การผลิตไบโอดีเซลจากปฏิกิริยาทรานส์เอสเทอริฟิเคชันที่เร่งโดยไลเพสตรึงรูป

จาก *Candida rugosa* บน Amberlite XAD7 HP

นางสาววศินี ไทพาณิชย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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PRODUCTION OF BIODIESEL FROM TRANSESTERIFICATION CATALYZED

BY IMMOBILIZED *Candida rugosa* LIPASE ON AMBERLITE XAD7 HP

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A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	PRODUCTION OF BIODIESEL FROM TRANSESTERIFICATION CATALYZED BY IMMOBILIZED *Candida rugosa* LIPASE ON AMBERLITE XAD7 HP
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Field of Study	Biotechnology
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วศินี ไทพาณิชย์ : การผลิตไบโอดีเซลจากปฏิกิริยาทรานส์เอสเทอริฟิเคชันที่เร่งโดยไลเพสตรึงรูปจาก *Candida rugosa* บ น Amberlite XAD7 HP (PRODUCTION OF BIODIESEL FROM TRANSESTERIFICATION CATALYZED BY IMMOBILIZED *Candida rugosa* LIPASE ON AMBERLITE XAD7 HP) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทิฆัมพร ยงวณิชย์, 93 หน้า.

การผลิตไบโอดีเซลซึ่งเป็นพลังงานหมุนเวียน สามารถทำได้ผ่านทรานส์เอสเทอริฟิเคชันโดยใช้ไลเพส เป็นตัวเร่งปฏิกิริยา เมื่อไม่นานมานี้ ไลเพสจากยีสต์ *Candida rugosa* (CRL) ได้รับการเปิดเผยว่าเป็นหนึ่งใน เอนไซม์ที่นิยมนำมาใช้มากที่สุดสำหรับอุตสาหกรรม อย่างไรก็ตาม ราคาที่สูงของเอนไซม์ยังคงเป็นอุปสรรคที่ สามารถแก้ไขทางอ้อมได้โดยการตรึงรูป ในงานวิจัยนี้สามารถหาภาวะเหมาะสมสำหรับการตรึงรูป CRL บนเรซิ นพอลิเมอร์ชนิดไฮโดรโฟบิค Amberlite XAD 7HP ด้วยวิธีดูดซับทางกายภาพได้ โดยการนำ CRL 3 มิลลิกรัม ต่อมิลลิลิตร ใน 20 มิลลิโมลาร์ฟอสเฟตบัฟเฟอร์ที่ความเป็นกรดด่างเท่ากับ 6 ตรึงบน Amberlite XAD7HP 1 กรัม และกวนผสมต่อเนื่องที่ 20 องศาเซลเซียสเป็นเวลา 4 ชั่วโมง ได้ค่าการทำงานของ CRL ตรึงรูป เท่ากับ1.64 ไมโครโมลต่อนาทีต่อกรัมตัวค้ำจุน เมื่อใช้ CRL ตรึงรูปนี้ เร่งทรานส์เอสเทอริฟิเคชันสำหรับการผลิตไบโอดีเซล โดยใช้น้ำมันปาล์มเป็นสารตั้งต้นที่ 40 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง พบว่าได้ผลิตภัณฑ์จากการ วิเคราะห์ด้วยโครมาโทกราฟีของเหลวสมรรถนะสูงประมาณร้อยละ 50 จากนั้น สามารถหาภาวะเหมาะสม สำหรับทรานส์เอสเทอริฟิเคชันที่เร่งด้วย CRL ตรึงรูปเพื่อการผลิตไบโอดีเซลได้ โดยการใช้ อัตราส่วนโมล 3 ต่อ 1 ของเมทานอลต่อน้ำมันปาล์ม ด้วยการเติมเมทานอลแบบ 4 ขั้น เร่งด้วยปริมาณ CRL ตรึงรูป ร้อยละ 20 ของ น้ำหนักน้ำมัน และกวนผสมต่อเนื่องที่อุณหภูมิ 40 องศาเซลเซียส เป็นเวลา12 ชั่วโมง พบว่าสามารถผลิตไบโอ ดีเซลได้เพิ่มขึ้นเป็นร้อยละ 74 CRL ที่ตรึงรูปบน Amberlite XAD7HP มีความเสถียรที่ 40 องศาเซียส และยังคง ค่าการทำงานสัมพัทธ์สำหรับทรานส์เอสเทอริฟิเคชันได้ถึงร้อยละ 45 ภายหลังจากการนำกลับมาใช้ซ้ำอีก 3 ครั้ง นอกจากนี้ เพื่อลดต้นทุนให้มากขึ้น ยังได้ศึกษาการผลิตไบโอดีเซลแบบต่อเนื่องโดยเครื่องปฏิกรณ์แบบเบดบรร จุเพื่อเปรียบเทียบกับการผลิตแบบกะ โดยการใช้ภาวะอัตราส่วนโมล 1 ต่อ 1 ของเมทานอลต่อน้ำมัน ปาล์ม อัตราการไหล 0.2 มิลลิลิตรต่อนาที ปริมาณ CRL 1 กรัม โดยมีอัตราส่วนโดยน้ำหนักของปริมาณ CRL ตรึงรูปต่อเม็ดแก้ว 1 ต่อ 4 ที่ 50 องศาเซลเซียสเป็นเวลา 48 ชั่วโมง พบว่าสามารถผลิตไบโอดีเซลได้ประมาณ ร้อยละ 10 จากผลทั้งหมดชี้นำว่าสามารถผลิตไบโอดีเซลได้โดยทรานส์เอสเทอริฟิเคชันที่เร่งด้วย CRLตรึงรูปบน Amberlite XAD7HP

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต	_____
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WASINEE THAIPANICH: PRODUCTION OF BIODIESEL FROM TRANSESTERIFICATION CATALYZED BY IMMOBILIZED *Candida rugosa* LIPASE ON AMBERLITE XAD7 HP. ADVISOR: ASSOC. PROF. TIKAMPORN YONGVANICH, 93 pp.

