment
- Carrier binding

2.3.1.1 Cross-linking

It can be defined as the intermolecular cross-linking of enzymes by bifunctional or multifunctional reagents and it is based on the formation of chemical bonds, as in the covalent binding method, but water-insoluble carriers are not used. The immobilization is performed by the formation of intermolecular cross-linkages between the enzyme molecules by means of bi or multifunctional reagents. The most common reagent used for cross-linking is glutaraldehyde. Cross-linking reactions are carried out under relatively severe conditions. These

harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity.

2.3.1.2 Entrapping

The entrapment for immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane in such a way as to retain protein while allowing penetration of substrate. This method differs from the covalent binding and cross-linking in that enzyme itself does not bind to the gel matrix or membrane; and thus, has a wide applicability. The conditions used in the chemical polymerization reaction are relatively severe, resulting in the loss of enzyme activity. Therefore, careful selection of the most suitable conditions for the immobilization of various enzymes is required.

Jegannathan *et al.* immobilized lipase PS from *Burkholderia cepacia* by encapsulating within a biopolymer, k-carrageenan. The optimal conditions for processing 10 g of palm oil was: 30 $^{\circ}$C, 1:7 oil/methanol molar ratio, 1 g water, 5.25 g immobilized lipase, 72 hours of reaction time. At the optimal conditions, triglyceride conversion of up to 100% could be obtained. The immobilized lipase was stable and retained 82% relative transesterification activity after five cycles (Jegannathan *et al.,* 2010).

2.3.1.3 Carrier binding

Carrier binding method is the oldest immobilization method for enzymes and is defined as the binding of enzymes to water-insoluble carriers. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depends on the nature of the carrier.

2.3.1.3.1 Covalent binding

The most intensely studied of the immobilization techniques is the

formation of covalent bonds between the enzyme and the support matrix. When trying to select the type of reaction by which a given protein should be immobilized, the choice is limited by two characteristics: (1) the binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and (2) the active site of the enzyme must be unaffected by the reagents used. The covalent binding method is based on the binding of enzymes and water-insoluble carriers by covalent bonds. This method can be further classified into diazo, peptide and alkylation methods according to the mode of linkage. The conditions for immobilization by covalent binding are much more complicated and less milder than in the cases of physical adsorption. Therefore, covalent binding may alter the conformational structure and active center of the enzyme, resulting in major loss of activity and/or changes of the substrate. However, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength.

Wang *et al.* immobilized recombinant *Rhizopus oryzae* lipase on anion exchange resin Amberlite IRA-93 by covalent binding technology. The highest biodiesel yield of 90.5% was achieved under the optimum conditions (enzyme dosage 24 U/g oil, methanol to oil molar ratio 4.8:1, water content 60% by weight of oil, temperature 37^{o}C). There was no obvious loss in immobilized lipase activity after being consecutively used for 7 cycles in the transesterification reactions (Wang *et al.,* 2010).

2.3.1.3.2 Physical adsorption

Physical adsorption of an enzyme onto a solid is probably the simplest way of preparing immobilized enzymes. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix, brought about by mixing a concentrated solution of enzyme with the solid. A major advantage of adsorption as a general method of insolubilizing enzymes is that usually no reagents and only a minimum of activation steps are required. As a result, adsorption is cheap, easily carried out, and tends to be less disruptive to the enzymatic protein than chemical means of attachment, being mainly by

hydrogen bonds, hydrophobic interaction, and Vander Waal's forces. In this respect, the method bears the greatest similarity to the situation found in biological membranes *in vivo* and has been used to model such systems.

2.4 Amberlite XAD761

Amberlite XAD761 is a highly porous phenolic adsorbent in granular form, designed to remove organic impurities from solution by adsorption. Its large active surface and defined pore size distribution is achieved by a unique method of synthesis. The phenolic, hydroxyl and methyl groups of Amberlite XAD761 account for its hydrophilic properties.

CHAPTER III
MATERIALS AND METHODS

3.1 Equipments

Balance	(Satorius, Germany)
Centrifuge tubes	(Oxygen scientific, USA)
Digital balance	(Satorius, Germany)
Desiccator	(Sigma-Aldrich, USA)
Heater	(Chatcharee holding, Thailand)
High performance liquid chromatography	(Shimudzu, Japan)
Magnetic stirrer	(KIKA Labortechnik, Malaysia)
Magnetic bar	(Lio Lab limited partnership)
Microplate reader spectrophotometer	(Biochrom, UK)
Microrefrigerated centrifuge: model 5417	(Eppendrof, UK)
pH meter	(Model 250, Dever Instrument)
Vacuum pump	(Scientific industries, USA)
Vortex	(Scientific industries, USA)
Whatman No.1	(Whatman, UK)
Water bath	(T.S. Instrument, Thailand)

3.2 Chemicals

Acetone	(Carlo erba, Italy)
Acylglycerols standard (Triolein, diolein and monoolein)	(Sigma, USA)
Amberlite XAD761	(Rohm and Haas company, USA)
Bovine serum albumin (BSA)	(Merck, Germany)
Bradford's reagent	(Biorad, USA)
Butanol	(Carlo erba, Italy)
Candida rugosa lipase type VII	(Sigma, USA)
Dipotassium hydrogen phosphate	(Scharlau, Spain)
Eicosane	(Aldrich, Gernany)
Ethanol	(Lab scan, Thailand)
Ethyl acetate	(Carlo erba, Italy)

Ethylene glycol	(Lab scan, Thailand)
Formic acid	(Lab scan, Thailand)
Glacial acetic acid	(Lab scan, Thailand)
Hexane	(Lab scan, Thailand)
Isopropanol	(Lab scan, Thailand)
Methanol	(Lab scan, Thailand)
Oleic acid	(Sigma, USA)
Oleic acid methyl ester (oleate)	(Sigma, USA)
Palm oil	(Morakot industry, Thailand)
ρ – nitrophenyl palmitate	(Sigma, USA)
Potassium dihydrogen phosphate	(Merck, Germany)
Sodium acetate anhydrous	(Sigma, USA)
Sodium dodecyl sulfate (SDS)	(Sigma, USA)
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)
HPLC data analysis program	(LC solution software)

3.4 Research methodology

All experiments were performed at least in tripliicates 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 minutes, 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 minutes 3 times. Then, the supports were immediately used for immobilization.

3.4.1.2 Immobilization of enzyme

The enzyme solution was prepared by dissolving 240 mg crude *Candida rugosa* lipase in 4 ml of 20 mM phosphate buffer solution, pH 7.5. The solution was centrifuged at 5,000 rpm, 4°C for 15 minutes 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 determined from standard curve of BSA shown in Appendix C. Finally, the concentration of lipase 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 5 hours 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 minutes 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 the immobilization. All solutions were quantitated for protein contents by Bradford method as described in section 3.4.3.2. Then, the immobilized lipases were dried at room temperature in desiccator and the enzyme was finally assayed for activities as described in section 3.4.3.1.

3.4.2 Optimization of immobilization procedure

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, concentration of enzyme, temperature and others. The factors studied were as follows: pH, ionic strength, protein loading, time, temperature and adjuvant.

3.4.2.1 Effect of pH

The effect of pH on the immobilization of lipase was studied at different pH values of buffer ranging from 5.0 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 and later used to dissolve crude lipase for immobilization. The immobilized lipase was assayed by the method described in section 3.4.3. The result was expressed as immobilization efficiency.

3.4.2.2 Effect of ionic strength

After the optimal pH was obtained, the following concentrations of buffer at that pH were prepared: 10, 20, 200 and 500 mM from the stock solution to dissolve crude lipase. The immobilized lipase was assayed by the method described in section 3.4.3. The result was expressed as immobilization efficiency.

3.4.2.3 Effect of protein loading

The effect of protein loading on the immobilization lipase was assayed by adjusting protein loading values such as 1, 3, 4.5, 6 and 8 mg/ml. Amount of proteins in lipase solution were prepared by dissolving commercial crude *Candida rugosa* lipase in the proper buffer solution from section 3.4.2.1 and ionic strength from section 3.4.2.2. The immobilized lipase was assayed by the method described in section 3.4.3. The result was expressed as immobilization efficiency.

3.4.2.4 Effect of time and temperature

After the optimal pH, ionic strength and enzyme loading were obtained as described in section 3.4.2.1-3.4.2.3, 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 5 hours at

various temperatures namely, 10, 20, RT, 30 and 40°C. The results were expressed as the percentage of the residual activity of the activity at room temperature. From the experiment described above, after the optimal period of time 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 3.4.3. The result was expressed as immobilization efficiency.

3.4.2.5 Effect of adjuvant

After the optimal pH, ionic strength, enzyme loading, time and temperature were obtained as described in section 3.4.2.1-3.4.2.4, the effect of adjuvant was investigated.

3.4.2.5.1 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 and 5% (v/v) except iso-propanol used was 0, 1, 2.5, 5, 10 and 20 (%v/v) and those of detergents were 0, 0.1, 0.25, 0.5, 0.75, 1 and 2.5 (%v/v), SDS used was 0, 0.1, 0.25, 0.5 and 0.75 (%v/v) and trition X-100 used was 0, 0.1, 0.25, 0.5, 0.75 and 1 (%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 3.4.2.4 and was assayed according to the protocol described in section 3.4.3.1.

3.4.2.5.2 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 to the enzyme solution for 2 minutes 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 methylester was determined by high performance liquid chromatography as described in section 3.4.7.2.

3.4.3 Enzyme activity and protein assays

3.4.3.1 Enzyme activity assay

Activities of the free and immobilized lipase were assayed using 0.5% (w/v) ρ-nitrophenyl palmitate (ρ-NPP) in ethanol as substrate. The reaction mixture consisting of 0.25 ml of 50 mM Tris-HCl buffer, pH 8.0 containing 10 mg immobilized lipase or 25 µl free lipase was initiated by adding 0.25 ml of substrate and mixed for 5 minutes at room temperature. The reaction was terminated by adding 0.5 ml of 0.25 M Na_2CO_3 followed by centrifugation at 14,000 rpm at 4^{o}C for 5 minutes. The increase in the absorbance at 410 nm produced by the release of ρ-nitrophenol in the enzymatic hydrolysis of ρ-NPP was then measured.

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

3.4.3.2 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 plate and was incubated at room temperature for 5 minutes, 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.

3.4.3.3 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.

3.4.4 Transesterification catalyzed by immobilized *Candida rugosa* lipase

After the optimal conditions for immobilization of *Candida rugosa* lipase were obtained as described in section 3.4.2.1-3.4.2.4, the activity of immobilized lipase was investigated. The lipase activities were determined by using immobilized lipase catalyzed transesterification. Transesterification reactions were carried out in 20 ml screw-capped vials containing 0.5 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 water bath for 24 hours at 40^{o}C. After completion of reaction, samples were taken from the reaction and analyzed by HPLC as described in section 3.4.7.1.

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

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

3.4.5.1 Effect of oil to methanol molar ratio

The effect of oil to methanol molar ratio 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% by weight of the immobilized lipase and 0.5 g palm oil were mixed and different ratios of methanol were added into the reaction using 7 steps. Experiments were carried out for the reaction periods of 24 hours and magnetically stirred in a water bath at 40^{o}C. The calculation for the molar ratio was shown in Appendix E.

3.4.5.2 Effect of addition mode of methanol

When the proper oil to methanol molar ratio from the method described above was obtained, the effect of addition mode of methanol on biodiesel production from palm oil using 1, 2, 3, 4, 5, 6 and 7 steps was studied. The reaction medium was magnetically stirred in a water bath for 24 hours at 40°C.

3.4.5.3 Effect of enzyme loading

When the proper oil to methanol molar ratio, addition mode of methanol 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 palm oil with each amount of the immobilized lipase for transesterification. Then, the optimal ratio of oil and methanol was added using the addition mode of methanol obtained from section 3.4.5.1 and 3.4.5.2, respectively. The reactions were magnetically stirred in a water bath for 24 hours at 40°C.

3.4.5.4 Effect of water content

When the proper oil to methanol molar ratio, addition mode of methanol and enzyme loading from the method described above were obtained, the effect of water content on lipase activity in transesterification reaction was examined at 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4 and 5% (v/v) of the oil. The reaction mixtures were magnetically stirred in a water bath for 24 hours at 40°C.