The production of biodiesel, a renewable energy, can be achieved through the transesterification using lipase as the catalyst. Recently, lipase from yeast, *Candida rugosa* (CRL) has been revealed as one of the most frequently used enzymes for industrial applications. Nevertheless, the high cost of the enzyme remains the barrier which can be indirectly addressed by the immobilization. In this work, the optimal conditions for the immobilization of CRL on hydrophobic polymeric resin, Amberlite XAD7HP by physical adsorption were obtained. 3 mg/ml of CRL in 20 mM phosphate buffer at pH 6 were immobilized on 1 g of Amberlite XAD7HP and continuously stirred at 20°C for 4 hours. The activity of the immobilized CRL was 1.64 µmole/min/g-support. When this immobilized CRL was used to catalyze the transesterification for the production of biodiesel using palm oil as the substrate at 40°C for 24 hours, the percentages of the obtained product from the analysis by high performance liquid chromatography were found to be approximately 50. Then, the optimal conditions for transesterification catalyzed by the obtained immobilized CRL for the production of biodiesel were later obtained. 3 to 1 mole ratio of methanol to palm oil with 4 steps of addition of methanol were catalyzed with 20% (of oil by weight) of immobilized CRL and continuously stirred at 40°C for 12 hours. The production of biodiesel obtained was found to increase to approximately 74%. The immobilized CRL on Amberlite XAD7HP was stable at 40°C and still retained 45% of relative transesterification activity after three reuses. In addition, to further reduce the cost, the production of biodiesel by continuous packed bed reactor (PBR) was investigated to compare with the batch operation. The conditions were 1 to 1 mole ratio of methanol to palm oil, flow rate 0.2 ml/min, 1 gram of CRL with 1 to 4 ratio weight by weight of amount of immobilized CRL to glass beads at $50°$C for 48 hours. The biodiesel yield was obtained at approximately 10%. Overall results suggested that production of biodiesel could be accomplished by transesterification catalyzed by the immobilized CRL on Amberlite XAD7HP.

Field of Study: Biotechnology   Student's Signature _____

Academic Year: 2014   Advisor's Signature _____

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CHAPTER I INTRODUCTION

1.1 Statement of problem

Energy consumption is inevitable for human existence. There are various reasons for the search of an alternative fuel that is technically feasible, environmentally acceptable, economically competitive, and readily available. The first reason is the increasing demand for fossil fuels in all sectors of human life, be it transportation, power generation, industrial processes, and residential consumption. This increasing demand gives rise to environmental concerns such as larger CO_2, other greenhouse gas emissions and global warming. The second reason is that fossil fuel resources are non-renewable and they will be exhausted in the near future. Some reports claimed that oil and gas reserves will be depleted in 41 and 63 years, respectively if the consumption pace remains constant (Cesarinia & Diaz, 2013; Kiakalaieh et al, 2013). The last reason is the price instability of fuels such as crude oil which is a serious threat for countries with limited resources. Several alternatives such as wind, solar, hydro, nuclear, biofuels such as biodiesel have been increasingly implemented worldwide. Biodiesel or fatty acid methyl esters (FAME) are renewable in nature which can be used as blends with petroleum diesel (Chattopadhyay & Sen, 2013). It has many advantages over petroleum based diesel from its ecofriendly production, biodegradability, and renewability.

Rudolph Diesel, the inventor of the internal compression ignition engine now known as the diesel engine, first used vegetable oils (such as peanut oil) to fuel his engine at the Paris Exposition of World's Fair in 1900 (Shay, 1993). The vegetable oils performed well in short term engine tests but failed in long term operations. Problems such as clogging of injectors, carbon deposits, oil ring sticking, thickening and gelling of the lubricating oils were encountered. High viscosity and a tendency for polymerization within the cylinder appeared to be the root causes for many problems associated with the direct use of vegetable oils as fuel (Openshaw, 2000). In order to reduce these difficulties, the idea of converting the vegetable oils into their ester derivatives was introduced. Reacting triglycerides (TAG) in oil with an alcohol in the presence of a

catalyst, transesterification, can produce esters of free fatty acids (FFA) such as FAME or biodiesel. This process is the best method for producing higher quality biodiesel (Meher et al, 2006). In general, the catalysts used for transesterification of biodiesel are alkali and acid which gives high conversion of TAG at short reaction times. However, the foremost drawback of the alkali process is its sensitivity towards FFA in oils that leads to soap formation during the transesterification process. On the other hand, the acid catalyzed processes although are insensitive to FFA, a large number of salt interaction can result in the corrosion. Furthermore, the use of chemical catalysts can cause technical problems related to biodiesel purification from catalysts and the glycerol byproduct. Recently, biological catalyst such as lipase can be used in transesterification to overcome the problems. No byproduct, easy product removal, reusability without any separation step and lower operating temperature are the key advantages of this method.

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are hydrolases that catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at an oil–water interface. However, under certain conditions, they catalyze synthetic reactions like acidolysis, alcoholysis, esterification and transesterification. The biocatalytic properties of lipases in both aqueous and nonaqueous media enable its use in food process industries, organic chemistry and in treatment of wastewater. Among various lipases, yeast lipases have been widely used in polymer degradation (Masaki et al, 2005), as an additive in detergent formulation (Thirunavukarasu et al, 2008), flavor and cosmetic industries (Ozyilmaz & Gezer, 2010), food industry, ester synthesis (Ferrer et al, 2005) biocatalytic resolution of pharmaceuticals wastewater treatment (Tsuji et al, 2013) and also production of biodiesel through the transesterification. Lipases are produced by animals, plants and microorganisms. Most of the plant lipases are not commercially used while the lipases from animal and microbial origins have wider applications from their broad pH and thermal stability. Among them, microbial lipases have gained special industrial attention resulting from its stability, broad substrate specificity, possible high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. They share

about 5% of the world enzyme market after proteases and carbohydrases (Christopher et al, 2014).

From previous studies by Biofuels by Biocatalysts research unit, the productions of biodiesel obtained from lipase producing microorganisms namely: bacterium (*Staphylococcus warneri*), filamentous fungus (*Fusarium solani*), wild type and UV irradiated mutant unicellular yeast (*Candida rugosa*) were compared. To reach higher level of lipase production, classical and reverse genetics have been used to mutagenize the yeast cells with ultra violet light. From this work, *Candida rugosa* lipase (CRL) from both wild type and mutant yeasts were found to exhibit higher hydrolytic activity than bacteria and fungi. Besides, CRL has also been reported to show a broader range of specificities and displayed the highest transesterification activities suggesting that this microbial lipase is promising as a potential biocatalyst for production of biodiesel (Winayanuwattikun et al, 2011).