3.4.5.5 Effect of reaction time and temperature

After the reaction mixtures were mixed using the optimized conditions from section 3.4.5.1-3.4.5.4, the reaction medium was incubated at different temperature namely, 30, 40 and 50°C and stirred in a water bath for 24 hours to obtain the optimal time and temperature for the transesterification. Then, 50 µl of samples were taken from the reaction mixtures at 3, 6, 9, 12, and 24 hours.

3.4.6 Stability of immobilized lipase

3.4.6.1 Repeated use of the immobilized *Candida rugosa* lipase for transesterification

The reusability of immobilized enzyme was tested by duplicate trials of reactions under optimal conditions which were 0.5 g of palm oil, immobilized lipase content of 30% (w/w of oil), 1:4 oil: methanol ratio and magnetically stirred in a water bath for 24 hours. In the present study, the immobilized lipase was rinsed with water, t-butanol and hexane after each batch to remove glycerol and oil. The enzyme was then dried in desiccators at room temperature. The dried immobilized lipase was used in the next batch composed of new substrates. The residual activity determined after the complete reaction was expressed as relative conversion. The conversion achieved in the first batch was set to 100.

3.4.6.2 Thermal stability

The thermal stability of immobilized enzyme was tested by incubating 10 mg of immobilized lipase at 40^{o}C in the water bath for 1, 3, 6 and 9 hours. Then, the residual activities were determined for the half life of the immobilized lipase as the percentage yield of activity compared to the activity of untreated control. The calculation for half life ($t_{1/2}$) was shown in Appendix D. The results were expressed as the percentage of relative residual activity and time.

3.4.7 Analysis of the fatty acid methyl ester (biodiesel)

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

3.4.7.1 Analysis of the fatty acid methyl ester by HPLC

3.4.7.1.1 Preparation of sample

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

3.4.7.1.2 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 × 4.6 mm ×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 (85:10:10:0.1 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) A increasing to 98% (v/v) in 20 minutes. The final mixture (A:B, 98:2 v/v) was employed for 3 minutes. The system was restored to its initial condition by passing the A:B, 1:99 (v/v) mixture through the column for 15 minutes.

3.4.7.2 Calculation of biodiesel

Biodiesel yield was calculated as the percentage of the actual amount of methylester detected in the reaction process divided by the theoretical quantity of methylester. Calculation of the yield was described in Appendix E.

CHAPTER IV
RESULTS

4.1 Optimization of lipase immobilization

The effects of immobilization parameters namely pH, ionic strength, enzyme loading, time, temperature and adjuvants of the immobilization were investigated and the result of each factor was illustrated in Figure 4-1 to Figure 4-7.

4.1.1 Effect of pH

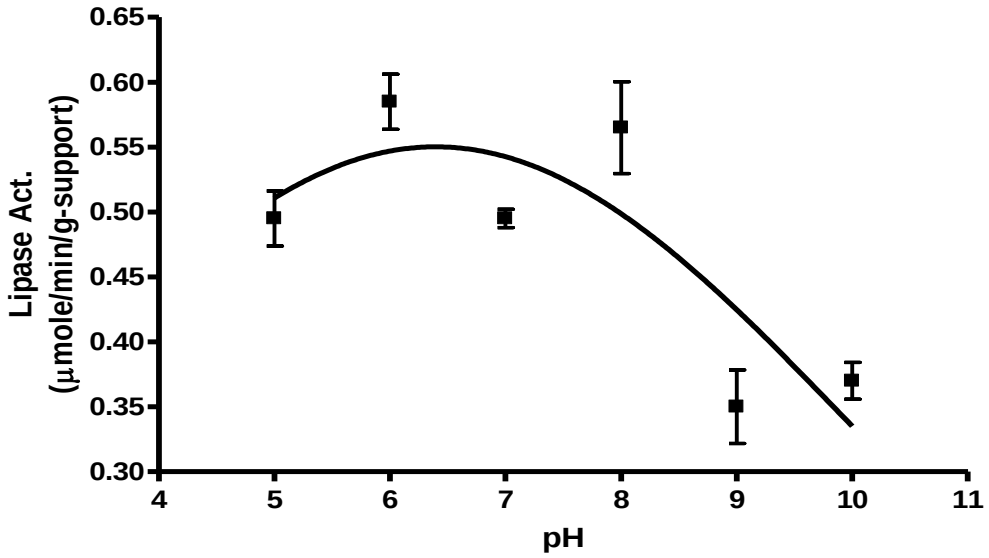


Figure 4-1. The effect of pH on lipase activity. Crude *Candida rugosa* lipase was dissolved in 3 ml 20 mM buffer solution at various pH. Then, 3 ml lipase solution (3 mg/ml) was added to 1 g of Amberlite XAD761 and magnetically stirred for 5 hours at room temperature. Activities shown on the y-axis are the means ± SD of three individual experiments.

The activities of lipase at various pH were studied and the results were shown in Figure 4-1. It can be seen that maximal activity (0.58 µmole/min/g-support) was obtained when pH of the system reached pH 6.0 and started to decrease by approximately 63% when the pH rose to 10 since denaturation tends to increase under basic condition. The results also indicated that immobilized lipase from *Candida rugosa* is quite stable in acidic environment. The optimum pH of lipase was therefore fixed as 6.

4.1.2 Effect of ionic strength

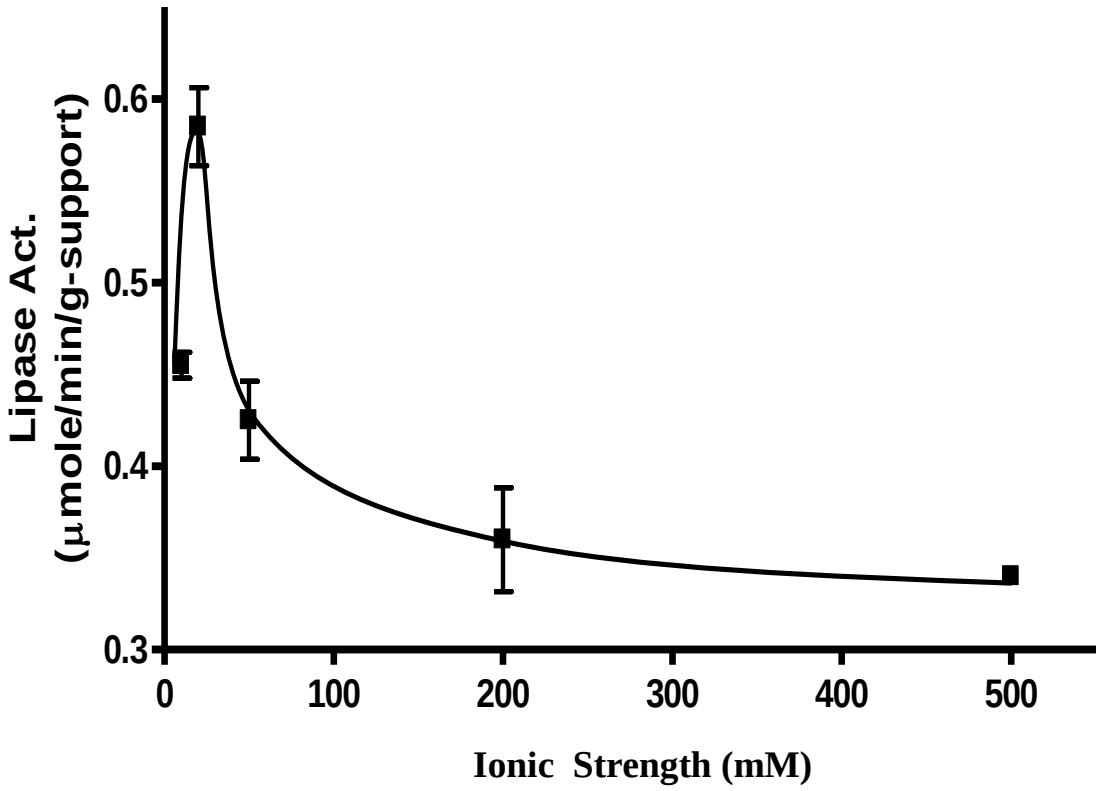


Figure 4-2. The effect of ionic strength on lipase activity. 3 ml lipase solution (3mg/ml) containing various concentrations of phosphate buffer, pH 6.0 was added to 1 g of Amberlite XAD761 and magnetically stirred for 5 hours at room temperature. Activities shown on the y-axis are the means ± SD of three individual experiments.

The effect of the ionic strength of enzyme solution on the adsorbed amount of protein was investigated and the results are shown in Figure 4-2. It can be seen that the activity of lipase increased from 0.45 to 0.58 umol/min/g-support with increasing ionic strength from 10 mM to 20 mM. On the other hand, when the ionic strength was elevated from 20 mM to 500 mM, the activity of lipase was decreased from 0.58 to 0.36 umol/min/g-support. Therefore, 20 mM of phosphate buffer pH 6.0 was used as optimal ionic strength for the immobilization of *Candida rugosa* lipase to study the effect of protein loading on activity of immobilized lipase.

4.1.3 Effect of enzyme loading

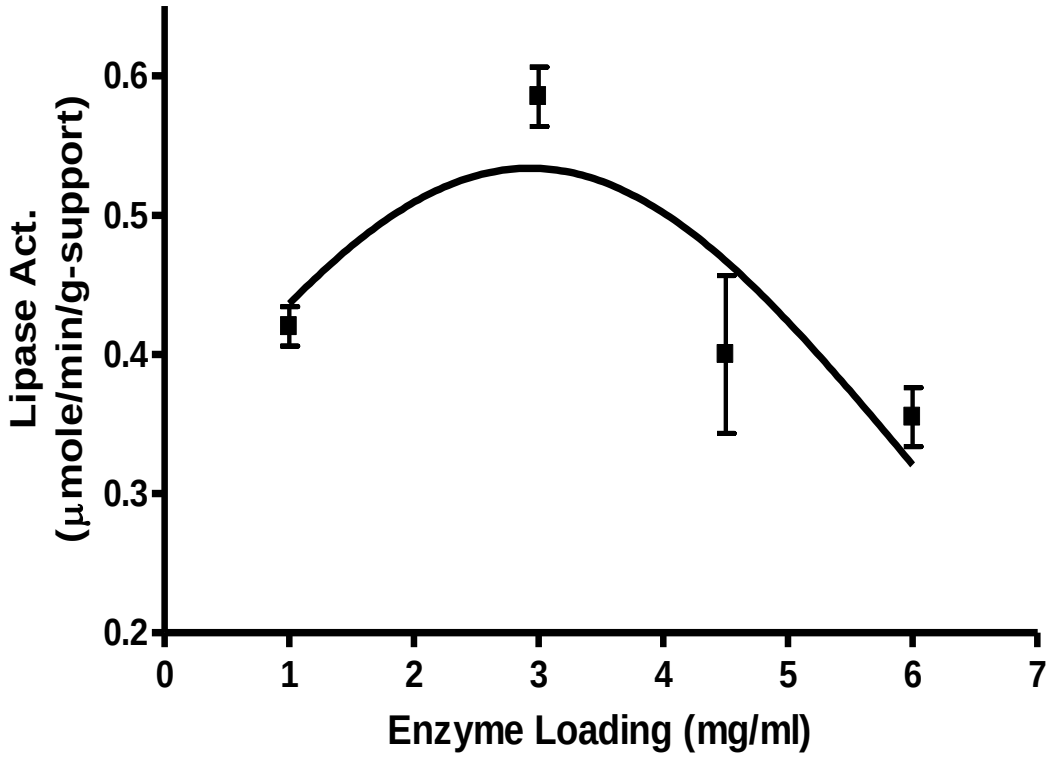


Figure 4-3. The effect of enzyme loading on lipase activity. Lipase solution containing various quantities of enzyme dissolved in 20 mM phosphate buffer, pH 6.0 was added to 1 g of Amberlite XAD761 and magnetically stirred for 5 hours at room temperature. Activities shown on the y-axis are the means ± SD of three individual experiments.

In this study, different quantities of enzyme were immobilized on the supports from 1 to 8 mg/ml and the results were shown in Figure 4-3. It can be seen that the activity significantly increased from 0.42 to 0.59 umol/min/g-support when the protein loading was increased from 1 to 3 mg/ml. On the contrary, when the protein loading was elevated from 3 to 6 mg/ml, the activity of lipase was decreased from 0.5 to 0.37 umol/min/g-support. Therefore, 3 mg/ml was used as optimal amount of protein loading. Hence, the optimal condition was selected as described in section 4.1.1-4.1.3 to study the effect of time of immobilization.

4.1.4 Effect of time

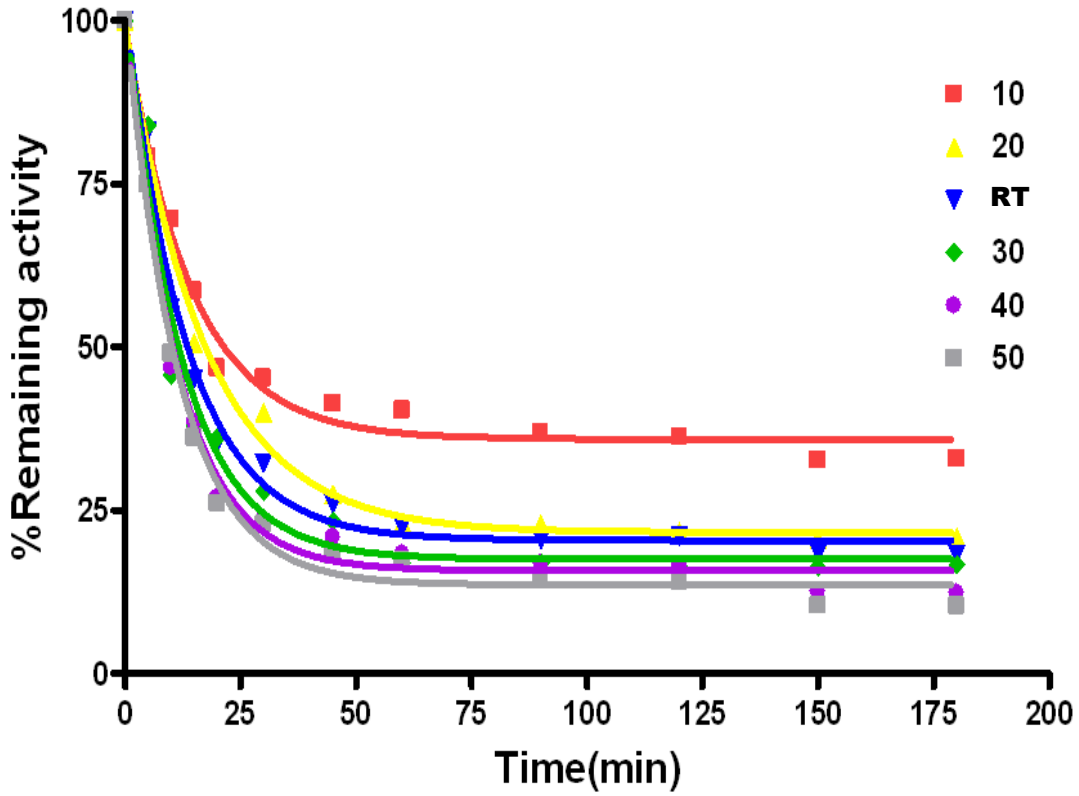


Figure 4-4. The effect of immobilization time on the residual activity of lipase. 3 mg/ml of crude *Candida rugosa* lipase solution were incubated at various temperatures. Residual activities on the y-axis are the means ± SD of three individual experiments.

The relationship of the residual activity with immobilization time at various temperatures was shown in Figure 4-4. The results were expressed as the percentage of the residual activity at room temperature. When lipase was incubated at 10°C for 180 minutes, the residual activity of lipase appeared unchanged at 36%. Furthermore, when lipase solution was incubated at 20°C and room temperature, the results showed that the residual activity leveled off in both temperatures at 18 and 16% respectively until 180 minutes. At 30 and 40°C, the residual activity of lipase solution gave the similar pattern that they significantly decreased from initial time of the incubation to around 25-75 minutes and stayed unchanged from then until 3 hours. Therefore, the optimal time for immobilization was 3 hours at 10 to 40°C and subsequently selected for the determination of the optimal temperature.

4.1.5 Effect of temperature

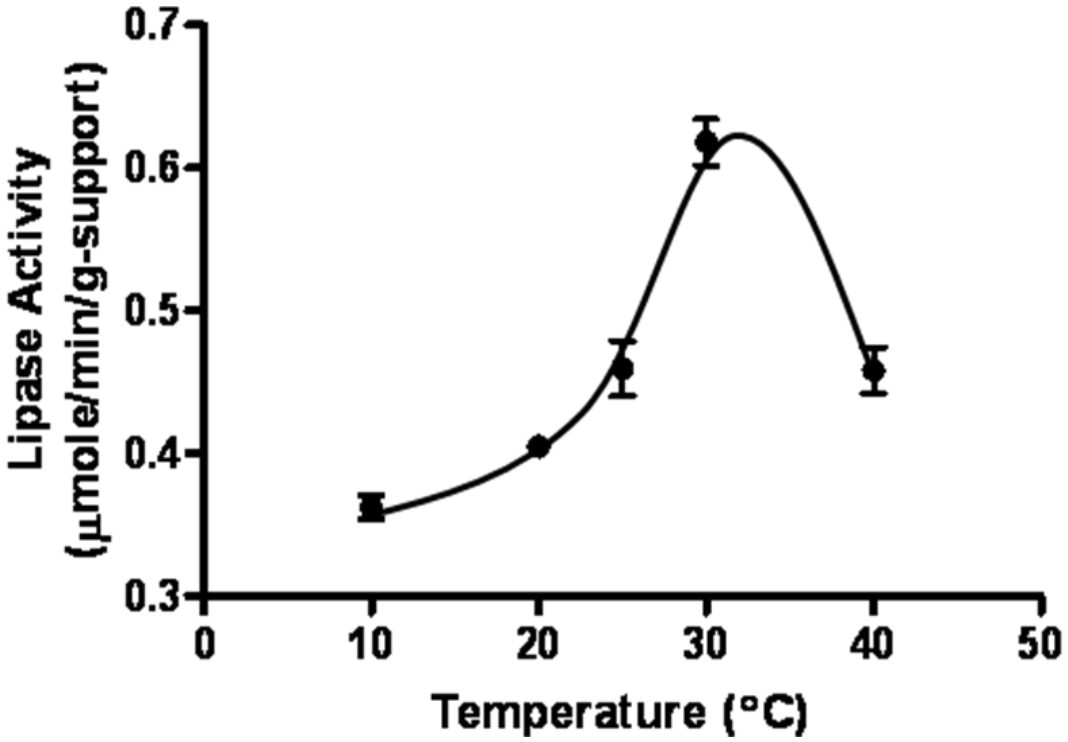


Figure 4-5. The effect of temperature on lipase activity. The immobilization was performed in 20 mM phosphate buffer, pH 6.0 with 3 mg/ml of lipase solution at specific time for each temperature. Activities on the y-axis are the means ± SD of three individual experiments.

Lipase was immobilized on Amberlite XAD761 at different temperatures from 10 to 40°C. The immobilization reaction was carried out under the optimal conditions obtained from all 4 previous parameters. In this experiment, the immobilization of *Candida rugosa* lipase was determined by checking the activity of immobilized lipase. From Figure 4-5, it was shown that when 10°C was used for immobilization, the activity of lipase was 0.36 umol/min/g-support. They considerably increased to 0.62 umol/min/g-support when temperature rose to 30°C. However, when temperature was increased to 40°C, the lipase activity decreased from 0.62 to 0.46 umol/min/g-support. From these results, 30°C was used as the optimal temperature to study the effect of adjuvant on *Candida rugosa* lipase immobilization.

4.1.6 Effect of adjuvant

Once the optimal conditions of 20 mM phosphate buffer, pH 6.0, 3 mg/ml of *Candida rugosa* lipase solution for immobilization at 30°C for 3 hours were obtained, the effect of adjuvant on the activity of immobilized *Candida rugosa* lipase was examined as described in section 3.4.2.5. The adjuvants were studied for both the effect of the types and the concentration from each type as well.

4.1.6.1 Effect of concentration of adjuvant

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

Type of adjuvant	Concentration of adjuvants (%v/v)
Methanol	5
Ethanol	5
Isopropanol	1
Butanol	5
t- Butanol	5
SDS	0.25
Tween 80	0.25
Ethylene glycol	2.5
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 categories of adjuvants with various different concentrations as described in section 3.4.2.5.1 and the results were tabulated in Appendix D. The results were expressed as the percentage of the activity of untreated lipase solution. Then, the various concentrations of each adjuvant 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.

4.1.6.2 Effect of the type of adjuvant

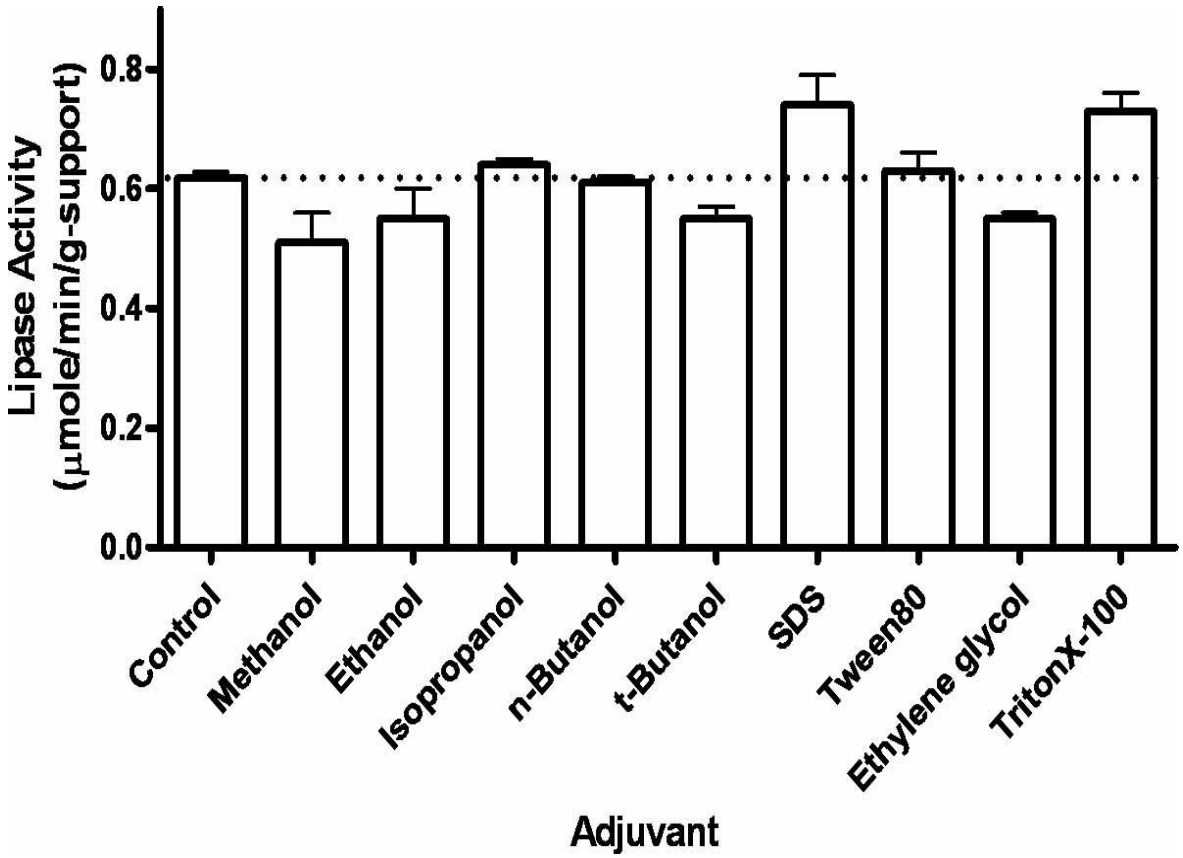


Figure 4-6. The effect of the type of adjuvant on lipase activity. The suitable concentration of each adjuvant was added to the enzyme solution (3 mg/ml) for 2 minutes before contact with support. The reaction mixture was magnetically stirred for 3 hours at 30°C.