Although the lipase catalyzed transesterification can produce more environmentally friendly product of biodiesel, the high cost and low stability of lipases limit its potential applications for industrial applications. Thus, immobilization of lipase has become more vital since the immobilized enzyme can be recovered easily and repeatedly used. There are various methods available for immobilization such as physical adsorption, covalent attachment, entrapment, or formation of crosslinked enzyme aggregates or whole cell biocatalysts (Nigam et al, 2014). One of the prominent methods is physical adsorption recommended by a majority of scientists from the reasons as it is nontoxic and inexpensive, able to retain activity and able to be regenerated (Tan et al, 2010). The efficiency of the immobilization depends on various factors such as types of supports, porosity of support and its surface area. In recent years, immobilized lipase by physical adsorption has been successfully applied for the catalysis of biodiesel production. Previous work has reported the high activity of CRL immobilized on Amberlite XAD 7HP with approximately 50 % yield of biodiesel from transesterification of palm oil (Winayanuwattikun et al, 2011). This work was therefore concentrated on the optimizations for both of the immobilization of CRL on Amberlite

XAD7HP and for the catalysis of transesterification using the palm oil as substrate for the production of biodiesel.

Furthermore, since the conventional transesterification processes for biodiesel production are carried out in batch operation resulting in some disadvantages such as poor adaptation to automation and labor-intensive, the continuous fixed bed reactor has been extensively applied in laboratory scale to overcome the problem (Severac et al, 2011). The packed bed reactor (PBR) has been designed to increase the micromixing efficiency and mass transfer by the utilization of great centrifugal force. In the continuous PBR, the reaction mixture is continuously pumped through the column and the enzyme can be effectively reused without a prior separation. In particular, it allows the continuous removal of glycerol and excess alcohol, and protects the enzyme particles from mechanical shear stress. The method is therefore considered superior to the batch due to automated control and operation, reduced labor costs, stable operating conditions, and easy quality control of products (Christopher et al, 2014). Hence, this research also investigated the production of biodiesel obtained from the continuous processes as well.

1.2 Objective

The purpose of this work was to produce biodiesel from transesterification catalyzed by the immobilized CRL on Amberlite XAD7HP.

1.3 Scopes of the investigation

1.3.1 To optimize the conditions for the immobilization of CRL on Amberlite XAD7HP

1.3.2 To optimize the conditions for the transesterification catalyzed by obtained immobilized CRL for the production of biodiesel

1.3.3 To investigate the stability and the reusablity of the immobilized CRL

1.3.4 To produce biodiesel from PBR catalyzed by immobilized CRL

1.4 Expected results

This research should provide the conditions for the optimal immobilization of CRL on Amberlite XAD7HP and optimal transesterification to produce the biodiesel for further industrial application.

1.5 Thesis organization

This thesis consists of five chapters as follows: Chapter 1 is the introduction. Chapter 2 gives the theoretical and literature reviews. Chapter 3 comprises material and methods. The results can be found in Chapter 4 and the final Chapter 5 presents the discussion and the conclusion.

CHAPTER II THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Biodiesel

Biodiesel is defined as the non-petroleum-based dieselfuel consisting of fatty acid alkyl esters, typically made by transesterification of vegetable oils or animal fats with alcohols, which could be used alone, or blended with routine petrodiesel in unchanged diesel-engine vehicles. Biodiesel has many benefits such as it is biodegradable, non-toxic, has a low emission profile and is a renewable resource. In addition, it does not contribute to the increase in carbon dioxide levels in the atmosphere and thus minimizes the intensity of the greenhouse effect.

2.1.1 Biodiesel production

There are 4 methods to produce the biodiesel namely, direct use and blending, pyrolysis, microemulsions and transesterification (Bisen et al, 2010).

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. Nevertheless, high viscosity, free fatty acid content, as well as gum formation due to oxidation and polymerization during storage and combustion are obvious problems (Ma & Hanna, 1999).

2.1.1.2 Pyrolysis

Pyrolysis is the chemical decomposition or conversion of condensed organic substances into another by means of heat alone or with the aid of a catalyst. The pyrolyzed material can be vegetable oils, animal fats, natural fatty acids and methyl esters of fatty acids. Disadvantages of this process include high equipment cost and need for separate distillation equipment for separation of various fractions. Also the product obtained was similar to gasoline containing sulfur which makes it less eco-friendly (Ma & Hanna, 1999).

2.1.1.3 Microemulsion

Various derivatives such as microemulsions or blends of various vegetable oils with conventional fuel have been proposed as alternative fuels for diesel engines (Stavarache et al, 2005). Microemulsions are isotropic, clear, or translucent thermodynamically stable dispersions of oil, water, surfactant, and often a small amphiphilic molecule, called co-surfactant (Fukuda et al, 2001). The formation of microemulsions (cosolvency) is one of the four potential solutions for solving the problem of vegetable oil viscosity. Microemulsions are defined as transparent, thermodynamically stable colloidal dispersions in which the diameter of the dispersed-phase particles is less than one fourth the wavelength of visible light.

2.1.1.4 Transesterification

Transesterification of triglycerides with alcohol gives fatty acid alkyl esters as main product and glycerol as by product in the presence of a catalyst. The first step is the conversion of triglycerides to diglycerides, which is followed by the conversion of diglycerides to monoglycerides and of monoglycerides to glycerol, yielding one methyl ester molecule from each glyceride at each step. The transesterification is accelerated in a number of ways such as using an acid catalyst, alkali catalyst and enzymatic catalyst.

2.1.1.4.1 Acid catalyst

Acid catalysts are insensitive to free fatty acids, and they have better results for vegetable oil with FFA greater than 1% (Freedman et al, 1984). However, acids can produce a large number of salt interaction, which is a cause of corrosion. Some researchers have used inorganic acids such as sulfuric acid, phosphoric acid, sulfonated acid and hydrochloric acid in the transesterification process.

2.1.1.4.2 Alkali catalyst

Many researchers have used alkali catalysts (NaOH, KOH, CH_3ONa) for production of biodiesel as these catalysts are cheap and readily available (Atapour & Kariminia, 2011). However, the process has some limitations such as high energy consumption which in turn causes a dramatic increase in capital equipment costs and

safety issues. In addition, this process is highly sensitive to water and free fatty acid (FFA) content in the feedstock. High water content can change the reaction to saponification, which causes reductions of ester yield, difficult separation of glycerol from methyl ester, increment in viscosity, and the formation of emulsion. All create many problems in downstream purification and methyl ester recovery.