When the proper concentration of each adjuvant as described was obtained, the effect of the type of adjuvant on the immobilization of *Candida rugosa* lipase was then determined as described in 3.4.3.1. The relationship between the lipase activity with the type of adjuvant was shown in Figure 4-6. The result showed that the highest lipase activity (0.74 (±0.05) μmol/min/g-support) was achieved when SDS was used as adjuvant but was not apparently different from control. Therefore, the immobilized lipase was then tested for transesterification to determine the optimal type of adjuvant by the method described in section 3.4.4.

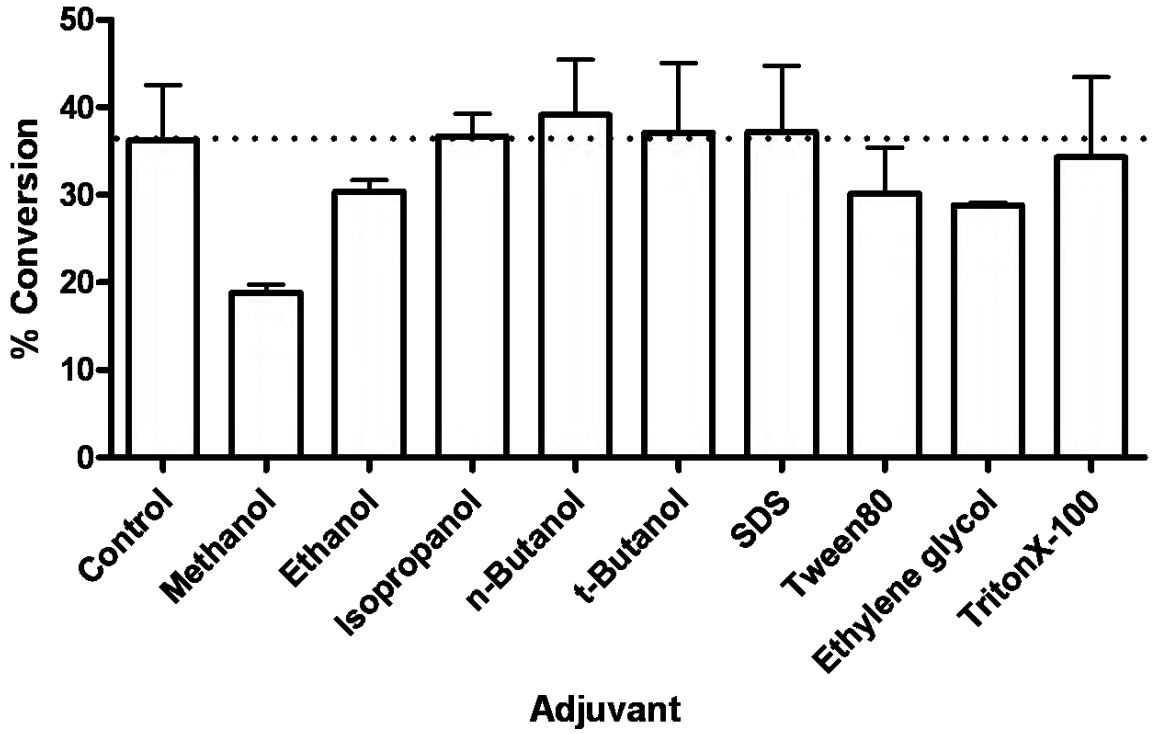


Figure 4-7. The effect of immobilized lipase with added various adjuvants on percentage of conversion to biodiesel. The reactions carried out in a mixture of 0.5 g of palm oil, 1:3 oil to methanol, 3 step addition mode of methanol and 20% (w/w of oil) immobilized lipase were magnetically stirred in a water bath for 24 hours at 40°C. Percent conversion shown in the y- axis is the means ± SD of three individual experiments.

From Figure 4-7, the results showed that the conversion was highest when n-butanol was used as adjuvant but was not much different from control. Therefore, in order to reduce the cost for further applications, no adjuvant was required.

Table 4-2 tabulated the obtained optimal conditions for the immobilization of *Candida rugosa* lipase on Amberlite XAD761.

Table 4-2. The overall optimal conditions for the immobilization of *Candida rugosa* lipase on the Amberlite XAD761

pH	6.0
Ionic strength (mM)	20
Protein loading (mg/ml)	3
Time (hr)	3
Temperature (^{o}C)	30

4.2 Transesterification catalyzed by immobilized *Candida rugosa* lipase

When the optimal conditions for immobilization of *Candida rugosa* lipase from the section 4.1.1-4.1.6 were obtained, the immobilized lipase was further investigated for the production of biodiesel. The transesterifications were carried out in the incubation medium containing 0.5 g of palm oil, 1 to 3 ratio of oil to methanol with 3 step additions catalyzed by 20% (w/w of oil) immobilized lipase. The sample was magnetically stirred for 24 hours at 40^{o}C and the product was quantitated by HPLC. The result revealed that approximately 35% of biodiesel was obtained.

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

The effect of oil to methanol molar ratio, addition mode of methanol, amount of enzyme, water content, time and temperature for the biodiesel production were investigated. The result for each factor was illustrated in Figure 4-8 to 4-12.

4.3.1 Effect of oil to methanol molar ratio

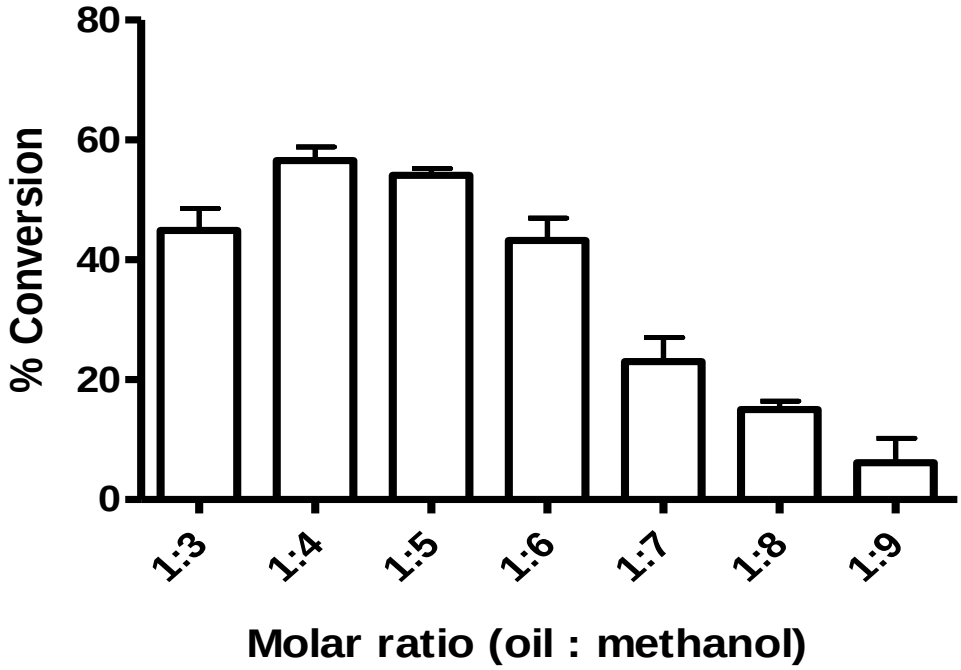


Figure 4-8. The effect of oil to methanol ratio on percentage of conversion to biodiesel. The reactions were carried out with 0.5 g of palm oil, 20% (w/w of oil) immobilized lipase using 7 steps addition mode of methanol and continuously stirred at 40°C for 24 hours. Percent conversion shown on the y-axis is the means ± SD of three individual experiments.

The effect of oil to methanol molar ratio on the conversion was performed at 40°C, with the immobilized lipase concentration of 20% (w/w of oil), and varying the oil to methanol molar ratio ranging from 1:3 to 1:9. Figure 4-8 showed the fatty acid methyl esters conversion obtained. As can be seen from this figure, the highest conversion (57%) was achieved at the molar ratio of 1 to 4. However, when the ratio was increased to 1 to 7, the yield of biodiesel was dramatically decreased for nearly 2.5 folds to 23%. Moreover, when the ratio was increased to 1 to 8 and 1 to 9, the yield of biodiesel was continuously decreased for 3.8 folds to 15% and 9.5 folds to 6% respectively. As a result, 1 to 4 molar ratio of oil to methanol was used as optimal conditions for transesterification to further study the effect of addition mode of methanol on transesterification.

4.3.2 Effect of addition mode of methanol

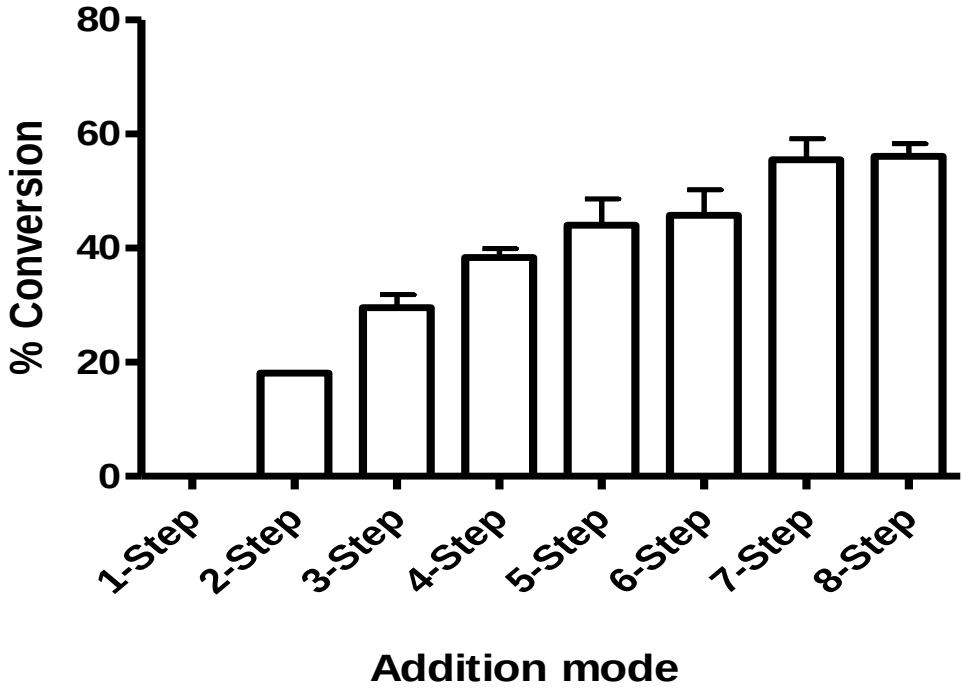


Figure 4-9. The effect of addition mode on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g of palm oil, 1:4 oil to methanol molar ratio with 20% (w/w of oil) immobilized lipase and continuously stirred at 40°C for 24 hours. Percent conversion shown on the y-axis is the means ± SD of three individual experiments.

The effect of addition mode of methanol from one 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 to 4 mole ratio of oils. From Figure 4-9, it was found that when four moles of methanol were added in one step, no biodiesel was obtained. The yield suddenly rose from 0% to 18% when the adding step of methanol increased from one to two steps and increased further from 18% to 57% at the seventh step and remained steady until eight step. The optimal addition mode of methanol for the maximum yield of biodiesel was therefore fixed at 7 steps and subsequently selected for the next experiment namely: optimal condition for the amount of enzyme on transesterification.