2.1.1.4.3 Enzymatic catalyst

Recently, it has been found that enzymatic catalyst (immobilized lipase) can be used in transesterification reaction. No byproduct, easy product removal, reusability without any separation step and lower operating temperature are the key advantages of this method. However, it is found to be very expensive (Robles et al, 2009). Ranganathan *et al.*, 2008 compared the alkali and enzymatic transesterification process in biodiesel production. They construed that the utilization of biocatalyst can produce very high purity FAME with lower or no downstream process compared to alkali catalysts. Some factors have significant influence on the biodiesel production with lipase such as number of cycles, types of alcohol, ratio with oil and type of lipase. There are different types of lipases that can be used as the catalyst such as: *Rhizopus oryzae, Candida rugosa, Psuedomonas fluorescens, Burkholderia cepacia, Aspergillus niger, Thermomyces lanuginose* and *Rhizomucor miehei* (Vasudevan & Briggs, 2008).

2.2 Lipase

Lipases are enzymes which carry out the hydrolysis of triglycerides to glycerol and fatty acids and hence categorized in the class of hydrolases (EC 3.1.1.3). They are best defined as carboxylesterases that catalyze both the hydrolysis and synthesis of long-chain acylglycerols.

2.2.1 Sources of lipase

These enzymes are ubiquitously present and based on their origin are classified as plant, animal or microbial lipases. Plant lipases have been reported from papaya latex, rapeseed, oat and castor seeds. Plant lipases are not commercially used

whereas the animal and microbial lipases are used extensively. Sources of the animal lipases are pancreas of cattle, sheep, hogs and pigs. As a matter of fact, microbial lipases have gained wide industrial importance and they now share about 5% of the world enzyme market after proteases and carbohydrases (Treichel et al, 2010). Lipases of microbial origin are more stable than plant and animal lipases and are available in bulk at lower cost compared to lipases of other origin. Thus, microbial lipases have gained special industrial application from its stability, broad substrate specificity, possible high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media.

2.2.2 Lipase producing microorganisms

Lipase can be found widely in nature, but microbial lipase are commercially significant because of low production cost, greater stability and wider availability than plant and animal lipase. They may originate from fungi, yeasts or bacteria.

A large number of microbial strains have been used for lipase production, however, the most frequently reported enzyme sources are *Candida* sp., *Pseudomonas* sp. and *Rhizopus* sp. (Benjamin & Pandey, 1998). Lipase producing microorganisms have been screened from various sources including soil, marine water, wastewater and industrial wastes. Soil isolates of *Aspergillus*, *Mucor*, *Candida* and *Sclerotina* species were reported to produce lipase. Yeasts lipases are easy to handle and culture compared to bacterial lipases. Among the yeast lipases, *Candida rugosa* has been found as good commercial importance (Kademi et al, 2003). The most commonly used biocatalyst for biodiesel production are the microbial lipases that are produced by a number of fungal, bacterial and yeast species (Lee et al, 2013).

2.2.3 Properties of lipase

Lipases display the unique feature of acting on the interface between aqueous and non-aqueous phases. This is the reason why they can catalyze many reactions including hydrolysis, inter-esterification and alcoholysis. The mode of action for lipases in substrate transesterifications to biodiesel depends on their origin and specific

properties. Lipases catalyze the transesterification between triglyceride and acyl acceptors (alcohols) through the formation of acyl-enzyme intermediates that subsequently donate the acyl moiety to produce FAME. The overall structure of the lipases can be described as a structure with a central L-sheet with the active serine placed in a loop called catalytic elbow. The activation which is often necessary for lipase is the movement of a lid. Some enzymes such as *T. lanuginosus* lipase have an active site and a lid on the surface of the enzyme. Others like *Candida rugosa* lipase (CRL) have an active site at the end of a tunnel containing the lid in its external parts. The structural properties of lipase from different sources might be the reason for showing different activity on different oil substrates, hence, the need to optimize the process based on the selected enzymes and substrates for biodiesel production. Based on their substrate specificity, lipases can be divided into three groups: 1,3-specific, fatty acid-specific, and non-specific lipases. The 1,3-specific lipases release and hydrolyze ester bonds in the position 1 and 3 of triglycerides. The fatty acid-specific lipases are known to hydrolyze esters of long chain fatty acids with double bonds in cis-position at C9, whereas the non-specific lipases randomly cleave the acylglycerolsin FFA. For optimal biodiesel production, lipases should be able to convert all three forms of glycerides (mono-, di-, and tri-glycerides) to biodiesel, hence, they need to be non-stereospecific and to efficiently catalyze the esterification of FFA.

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.

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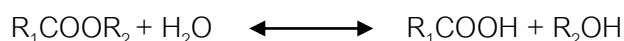
2.2.4.2 Esterification

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

$$R_1COOR_2 + R_3COOH \longleftrightarrow R_3COOR_2 + R_1COOH$$

2.2.4.3.2 Interesterification

$$R_1COOR_2 + R_3COOR_4 \longleftrightarrow R_3COOR_2 + R_1COOR_4$$

2.2.4.3.3 Alcoholysis

$$R_1COOR_2 + R_3OH \longleftrightarrow R_1COOR_3 + R_2OH$$
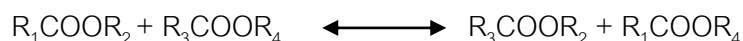
Figure 2- 1 Reactions catalyzed by lipases.

2.2.5 Applications of lipases

Lipases are a class of enzymes which catalyse the hydrolysis of long chain triglycerides. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology. Lipases are widely used in various industrial applications for example, the detergent, food, flavor, perfumery and cosmetics,

biocatalytic resolution of pharmaceuticals, production of esters, amino acid derivatives, fine chemicals, agrochemicals, biosensor, bioremediation and also the production of biodiesel.

2.3 Immobilization of lipase

In the industrialization of biodiesel production processes, immobilization of lipase has become vital because it could be recovered easily and used in continuous reactions. Immobilized enzymes are more stable towards temperature, chemicals as well as shear denaturation. In addition, they are easy to handle and easier process for the recovery and recycling of the biocatalyst. The most widely used immobilized lipases are extracellular and commercially available as Novozyme 435, *C. antarctica* lipase, immobilized on a macroporous acrylic resin, Lipozyme RM IM, *Rhizomucor miehei* lipase, immobilized on an anionic resin, and Lipozyme TL IM in which *Thermomyces lanuginosus* lipase can be immobilized on a gel of granulated silica, respectively.