4.3.3 Effect of amount of enzyme

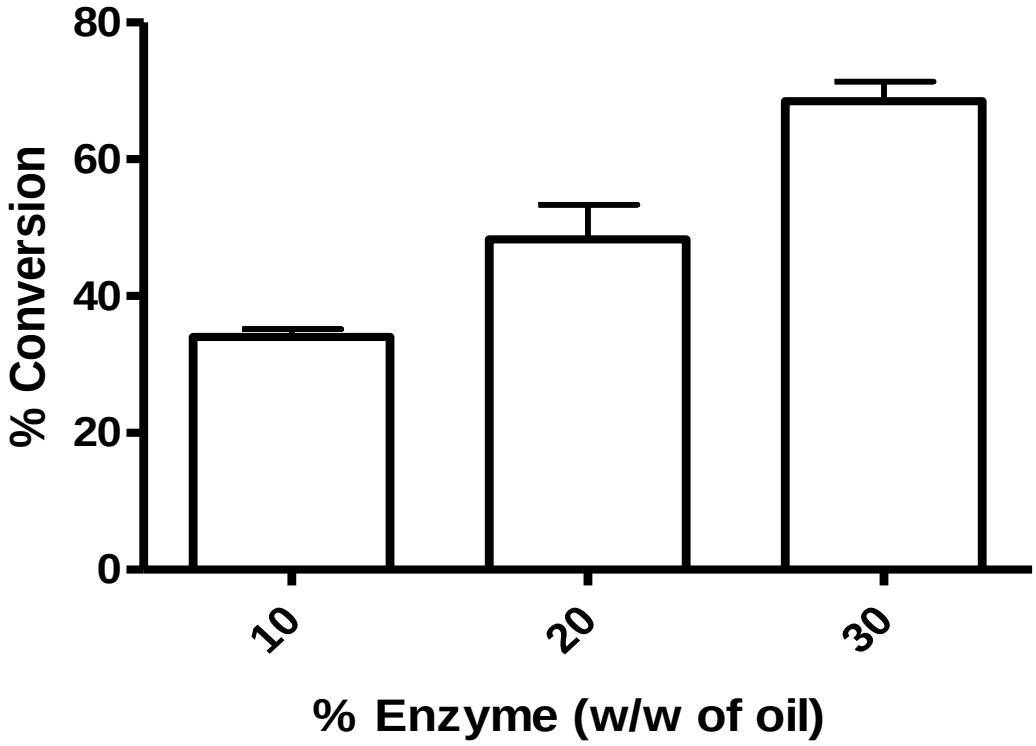


Figure 4-10. The effect of amount of enzyme on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g palm oil, 1 to 4 oil to methanol molar ratio, 7 step addition mode of methanol and magnetically stirred in a water bath at 40°C for 24 hours. Percent conversion shown on the y-axis is the means ± SD of two individual experiments.

The effect of immobilized lipase concentration on conversion was performed with enzyme concentration of 10%, 20% and 30% w/w of oil , the ratio of oil to methanol of 1 to 4, and 7 steps of methanol addition at the temperature of 40°C. Figure 4-10 illustrated the conversion of biodiesel when various amounts of immobilized lipase were used. From this figure, when 10 and 20% immobilized enzyme were used, the conversion of fatty acid methyl ester increased from 34 – 48%. However, the maximal yield of FAME at 68.49% was achieved

with the concentration of 30% immobilized lipase Therefore, the following optimal conditions: 1 to 4 molar ratio of oil to methanol, 7 steps addition mode of methanol and 30% of enzyme were subsequently applied for the next optimal condition namely; water content.

4.3.4 Effect of water content

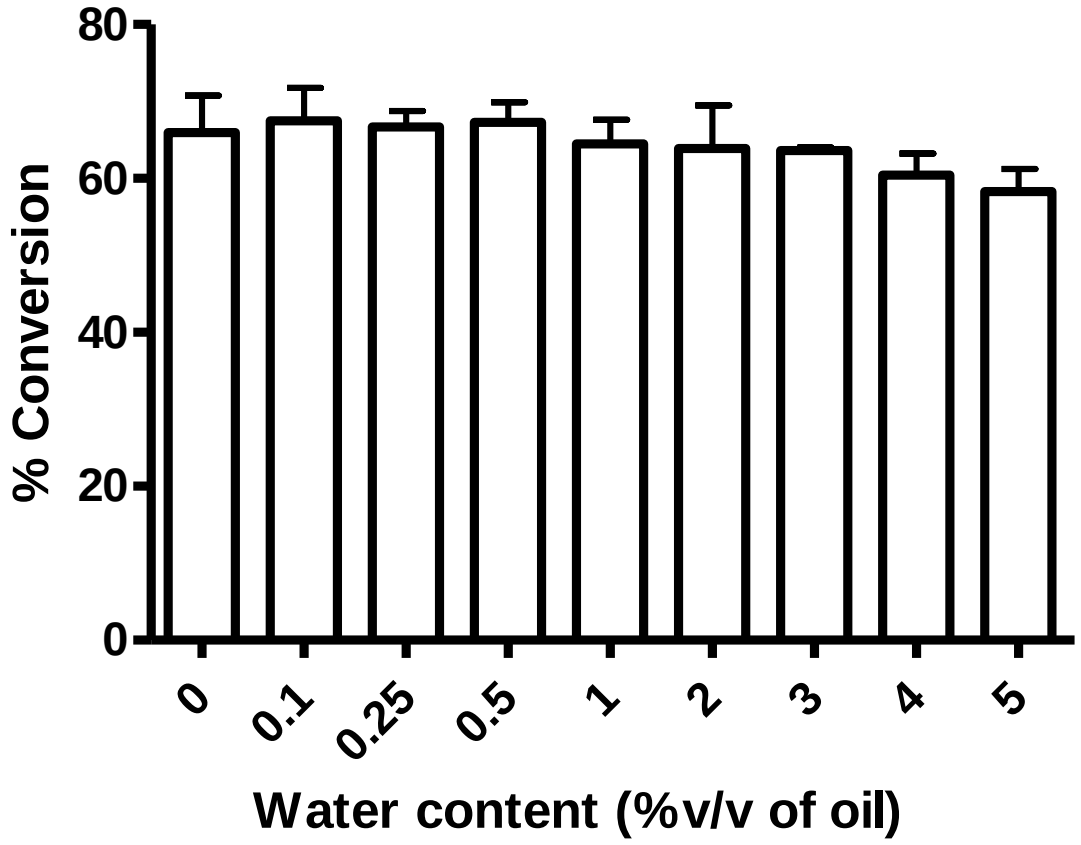


Figure 4-11. The effect of water content on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g of palm oil, 30% (w/w of oil) immobilized lipase, 1 to 4 molar ratio of oil to methanol, 7 steps addition mode of methanol and continuously stirred at 40°C for 24 hours. Percent conversion shown on the y-axis is the means ± SD of two individual experiments.

The reaction was carried out by adding water ranging from 0% – 5 % (%v/v) 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, 94 µl methanol (added at seven steps, each 13.5 µl) and magnetically stirred in a water bath for 24 hours at 40°C. The results were shown in Figure 4-11.

As indicated in Figure 4-11, it can be seen that the FAME content with the addition of water were approximately 69% which was indifferent from the control. So, the reaction was subsequently carried out in the absence of water.

4.3.5 Effect of time and temperature

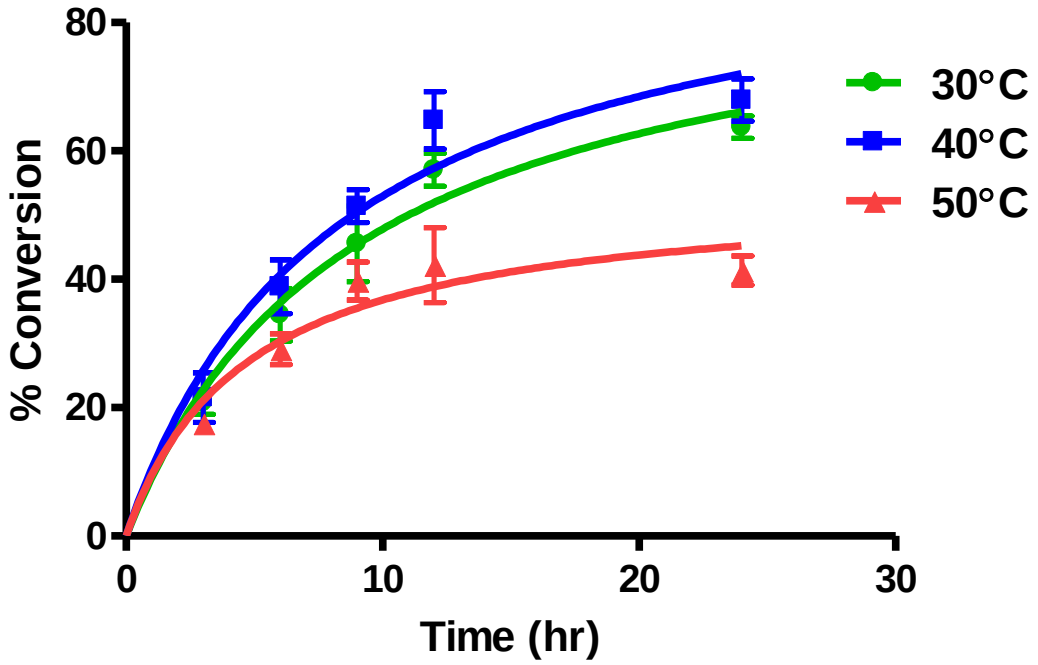


Figure 4-12. The effect of time and temperature on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g palm oil, 30% (w/w of oil) immobilized lipase,

1 to 4 of oil to methanol molar ratio, 7 steps addition mode of methanol and magnetically stirred in a water bath at each temperature for 24 hours. Percent conversion shown on the y-axis is the means ± SD of two 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 50°C shown in Figure 4-12.

Experimental results showed that the transesterification reaction could proceed within the temperature range studied. Nevertheless, the reaction time to complete the reaction varied significantly with reaction temperature. It can be seen that the highest yield of 72% biodiesel was obtained at 40°C for the period of 24 hours.

The results in Figure 4-12 showed that the percentage yield of biodiesel gradually rose when the reaction time was increased from 0 to 24 hours. When the reaction temperature increased to 50°C, it was found that the product yield started to decrease from the loss of the enzyme activity.

After all of the optimal conditions for transesterification were applied, it can be seen that the yield of biodiesel was increased from 35 to 72%.

4.4 Stability of immobilized lipase

4.4.1 Repeated use of the immobilized *Candida rugosa* lipase for transesterification

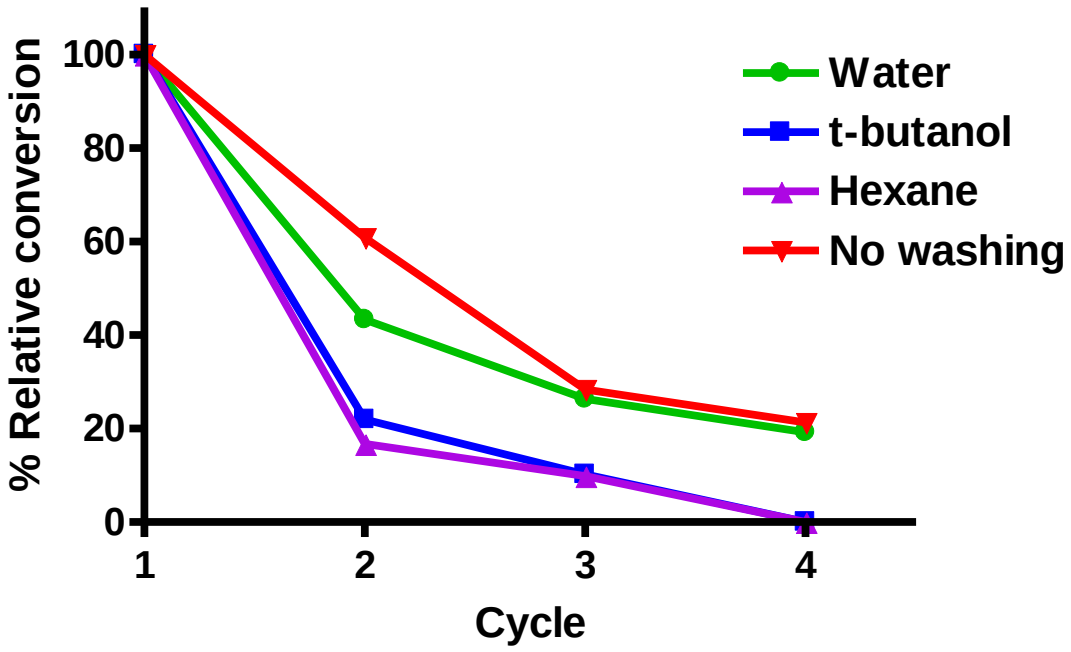


Figure 4-13 Operational stability of immobilized *Candida rugosa* lipase catalysis for transesterification. The reactions were carried out in a mixture of 0.5 g of palm oil, 30% (w/w of oil) immobilized lipase, 1 to 4 of oil to methanol molar ratio, 7 steps addition mode of methanol and continuously stirred at 40°C for 24 hours. The lipase was transferred into the same system for a new cycle after completion of former reaction in 24 hours.