2.3.1 Methods of enzyme immobilizations
2.3.1.1 Adsorption

Adsorption is the attachment of lipase on the surface of the carrier by weak forces, such as van der Walls, hydrophobic interactions or dispersion forces. Adsorption can be prepared under mild conditions without major activity loss and the associated process is relatively easy and cheap. Moreover, the carrier can easily be recovered for repeated immobilization. With these advantages, adsorption is still the most widely employed method for lipase immobilization. The immobilized lipases employed for biodiesel production are derived from different sources, for example: *Candida antarctica* (Watanabea et al, 2000), *Candida* sp. 99–125 (Lu et al, 2010; Nie et al, 2006), *Pseudomonas fluorescens* (Salis et al, 2008), *Pseudomonas cepacia* (Salis et al, 2005), *Porcine pancreatic*, *Rhizomucor miehei* and *Chromobacterium viscosum* (Yesiloglu & Kilic, 2004) etc. The carriers used include acrylic resin, textile membrane, polypropylene, celite and diatomaceous earth. There are two kinds of lipase used most

frequently, especially for large scale industrialization. One is the *C. antarctica* lipase immobilized on acrylic resin, which is known by its commercial name Novozyme 435. Novozyme 435 can catalyze vegetable oil and cooking oil with yield higher than 90%, and the lipase shows quite excellent catalytic properties in t-butanol solvent (Royon et al, 2007). The other is the *Candida* sp. 99–125 lipase immobilized on cheap textile membrane. This immobilized lipase textile can catalyze lard, waste oil and various vegetable oil with yield higher than 87%. Although adsorption has its special commercial advantages for its high activity towards biodiesel production at low cost, the lipase may be stripped off from the carrier because of the weak adhesion forces between the enzyme and support. The immobilized lipase may not be stable enough to prevent lipase from desorption during the catalytic process, though enzyme inactivation is not the main reason for lower activity.

2.3.1.2 Covalent

Lipase immobilization by covalent bonding to the solid carrier has the expected advantage of irreversible binding of the lipase to the support matrix. With the covalent bond method, the strong interactions between the lipase and the support make enzyme leaching uncommon during the catalytic process. Lipase from *T. lanuginosus* was immobilized by covalent attachment onto polyglutaraldehyde activated styrene-divinylbenzene (STYDVB) copolymer, which was used to catalyze methanolysis of canola oil for biodiesel synthesis. Under the optimized conditions, the maximum biodiesel yield was 97% at 50 °C in 24 h reaction. The immobilized enzyme retained its activity during the 10 repeated batch reactions (Dizge et al, 2009).

2.3.1.3 Entrapment

Entrapment entails capture of the lipase within a matrix of polymer. In theory, the entrapped enzyme is not attached to the polymer; its free diffusion is merely restrained. Virtues of the entrapment method for immobilizing lipase are that it is fast, cheap, very easy and usually involves mild conditions. *P. cepacia* was entrapped in hydrophobic sol–gel, and the immobilized lipase could catalyze biodiesel production with soybean oil as feedstock. The final conversion was around 67% (Noureddini et al, 2005). Lipase (NS44035) from Novozymes North America Inc. was entrapped in a celite supported

sol–gel to prepare the immobilized lipase. Then the lipase was then employed for methanolysis of triolein with an approximately 60% conversion (Meunier & Legge, 2010). The conversion catalyzed by entrapped lipase has so far proven to be relatively low. The reason might be due to the poor diffusion and erosion of lipase from the surface of the support during the processing procedures.

2.3.1.4 Cross link

The cross-linking method is based on the formation of intermolecular cross-linkages between the enzyme molecules by means of bifunctional or multifunctional reagents such as glutaraldehyde, bisdiazobenzidine, hexamethylenediisocyanate etc. Cross-linked enzyme aggregates are matrix-free immobilized preparations. Generally, the first step of the immobilization is to precipitate the enzyme using acetone to produce physical aggregates of the enzyme. These aggregates are then cross-linked with glutaraldehyde to form a more robust structure. The application of this biocatalyst designed for the production of biodiesel has been explored. The use of cross-linked enzyme aggregates accelerated the rate of transesterification and a conversion of 92% has been obtained. Lipase from *B. cepacia* was immobilized on modified attapulgite by cross-link reaction for biodiesel production with jatropha oil as feed stock. The best conditions for biodiesel production preparation were 10 g jatropha oil, 2.4 g methanol being added at 3 h intervals, 7 wt% water, 10wt% immobilized lipase temperature 35°C and time 24 h. under these conditions, the maximum biodiesel yield reached 94% (You et al, 2013). However, one of the instrinsic drawbacks for cross-linked enzyme aggregates is that their particle size is usually below 10 µm. Thus, difficulties arise when they are used in heterogeneous reaction systems, where the substrate particle and the cross-linked enzyme aggregates particles might be in the same range. This can create problems in separation of immobilized enzyme from the product for the continuous use.

2.4 Amberlite XAD7HP

Amberlite XAD7HP is a polymeric adsorbent, supplied as white in soluble beads. It is a nonionic aliphatic acrylic polymer which derives its adsorptive properties

from its patented macroreticular structure (containing both a continuous polymer phase and a continuous pore phase), high surface area and the aliphatic nature of its surface. This macrorecticular structure also gives AmberliteXAD7HP polymeric adsorbent excellent physical and thermal stability. Due to its aliphatic nature, AmberliteXAD7HP adsorbent can adsorb non polar compounds from aqueous systems.

2.5 Continuous process for biodiesel production

In general, there are three types of operation mode namely batch, continuous and fed batch modes. Commonly used reactors for these operation modes are stirred tank reactors, packed bed reactor (PBR), fluidized reactors and membrane reactors. The type of reactors to be selected using immobilized lipase depends on the nature of the immobilized enzyme. The mechanical stability of the immobilized enzyme must be considered when using a stirred tank reactor whereas the pressure drop created across the reactor packing and the interparticle diffusion limitations should be considered when using PBR (Chattopadhyay & Sen, 2013). It has traditionally been used for most large-scale catalytic reactors because of its high efficiency, low cost and ease of construction and operation. In addition, it allows for continuous removal of glycerol and excess alcohol, and protects the enzyme particles from mechanical shear stress (Ko et al, 2012). Nie *et al*. conducted continuous transesterification on PBR with lipase from *Candida* sp. 99-125 and hydrocyclone was set after PBR to separate glycerol. The final conversion to FAME from plant oil and waste oil under the optimal condition was 90% and 92%, respectively. The life of the immobilized lipase was more than 10 days (Nie et al, 2006). Chen *et al*. tested a PBR for continuous biodiesel production using methanolysis of soybean oil in a t-butanol solvent system catalyzed by Novozyme 435. A molar conversion of 83% was attained with no considerable decline in lipase activity in continuous operation for 30 days at a flow rate of 0.1 ml/min, 52°C and a 4:1 methanol to oil molar ratio (Chen et al, 2011).