From the results, the yield of FAME obtained was 61% from the first usage and was set as 100% relative conversion. Then, rapid decrease of conversion; 43%, 23% and 16% were obtained after the immobilized lipase was rinsed with water, t-butanol, and hexane for the second usage respectively. Furthermore, the enzyme apparently lost all of its activity after the immobilized lipase was rinsed with t-butanol, and hexane in the 4th cycle. However, both of the

unwashed immobilized lipase and the enzyme washed with water could still retain approximately 20 % of its initial activity. This result indicated that unwashed immobilized lipase could be used repeatedly over an extended period of time.

4.4.2 Thermal stability

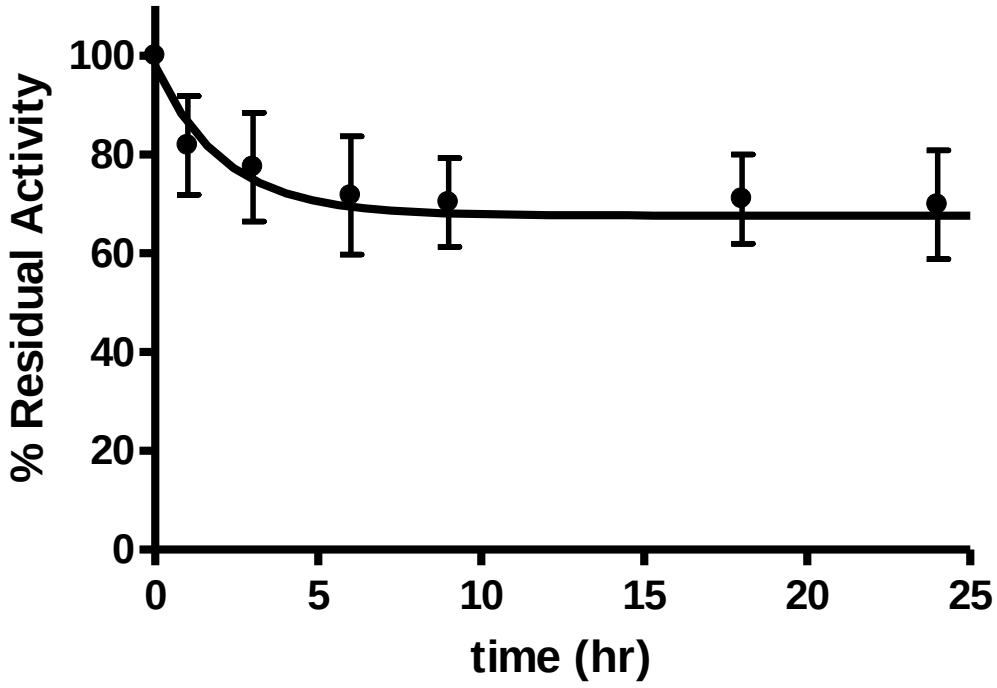


Figure 4-14 The thermal stability of immobilized *Candida rugosa* lipase. 10 mg of immobilized lipase were incubated at 40^{O}C for 1, 3, 6, 9, 18 and 24 hours. The results were average values of triplicate experiments.

The thermal stability of immobilized lipase was carried out by incubating 10 mg of immobilized lipase for the period of 24 hours at 40^{o}C, optimal temperature for transesterification. The samples were periodically taken and the residual activities were determined as the percentage yield of activity compared to the activity of untreated control. From Figure 4-14, the results showed that the percentage of remaining activity decreased from 100% to 70% within

6 hours and stayed unchanged until 24 hours. Then, the half life ($t_{1/2}$) of the immobilized enzyme was determined (as described in Appendix D). The results were illustrated in Figure 4-15.

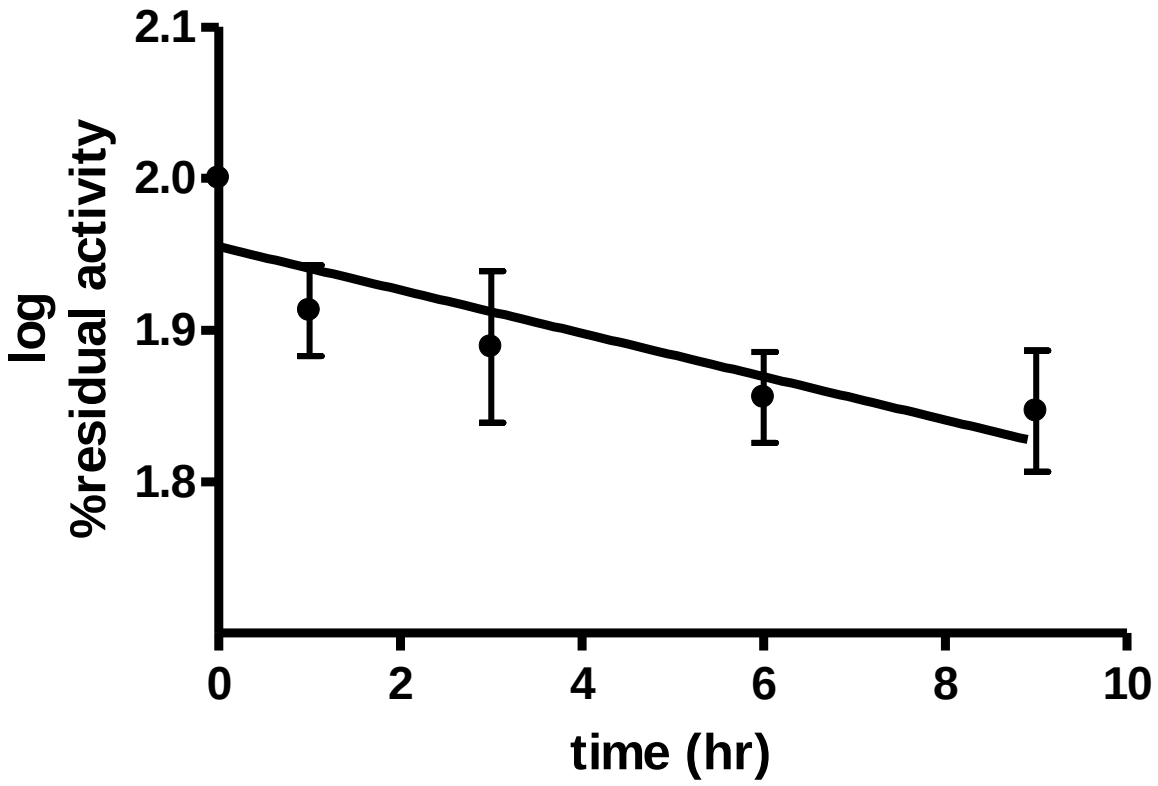


Figure 4-15. Half life of immobilized *Candida rugosa* lipase. 10 mg of immobilized enzyme was incubated at 40°C for 1, 3, 6 and 9 hours. Log of the residual activity shown on the y-axis is the means ± SD of three individual experiments.

When the log of the residual activity was plotted against time at optimal temperature for transesterification (40°C), the straight line was obtained as shown in Figure 4-15 .The calculated half-life of the immobilized enzyme was 21.08 ± 3.48 hours as described in Appendix D.

CHAPTER V
DISCUSSION

5.1 Enzyme immobilization

There are many factors that influence the performance of an immobilized enzyme preparation. Some of the most important factors are the choice of a carrier, the properties that an enzyme carrier should have can be listed as follows: large surface area, permeability, insolubility, chemical, mechanical and thermal stability, high rigidity, suitable shape and particle size, resistance to microbial attack and cost-effectiveness. Amberlite XAD761 selected for this work as the carrier materials for optimal immobilization of *Candida rugosa* lipase are highly porous phenol-formaldehyde matrix granular, with a high hydrophobicity for enzyme immobilization. The immobilized enzymes with these supports were used to catalyze transesterification. 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^{o}C for 24 hours with continuous stirring by magnetic stirrer. It could be seen that Amberlite XAD761 gave the approximate conversion of 35%. 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 lipase immobilization

The optimal conditions for immobilization of *Candida rugosa* lipase were determined as follows: pH, ionic strength, enzyme loading, time, temperature and adjuvant. The effects of these factors on activity of immobilized lipase were described.

5.2.1 Effect of pH

The optimum pH for lipase activity varies with the enzyme species. From the results, the effect of changing pH and the lipase activity of immobilized lipase were shown in Figure 4-1. It could be seen that the shape of the graph is bell-shaped curve and 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 altered functional groups are dependent upon the pH of the solution (Lei *et al*., 2009). Since isoelectric point of lipase from *Candida rugosa* is 4.6, overall net change 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 environment 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 optimum of immobilized *Candida rugosa* lipase can be slightly higher and lower than the free enzyme (Pereira *et al*., 2001, Blanco *et al*., 2004 and Yesloglu Y, 2005). However, the lipase activity started to decrease when the pH subsequently rose to more than 10. At higher or alkali pH, the denaturation of lipase tends to be increasing like other protein.

5.2.2 Effect of ionic strength

From the result of the ionic strength on activity of immobilized lipase in section 4.1.2 (Figure 4-2), it was found that activity of immobilized lipase was highest in 20 mM phosphate buffer pH 6.0. The activity of lipase increased with the increasing ionic strength from 10 mM to 20 mM. Conventionally, adsorption of proteins by hydrophobic interaction is stronger when the strength is increased (Bastida *et al.,* 1998). Since lipase will exhibit net negative charge at pH 6.0, therefore the increase of ionic strength will gradually decrease the charges on enzyme molecules until the net charge approaches zero. This results in more pronounced hydrophobic interaction between enzyme and the support. In contrast, when the ionic strength was elevated from 20 mM to 500 mM, the activity of lipase was decreased indicating that the higher ionic strength initiates more hydrophobic environment around the active sites. Accordingly, the numbers of active enzyme were further reduced.

5.2.3 Effect of protein loading

The amount of lipase immobilized on carrier was limited because the porous sites as the carriers were saturated (Jian *et al.*, 2005). In order to determine the optimal amount of lipase, the immobilization of various amounts of lipase on 1.0 g of Amberlite XAD761 was studied. The effect of enzyme loading on lipase activity was shown in Figure 4-3. The lipase activity significantly increased when enzyme solution was increased from 1 to 3 mg/ml. From these results, it could be realized that 3 mg/ml of *Candida rugosa* lipase solution was the maximum enzyme loading and the lipase activity was dependent on the enzyme concentration. However, the lipase activity started to decrease when the enzyme quantitatively rose to more than 8 mg/ml. Therefore, 3 mg/ml of *Candida rugosa* lipase solution was selected as the optimum enzyme loading for subsequent experiment.

5.2.4 Effect of time

The residual activity of lipase solution was checked for each time of immobilization at various temperatures as described in 3.4.2.4. The relationship of the residual activity with immobilization time at various temperatures was shown in Figure 4-4. The amount of soluble protein was rapidly decreased with the increment of the immobilization time. When lipase was incubated at 10^{o}C for 3 hours, the residual activity of lipase appeared unchanged. Furthermore, when lipase solutions were incubated at 20^{o}C and room temperature, the results showed that the residual activity leveled off until 3 hours of the incubation. At 30 and 40^{o}C, the residual activity of lipase solution gave the similar pattern that they significantly decreased from initial time of the incubation to around 25-75 minutes and stayed unchanged from then until 3 hours. From the results, it could be explained that the immobilization is the chemical reaction. Higher temperature usually renders shorter time for the reaction to completion than low temperature. However, enzyme denaturation causing activity loss is inevitable. Thus, the activity of the enzyme immobilized at various temperatures were further checked. Nevertheless, at low temperature, the enzyme is less denatured but possibly requires longer interaction time to

permit the correct alignment between reactive groups, and partially rigidified enzyme (Guisan, 2006; Mateo *et al.,* 2007). As a consequence, the optimal immobilization time is compromised between the shortest time that provides both the bonded protein on the support and enzyme activity. Hence, the decreasing lipase activity in solution indicated that the reacted amount of enzyme to enzyme carrier with equilibrium were different at specific time of each temperature.