CHAPTER III MATERIALS AND METHODS

3.1 Lipase sources

Both wild type and mutant of *Candida rugosa* (CRL) were obtained from Biofuels by Biocatalysts Research unit. The crude commercial CRL type VII for immobilization was purchased from Sigma Aldrich Co. Ltd. (USA).

3.2 Equipments

Apollo Silica 5U column	(Shimadzu, Japan)
Balance	(Satorius, Germany)
Digital balance	(Satorius, Germany)
Desiccator	(Sigma-Aldrich, USA)
Evaporative Light Scattering Detector	(Shimadzu, Japan)
High performance liquid chromatography (HPLC)	(Shimadzu, Japan)
Magnetic stirrer	(KIKA Labortechnik, Malaysia)
Microplate reader spectrophotometer	(AnthosZenyth200, USA)
Microcentrifuge	(HettichZentrifugen, Germany)
Peristaltic pump	(Ismatec, Switzerland)
pH meter	(Model 250, Dever Instrument)
Refrigerated centrifuge	(Heto, Denmark)
Vacuum pump	(Scientific industries, USA)
Vortex	(Scientific industries, USA)
Whatman No.1	(Whatman, UK)
Water bath	(T.S. Instrument, Thailand)

3.3 Chemicals

Acetone	(Carlo erba, Italy)
Acylglycerol standards (Triolein, diolein and monoolein)	(Sigma, USA)
Amberlite XAD7HP	(Sigma, USA)
Bovine serum albumin (BSA)	(Merck, Germany)
Bradford's reagent	(Biorad, USA)

Butanol	(Carlo erba, Italy)
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)
Palm oil	(Morakot industry, Thailand)
ρ–nitrophenylpalmitate	(Sigma, USA)
Potassium dihydrogen phosphate	(Merck, Germany)
Sodium acetate anhydrous	(Sigma, USA)
Sodium dodecyl sulfate (SDS)	(Sigma, USA)
Triton x-100	(Scharlau, Spain)
t-butanol	(Carlo erba, Italy)
Tween-80	(Lab scan, Thailand)

3.4 Data analysis

Graph analysis program	(Graph Pad Prism4)
HPLC data analysis program	(LC solution software)

3.5 Research methodology

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

3.5.1 CRL preparations

3.5.1.1 CRL from wild type and mutant

The samples of wild type *C. rugosa* and the mutants obtained by UV radiation were kindly provided from Biofuels by Biocatalysts Research unit as culture stock and

maintained in glycerol at -8 0 °C. They were both initially streaked on yeast malt (YM) agar and incubated at 30°C for 3 days. A single colony was later inoculated into 5 ml of YM media before cultured at 30°C, 2 0 0 rpm overnight. Then, 5 0 0 µl of cell culture was transferred into 5 0 ml liquid production media (Appendix B) and later grown at 3 0 °C, 2 0 0 rpm, for 7 days. After that, the cell solution was sampled at intervals to determine the growth curve and lipase production.

3.5.1.2 Commercial CRL

The commercial CRL solution was prepared by dissolving 2 4 0 mg crude CRL in 4 ml of 2 0 mM phosphate buffer solution, pH 7.5. The solution was centrifuged at 5 0 0 0 rpm, 4 °C for 1 5 minutes. After centrifugation, supernatant was collected and the protein content of CRL was determined by 3.5.4.2.

3.5.2 Immobilization of CRL

3.5.2.1 Preparation of supports

The supports, Amberlite XAD7 HP, were prepared by suspending 1 g of support powder in 3 ml methanol and then stirred at room temperature at 3 5 0 rpm. After 3 0 minutes, methanol was removed and the supports were then washed 3 times with 2 0 mM phosphate buffer, pH 7 . 5 under the same condition for immediate use.

3.5.2.2 Immobilization of enzyme

The CRL solution was prepared as described in 3 . 5 . 1 . 1 . The concentration of 3 mg/ml were prepared by mixing with1 g of Amberlite XAD7 HP. The immobilization of lipase onto the support was performed by stirring at 3 5 0 rpm for 5 hours at room temperature (Piriyakananon, 2008). After the immobilization, the solution was removed from the obtained immobilized CRL for protein determination. The immobilized CRL was later washed with 3 ml of 2 0 mM phosphate buffer, pH 7 .5 for 5 minutes until no protein was finally detected in the washing solution. The amount of adsorbed protein on the support was estimated by calculating from the differences of protein content in the solution before and after the immobilization. All washing solutions were quantitated for protein contents by Bradford method as described in section

3.5.4.2. Then, the immobilized CRL were dried at room temperature in desiccator and finally assayed for activities as described in section 3.5.4.1.

3.5.3 Optimization of immobilization

There are many factors affecting the activity recovery of enzymes in immobilization process. Some of the important factors are pH, ionic strength, protein loading, time, temperature and adjuvant.

3.5.3.1 Effect of pH

The effect of pH on the immobilization of lipase was studied at different pH values of buffer ranging from 4.0 to 10.0. The stock of buffer solution was prepared as 1 M buffer solution. All these buffer solutions were diluted to 20 mM before dissolving the crude lipase for immobilization. The immobilized CRL activitiy was assayed by the method described in section 3.5.4.1. The result was expressed as the immobilization efficiency.

3.5.3.2 Effect of ionic strength

After the optimal pH was obtained, the following concentrations of buffer at that pH were prepared: 10, 20, 50, 100 and 250 mM from the stock solution to dissolve crude lipase. The immobilized CRL activitiy was assayed by the method described in section 3.5.4.1. The result was expressed as the immobilization efficiency.

3.5.3.3 Effect of protein loading

The effect of protein loading on the immobilization lipase was performed by adjusting protein loading values from 1, 2, 3, 4 and 5 mg/ml. The amount of proteins in lipase solution was prepared in the proper buffer solution from section 3.5.3.1 and the ionic strength from section 3.5.3.2. The immobilized CRL activity was assayed by the method described in section 3.5.4.1. The result was expressed as immobilization efficiency.

3.5.3.4 Effect of time and temperature

After the optimal pH, ionic strength and enzyme loading were obtained as described in section 3.5.3.1-3.5.3.3, the effect of time and temperature were investigated. The time of immobilization was 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, 25, 30, 40 and 50°C. The results were expressed as the percentage of the residual activity comparing to 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 the activity of immobilized lipase from each temperature of the immobilization. The assay methods for the free and immobilized lipase were described in section 3.5.4.1. The result was expressed as immobilization efficiency.