5.2.5 Effect of temperature

Lipase was immobilized on Amberlite XAD761 at different temperatures; 10, 20, RT, 30 and 40°C. The results in Figure 4-5 showed that the activity of lipase was initially increased under the temperatures from 10 to 30°C and the highest activity was obtained when the temperature of immobilization reached 30°C. As shown in Figure 4-5, 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 30 to 40°C, the activities were reduced. This can be simply explained that the temperatures were too high causing the thermal deactivation of the lipase. From the optimal immobilization time obtained in section 4.1.4, the activity of immobilized lipase was found to be suitable at 30°C. Therefore, the optimal immobilization time was 3 hours at 30°C.

5.2.6 Effect of adjuvant

Once the optimal conditions of 20 mM phosphate buffer, pH 6.0, 3 mg/ml of *Candida rugosa* lipase solution for immobilization at 30°C for 3 hours were obtained, the effect

of adjuvant on the activity of immobilized *Candida rugosa* lipase was examined by using two categories of adjuvant namely; alcohol and detergents. Both the concentration and the type of the adjuvants were studied.

5.2.6.1 Effect of concentration of adjuvant

The highest concentration of each type of adjuvant necessary for the desired effect of each type of adjuvant with no damage of the enzyme activity was determined. Table 4-1 showed the concentrations of various adjuvants with the activities of *Candida rugosa* lipase. 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 4-6. Lipases catalyze the hydrolysis of water insoluble triacylglycerols at the oil-water interface where the lipase activities are greatly increased by a mechanism of interfacial activation. On the molecular level, lipase-substrate-detergent interactions can be complex because of many parameters including micelle formation, concentrations of free and micellar substrate, their availability to enzyme, the degree and mode of the enzyme activation by the hydrophobic interactions, enzyme denaturation or inactivation by detergents, and the structure of the enzyme itself defining the location of the enzyme in the water-oil interphase. Lipase-detergent interactions are primarily hydrophobic but the nature of the hydrophilic group on the detergent contributes also to the contact. Hence, there can be a considerable divergence even between the behavior of anionic, cationic, or nonionic detergents with a lipase (Helistö and Korpela, 1998). Moreover, two forms of lipases show very different activity; closed form (inactive form) and open form (active form). If the enzyme is 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 substrates such as detergent and short chain alcohols 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 Sánchez-Otero *et al*. that improvement of lipase activity was found when the enzyme was prepared in the presence of Triton X-100 (Sánchez-Otero *et al*. 2008). 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, n-butanol, t-butanol, SDS, ethylene glycol, tween 80 and triton X-100. However, the result showed that 40% conversion was the highest when n-butanol was used as adjuvant but was not much different with control.

5.3 Transesterification catalyzed by immobilized *Candida rugosa* lipase

After the optimal conditions for immobilization of *Candida rugosa* lipase from section 4.1.1-4.1.6 were obtained, the transesterification activity of immobilized lipase was investigated . The reactions were conducted as described in section 3.4.4. The yield was quantitated by HPLC and approximately 35% was obtained. To increase yield of biodiesel production, the optimal conditions of transesterification catalyzed by immobilized lipase were investigated.

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

In this study, the effects of various factors on the transesterification catalyzed by *Candida rugosa* lipase immobilized on Amberlite XAD761 were investigated. Biodiesel yield was increased significantly by each sequential variable, namely, molar ratio of oil to methanol, addition mode of methanol, enzyme loading, water content, reaction time and temperature.

5.4.1 Effect of oil to methanol molar ratio

Although the high molar ratio of methanol results in greater methyl ester conversion in the shorter time but it can also inactivate the enzyme. So, the suitable ratio of oil to methanol molar ratio was examined and the results were shown in section 4.3.1 (Figure 4-8). From the results, the oil to methanol molar ratio of 1 to 4 gave the highest 57% conversion of fatty acid methyl ester which was quite similar to the obtained yield of biodiesel when the oil to methanol molar ratio of 1 to 5. This indicated that the 1:4 of oil to methanol molar ratio was suitable for the production of biodiesel in order to reduce the cost for further application. When the oil to methanol molar ratio increased to 1:6, 1:7, 1:8 and 1:9, the yield of biodiesel decreased gradually to 6%. From these results, it could be explained that the immobilized lipases probably were inactivated when the oil to methanol molar ratio was high. Besides, the higher concentration of methanol may distort the essential water layer that stabilizes the immobilized enzyme leading to their denaturation (Kose *et al*., 2002). In addition, the mole excess of methanol over oil results in the insolubility in the reaction (Antczak *et al*., 2009). Thus, the ratio at 1:4 was optimal for the production of biodiesel.

5.4.2 Effect of addition mode of methanol

The effect of addition mode of methanol has been shown to play important role in transesterification for the production of biodiesel because the excessive methanol content may inactivate the enzyme. Since the stoichiometric for transesterification requires three moles of alcohol (Ma and Hanna, 1999; Fukuda *et al*., 2001), the methanol was therefore divided and stepwisely added to maintain the methanol content at the desired appropriate level. The results were shown in section 4.3.2 (Figure 4-9). From the figure, it was found that when four moles of methanol were totally added all at once, no biodiesel was obtained indicating that the immobilized enzyme may strongly be denatured. However, the yield of fatty acid methyl ester gradually rose in two to six steps addition of methanol and maximum in seven steps. These results clearly showed that the stepwise addition of methanol is more effective than one step

because lipase can be protected from inactivation (Guan *et al.*, 2010). So, seven feeding steps of methanol was selected as the optimal addition mode to further study the effect amount of enzyme for the production of biodiesel.

5.4.3 Effect of amount of enzyme

After the optimal molar ratio and the addition step of methanol were obtained, the suitable content of the immobilized *Candida rugosa* lipase for high conversion of biodiesel was studied. The results of the effect of amount of enzyme were shown in section 4.3.3 (Figure 4-10). From the result, with the increasing lipase quantity, there was an increase in the fatty acid methyl ester yield. However, it was found that the highest 68% conversion rate of biodiesel was obtained when the amount of enzyme was 30% (w/w) of oil. The similar phenomena were also observed from the previous studies by Xie and Ma. During the enzymatic transesterification for biodiesel production, the soybean oil conversion was enhanced with an increase in the bound lipase loading. (Xie and Ma, 2010). So from this study, the 30 % immobilized lipase by weight of oil was selected for the optimal condition of transesterification.

5.4.4 Effect of water content

Activation of the lipase involves unmasking and restructuring of the active site through conformational changes of the lipase molecule, which requires the presence of oil–water interface (Zeng *et al.,* 2009). However, the excessive amount of water might lead to some unintended side-reactions such as hydrolysis (Lu *et al.,* 2009). As a consequence, the optimal water content was examined to compromise the minimizing hydrolysis and maximizing synthesis activity for the transesterification reaction.The results of the effect of water content on biodiesel yield were shown in section 4.3.4 (Figure 4-11).With the fixed optimal conditions obtained from previous experiments, the yields of fatty acid methyl ester were not significantly different when various content of water was added in the reaction. This indicated that water content did not display any effect on the production of biodiesel by transesterification and could

also be explained that the water content in the reaction mixture might have been sufficient for lipase catalysis. Hence, the following optimal conditions for transesterification; 30% (w/w of oil) enzyme, the ratio of oil to methanol of one to four, seven addition steps of methanol with no addition of water were used to further study the effect of reaction time and temperature.

5.4.5 Effect of time and temperature

Temperature is an important parameter in the enzyme synthetic process. Although higher temperature can give a faster transesterification but too high temperature denatures lipase and also leads to the loss of solvents through volatilization (Lu *et al*., 2007 and Nie *et al.,* 2006). Thus, the effect of reaction time and temperature on enzymatic transesterification for biodiesel production was studied using the obtained optimal conditions as shown in section 4.3.5 (Figure 4-12). From the result, it can be seen that highest yield 72% of biodiesel was obtained at 40^{o}C for the period of 24 hours. When the reactions were carried out at 50^{o}C, the yield of fatty acid methyl ester was approximately 40% for 24 hours indicating that the immobilized lipase was inactivated by heating. This result is in agreement with results obtained by Zheng *et al.* who conducted the biodiesel of soybean oil using the immobilized *Candida antarctica* lipase from 30 to 60^{o}C. The highest yield of 97% was observed at 40^{o}C when the reaction time was extended to 15 hours (Zheng *et al*., 2009). From Figure 4-12, it was shown that when the reaction temperature increased to 50^{o}C, the product decreased to 40%, which is in agreement with the previous literature report (Dizge *et al*., 2009 and Rashid *et al*., 2008). Moreover, the results showed that when increasing the reaction time to 24 hours, the percentage yield of biodiesel was increased to 72%. Thereafter, increase in the reaction time did not have the effect on the production of biodiesel. Nie *et al.* who studied the effect of temperature of the immobilized *Candida* sp. 99-125 lipase by adsorption. They found that the highest yield of fatty acid methyl ester of 87% was obtained at 12 hours and remained steady till 48 hours of reaction time, indicating that the optimum reaction time could be 12 hours (Nie *et al.,* 2006).

5.5 Stability of immobilized lipase

5.5.1 Repeated use of the immobilized *Candida rugosa* lipase for transesterification

The reusability of immobilized lipase is very important to the enzymatic transesterification in biodiesel production. After each batch, the immobilized lipase after each batch was washed with phosphate buffer and used in the next batch with fresh substrate. It was found that the immobilized recombinant *Rhizopus oryzae* lipase onto anion resin Amberlite IRA-93 almost maintained its full activity during 7 consecutive batch reactions (Wang *et al.,* 2010). The repeated use of lipase is a major issue. When some short chain alcohols were used as the acyl acceptor, glycerol, as one of the major by-products, can inhibit the enzymatic reaction and the lipase can lose activity rapidly during the repeated use without glycerol removal. Some efforts have been made to desorb glycerol from the lipase and usually large amount of hydrophilic solvents are needed for glycerol removal which is very complicated especially for large-scale production of biodiesel (Dossat *et al.* 1999). The decrease in methylesters conversion upon reuse might be due to the leakage of enzyme from the matrix (Yadav and Jadhav, 2005). The immobilized lipase was also washed by ethanol, propanol, n-hexane and water for restoring its activity (Rodrigues *et al.,* 2009). Immobilized enzyme presents the advantage that it can be reused for several times, but its activity decreases along repeated batches due to many factors such as desorption. Therefore, the reaction could improve the stability of immobilized *Candida rugosa* lipase by washing the system with different solvents after every batch of synthesis. The experiments were done using the optimal conditions previously obtained. Figure 4-13 shows the results of the yields obtained after the treatments with water, t-butanol, n-hexane and the control as percentage of the first batch reaction. For all polar solvents; water and t-butanol, the remaining enzyme activity after four batches was approximately of 20% and lost all of its activity respectively. The treatment with n-hexane, the only non-polar solvent tested resulted in loss of all the enzyme activity while dried unwashed immobilized lipase could still retain approximately 20 % of its initial activity after 4^{th} cycle.

However in 2nd cycle, the dried unwashed immobilized lipase still gave 60% conversion which was higher than the water washed immobilized enzyme. Therefore, dried immobilized lipase was repeatedly used for the study of biodiesel production transesterification.

5.5.2 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 thermostability were tested as described in 3.4.6.2. The thermal stability of immobilized lipase was carried out by incubating the immobilized lipase for the period of 24 hours at 40°C, optimal temperature for transesterification. Then, the residual activities were determined as percentage yield of activity at different time compared to the activity of untreated control. The results in Figure 4-14 revealed that the percentage of remaining activity decreased from 100% to 70% within 6 hours and stayed unchanged until 24 hours. When the half life ($t_{1/2}$) was calculated at 40°C, 21.08 ± 3.48 hours was obtained. Our results gave 4 times longer half life than the study reported by Santos *et al.* The half-life of the soluble free *Candida rugosa* lipase at 40°C was equal to 0.48 hours whereas the half life for their reported immobilized *Candida rugosa* lipase on chemically modified hybrid matrix of polysiloxane–polyvinyl alcohol by covalent immobilization was 5.79 hours (Santos *et al.*, 2008). Evidently, our 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 and helps preserve the enzyme structure from thermal inactivation (Huang and Cheng, 2008).

CONCLUSION

In this research, lipase from *Candida rugosa* was successfully immobilized on Amberlite XAD761 by adsorption. The optimal conditions for the immobilization obtained were as follows: pH 6.0, 20 mM ionic strength, 3 mg/ml enzyme loading at 30°C for 3 hours. When the immobilized lipase-catalyzed transesterification was carried out for the production of biodiesel, the maximal yield of 72% was achieved under the following optimal parameters: seven step addition mode, 1 to 4 molar ratio of oil:methanol, 30% (w/w) of oil enzyme loading at 40°C for 24 hours. Finally, the obtained immobilized *Candida rugosa* lipase could be used twice with the half life of approximately 21 hours at 40°C. Therefore, it can be concluded that the immobilized *Candida rugosa* lipase on Amberlite XAD761 can catalyze the transesterification for the production of biodiesel.

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APPENDICES

APPENDIX A

Properties of Amberlite XAD761

Figure A-1 Amberlite XAD761

Table A-1. Details of Amberlite XAD761

Matrix:	Crosslinked phenol-formaldehyde polycondensate
Functional groups:	Principally phenol
Physical form:	Ochre-colored granules
Moisture holding capacity:	62 - 70%
Shipping weight:	615 g/L (38.4 lb/ft^3)
Specific gravity:	1.070 to 1.130
Particle size	
Harmonic mean size:	0.560 to 0.760 mm
Uniformity coefficient:	$\leq$ 1.8
Fines content:	< 0.300 mm: 2.0% max
Coarse beads:	> 1.180 mm: 1.0% max
Porosity:	0.95 - 1.18 ml/g
Surface area:	150 - 250 m^2/g
Average pore diameter:	600Å

APPENDIX B

Hydrolysis assay

Preparation of solutions for hydrolysis assay

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.

50 mM Tris buffer, pH 8.0

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

2. ρ- nitrophenyl palmitate solution

ρ- nitrophenyl palmitate	50	mg
Absolute ethanol	10	ml

ρ- nitrophenyl palmitate was dissolved with absolute ethanol. Then, the solution was mixed and kept in a brown bottle (before use).

APPENDIX C

Protein determination

1. Preparation of solutions for protein assay

The assay reagent was prepared by diluting 1 volume of the dye stock with 4 volumes of distilled H_2O. Then, the solution was filtered by filter paper, Whatman No. 1. The solution should appear brown with pH around 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 at 10 mg/ml.

2. 10 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 (mg)	Reagent volume (µl)	
	Stock of BSA	dH_2O
0	-	1000
0.1	10	990
0.2	20	980
0.3	30	970
0.4	40	960
0.5	50	950
0.6	60	940

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

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

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

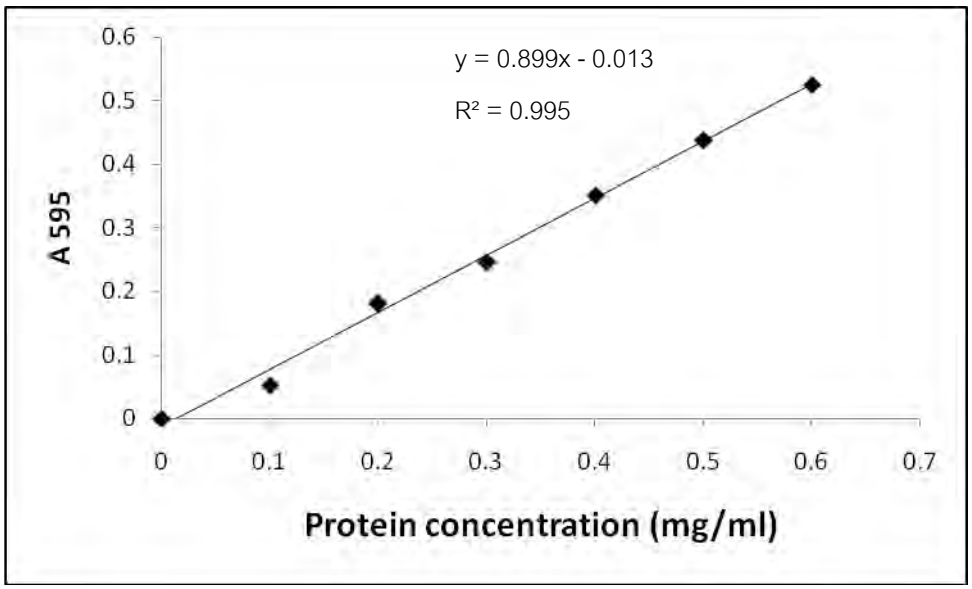

Figure C-1 Calibration curve for protein determination by Bradford's method

3. Calculation of total protein

The absorbance value at 595 nm was calculated by:

$$Y = aX + b$$

Where

X axis = Standard protein concentration (mg/ml)

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 p (mg/g) was calculated from the following formula:

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

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 *p*-NPP per minute under the test conditions. Lipase activity was calculated from

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

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

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

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

2. Calculation of thermal stability

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

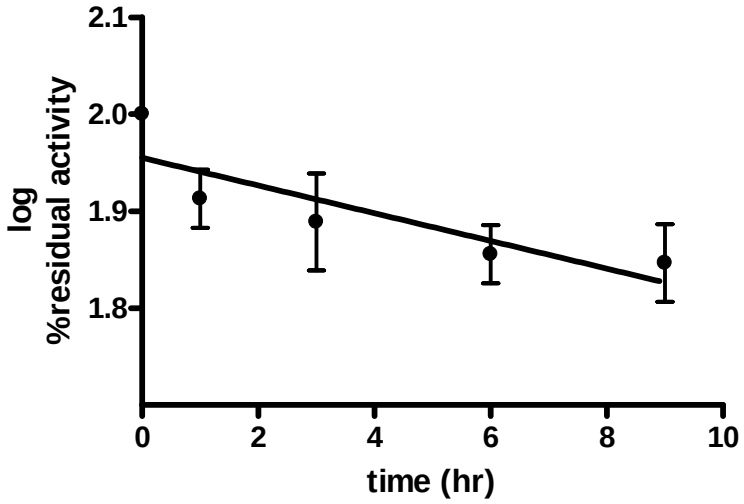

Figure D-1 Thermostability of untreated immobilized enzyme

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

$$t_{1/2} = \frac{\ln 2}{k_d} \quad \text{.....(2)}$$

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 $= \frac{k_d}{2.3}$ (From Fig D-1)

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

untreated immobilized enzyme

3. Effect of various adjuvant concentrations

%v/v	Alcohols					Detergents			
	MeOH	EtOH	Isopropanol	n-Butanol	t-Butanol	SDS	tween-80	Ethylene Glycol	Triton X-100
0.1						79.15	83.72	83.55	118.66
0.25						92.27	85.98	92.2	72.68
0.5						88.57	67.02	86.8	56
0.75						84.34	81.25	66.58	109.2
1	104	76.8	99.14	92.47	85.04		90.32	92.41	122.45
2.5	129.25	97.8	95.25	81.57	89.46		98.13	107.87	
5	126.15	104	90.59	92.16	136.22				
10			80.99						
20			70.65						

Table D-1 Effect of various adjuvant concentrations on the *Candida rugosa* lipase activities

APPENDIX E

Calculation of transesterification reaction

1. Molecular weight of palm oil

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

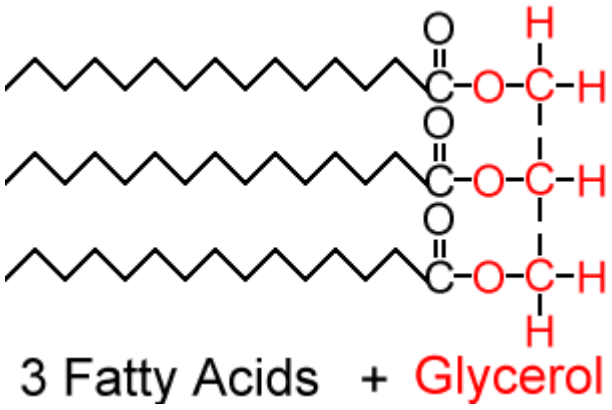

Figure E-1 Molecular structure of triglyceride

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

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

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-1)

Table E-1 Fatty acid composition of palm oil (Winayanuwattikun *et al.,* 2008)

Common	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

Molecular weight of palm oil can be calculated as follows;

$$R_{Ave} = \left(\frac{0.59}{100}\times 155\right)+\left(\frac{0.96}{100}\times 183\right)+\left(\frac{38.67}{100}\times 211\right)+\left(\frac{0.11}{100}\times 209\right)+\left(\frac{3.32}{100}\times 239\right)$$
$$+\left(\frac{45.45}{100}\times 237\right)+\left(\frac{10.87}{100}\times 235\right)+\left(\frac{0.20}{100}\times 233\right)+\left(\frac{0.23}{100}\times 267\right)+\left(\frac{0.02}{100}\times 295\right)$$

= 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 × 226.823) + 173

= 853.469

So, molecular weight of palm oil is 853.469

2. 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 Figure E-1. The applied volume of methanol was determined by using the molecular weight of palm oil equal to 853.47.

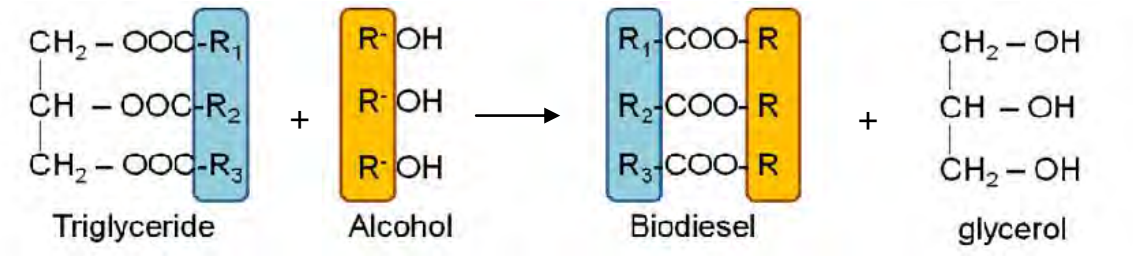

Figure E-1 Transesterification of palm oil and methanol

So, 0.5 g of palm oil = 0.5/853.47

$\approx 5.89 \times 10^{-4}$ mole

Thus, the volume of methanol = $3 \times 5.89 \times 10^{-4}$

= 1.76×10^{-3} mole

Since molecular weight of methanol is equal to 32

So, methanol 1.76×10^{-3} mole = $1.76 \times 10^{-3} \times 32 = 0.056$ g

From $D = \frac{M}{V}$

D = Density of methanol (0.792 g/ml)

M = Mass of methanol (0.056 g)

V = volume of methanol (ml)

$$V = \frac{0.056 \text{ g}}{0.792 \text{ g/ml}}$$

So, the volume of methanol is equal to 0.071 ml.

2. %conversion from HPLC analysis

All FAME obtained by HPLC can be calculated as follows;

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

FAME = Concentration of methyl ester

FFA = Concentration of free fatty acid

TAG = Concentration of triglyceride

DAG = Concentration of diglyceride

MAG = Concentration of monoglyceride

BIOGRAPHY

Miss Suthasinee Putha was born on September 18, 1984 in Ubonratchathani, Thailand. She graduated with the Bachelor Degree of Science from the Department of Biotechnology, Faculty of Science, Mahidol University in 2007. Later in 2008, she furthered her Master of Science in Biotechnology from Program in Biotechnology, Department of Biochemistry, Faculty of Science, Chulalongkorn University, at which she finished in April of 2011. During her studies, part of her work was presented as poster presentation at The 22nd Annual Meeting of the Thai Society for Biotechnology "International Conference on Biotechnology for Healthy Living" on the topic of "Optimal immobilization conditions of lipase from *Candida rugosa* on AMBERLITE XAD761 for biodiesel production" at Prince of Songkla University, Trang Campus, Thailand on 20-22 October, 2010.