After the optimal pH, ionic strength, enzyme loading, time and temperature were obtained as described in section 3.5.3.1-3.5.3.4, the effect of adjuvant was finally investigated.

3.5.3.5 Effect of adjuvant

The effect of types and concentrations of adjuvants were studied. The types of the adjuvants were divided into 2 groups namely, alcohol and detergents. The alcohol group consisted of methanol, ethanol, iso-propanol, butanol and t-butanol whereas the detergents were SDS, tween-80, ethylene glycol and triton X-100.

3.5.3.5.1 Effect of adjuvants on activity of immobilized lipase
3.5.3.5.1.1Effect of concentration

Firstly, the effect of the concentration of alcohols was studied at the range from 0, 1, 2.5, 5, 10 and 20% (v/v) while those of detergents were at 0, 0.1, 0.25, 0.5 and 0.75 (% v/v). The experimental procedure to determine catalytic activity of enzyme solutions containing adjuvants were as follows: 200 µl of soluble lipase from section 3.5.2.2 containing the corresponding amount of adjuvants were incubated at temperature for the period of optimal time described in section 3.5.3.4 and were assayed according to the method described in section 3.5.4.1.

3.5.3.5.1.2 Effect of type

When the proper concentration of each adjuvant was obtained, the effect of the types of the adjuvant on the immobilization was then determined. Each was then added with the enzyme solution from section 3.5.3.3 for 2 minutes before mixing with the support and incubated at time and temperature described in section 3.5.3.4. The results were expressed as immobilization efficiency of lipase and confirmed by using the immobilized lipase with added adjuvants to catalyze transesterification.

3.5.3.5.2 Effect of adjuvants on transesterification

Transesterification was later performed to confirm the activity of immobilized lipase with the addition of each selected adjuvants. The conditions for transesterification were described in 3.5.5. The yield of fatty acid methyl ester was determined by HPLC as described in section 3.5.9.

3.5.4 Immobilization efficiency

3.5.4.1 Lipase activity assay

Activities of the free and immobilized lipase were assayed using 0.5% (w/v) ρ-nitrophenylpalmitate (ρ-NPP) in ethanol as substrate. The reaction mixture consisting of 5 mg immobilized lipase or 25 µl free lipase was initiated by adding 500 µl of the mixture containing 250 µl of substrate and 250 µl 50 mM Tris-HCl buffer, pH 8.0. Then, the reaction mixture was incubated for 5 minutes at room temperature and was terminated by adding 0.5 ml of 0.25 M Na_2CO_3 followed by centrifugation at 14,000 rpm at 4°C for 5 minutes. The release of ρ-nitrophenol in the enzymatic hydrolysis of ρ-NPP in the supernatant was then measured for the absorbance at 410 nm. One international unit (U) of lipase was defined as the amount of enzyme needed to liberate 1 µmol of ρ-nitrophenol per minute under the described condition. Calculation of the unit of enzyme activity was described in Appendix D.

3.5.4.2 Protein determination

The protein content was determined by Bradford method. The reaction mixture consisted of 5 µl of sample and 300 µl of Bradford reagent in 96 well-

plate was incubated at room temperature for 5 minutes, and later measured for the absorbance at 595 nm. The calculation was shown in Appendix C.

3.5.5 Transesterification catalyzed by immobilized CRL

After the optimal conditions for immobilization of CRL were obtained by the method described in section 3.5.3.1-3.5.3.4, the capability to catalyze transesterification of the immobilized CRL was examined. 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 reaction mixture was magnetically stirred in water bath at 40°C for 24 hours (Chulalaksananukul et al, 1990). After completion of reaction, the samples were taken and analyzed by HPLC as described in section 3.5.9.

3.5.6 Optimization of the transesterification catalyzed by immobilized CRL

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

3.5.6.1 Effect of addition mode of methanol

The reaction mixture of 0.5 g palm oil and methanol was prepared in 20 ml screw-cap bottles containing 0.1 g immobilized CRL. Then, 3 moles of methanol (71.1 µl) were divided and added in the intervals of 1, 2, 3, 4, 5, 6 and 7 steps. The reactions were then magnetically stirred in a water bath at 40°C for 24 hours.

3.5.6.2 Effect of oil to methanol molar ratio

When the proper addition mode of methanol was obtained, the effect of oil to methanol mole concentration was studied at different ratios. In this study, 0.5 g palm oil and 20% (w/w of oil) of the immobilized CRL were mixed and the amount to prepare the ratios of the oil to methanol at 1:3, 1:4, 1:5, 1:6, 1:7, 1:8 and 1:9 was added into the reaction using the obtained optimal addition mode of methanol from

section 3.5.6.1. The samples were then magnetically stirred in a water bath at 40°C, for 24 hours.

3.5.6.3 Effect of enzyme loading

When the proper addition mode of methanol, oil to methanol molar ratio were obtained, the effect of enzyme loading of 10%, 20% and 30% (w/w of oil) on biodiesel production was studied. This experiment was conducted by adding 0.5 g of palm oil with each amount of the immobilized lipase for transesterification. Then, the optimal addition mode of methanol and ratio of oil and methanol was obtained from section 3.5.6.1 and 3.5.6.2, respectively. The reaction mixture was magnetically stirred in a water bath at 40°C for 24 hours.

3.5.6.4 Effect of water content

When the addition mode of methanol, proper oil to methanol mole ratio and enzyme loading were obtained, the effect of water content on immobilized CRL activity in transesterification reaction was examined at 0.5, 1, 2, 5 and 7% (v/v) of the oil. The reaction mixtures were magnetically stirred in a water bath at 40°C for 24 hours.

3.5.6.5 Effect of reaction time and temperature

The reaction mixtures were prepared using the optimized conditions from section 3.5.6.1 to 3.5.6.4. The samples were then incubated at different temperatures from 30, 40 and 50°C and stirred in a water bath for 24 hours to obtain the optimal time and temperature for transesterification. Finally, 50 µl of samples taken from the reaction mixtures at 3, 6, 9, 12, and 24 hours were later analyzed by HPLC as described in 3.5.9. The conversion of FAME was also monitored with the reaction time.

3.5.7 Stability of immobilized lipase

3.5.7.1 Thermal stability

The thermal stability of immobilized enzyme was determined by incubating 5 mg of immobilized lipase at 40°C. The samples were periodically taken and the percentage of the residual activity relative to the untreated control were analyzed together with the half life ($t_{1/2}$) as shown in Appendix D. The results were expressed as the percentage of relative residual activity and time.

3.5.7.2 Repeated use of the immobilized CRL for transesterification

The reusability of immobilized CRL was performed by triplicate trials of reactions under optimal conditions as follows: 0.5 g of palm oil, 20% (w/w of oil) of immobilized CRL, 1:3 oil: methanol ratio and the mixture was magnetically stirred in a water bath for 12 hours. In this work, the immobilized CRL 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 and later used in the next batch with the new substrates. The residual activity determined after the complete reaction was expressed as relative conversion. The conversion obtained in the first batch was set at 100.

3.5.8 Continuous operation

A schematic diagram of the equipment used for continuous synthesis of biodiesel is illustrated in Figure 3 -1 .The immobilized CRL was packed in a chromatography column (inner diameter 1.6 cm, height 20 cm, volume 40.2 cm^3) with a bed volume of 16.1 cm^3. Reaction mixture containing palm oil and methanol was fed continuously into the packed bed reactor from the bottom through a peristaltic pump with a flow rate of 0.2 ml/min. The ratio of immobilized CRL to glass beads was 1:4. Reactor temperature was maintained at 50 °C (Suptaweewut, 2007), and the eluent was collected at various times from the outlet provided at the top of the reactor. The concentrations of the substrate and the product were analyzed by HPLC as described in section 3.5.9.
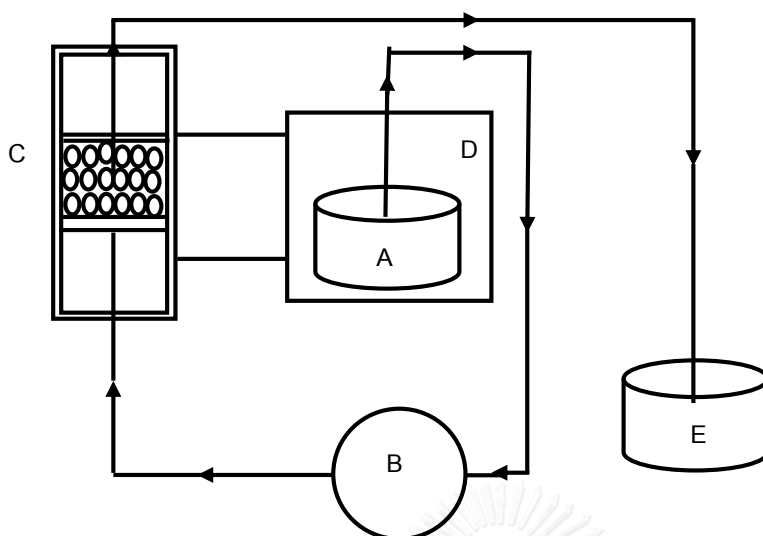
Figure 3- 1 Schematic diagram of the continuous packed bed reactor operation. A: substrate container, B: pump, C: enzyme column, D: water bath, E: product container.

3.5.9 Analysis of the FAME

The biodiesel content in the reaction mixture was analyzed using HPLC. Samples were taken from the reaction mixture at specified time and centrifuged at 12,000 rpm for 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. 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. 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 (Winayanuwattikun et al, 2008).

CHAPTER IV RESULTS

4.1 CRL preparation

4.1.1 CRL from wild type and mutant

The wild type and mutant of *Candida rugosa* were cultured at 30°C, 200 rpm for 7 days. The growth curves were obtained by measuring the absorbance at 600 nm and lipase production was determined by lipase activity as shown in Figure 4-1. It can be seen that similar growth patterns were obtained. For the first 12 hours, both samples were still in the lag phase. Then the growth was drastically increased into logarithmic phase within a day and later stayed unchanged at stationary phase. The lipase activity was assayed by using ρ-nitrophenylpalmitate as substrate and the liberated ρ-nitrophenol was measured at 410 nm. Unfortunately, the obtained lipase activities from both were very low. As a consequence, the commercial CRL was finally used for this work. In the experiment, the commercial CRL solution was prepared as the method described in 3.5.1.2.
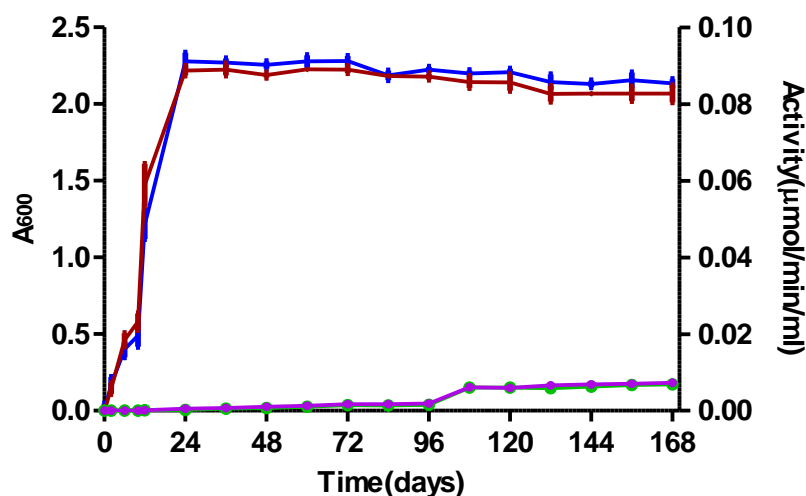
Figure 4- 1 Growth curves and lipase production of Candida rugosa wild type and mutant. Both wild type and mutant of *Candida rugosa* were grown in lipase inducing media and cultured at 30°C, 200 rpm for 7 days. Activity was assayed using ρ-nitrophenylpalmitate as substrate. The liberated ρ-nitrophenol was measured at 410 nm. Growth: (—◆—) wild type and (—■—) mutant; lipase activity: (—▲—) wild type and (—✕—) mutant. The results were shown on the y-axis are the means ± SD of three individual experiments.

4.2 Optimization of immobilization

The optimization for the immobilization of CRL on Amberlite XAD 7HP was studied. To achieve high activity of the immobilized CRL depends on many factors. In this work, the effects of immobilization parameters namely pH, ionic strength, enzyme loading, time, temperature and adjuvants of the immobilization were investigated.

4.2.1 Effect of pH

The activities of lipase at various pH were studied and the results were shown in Figure 4-2.
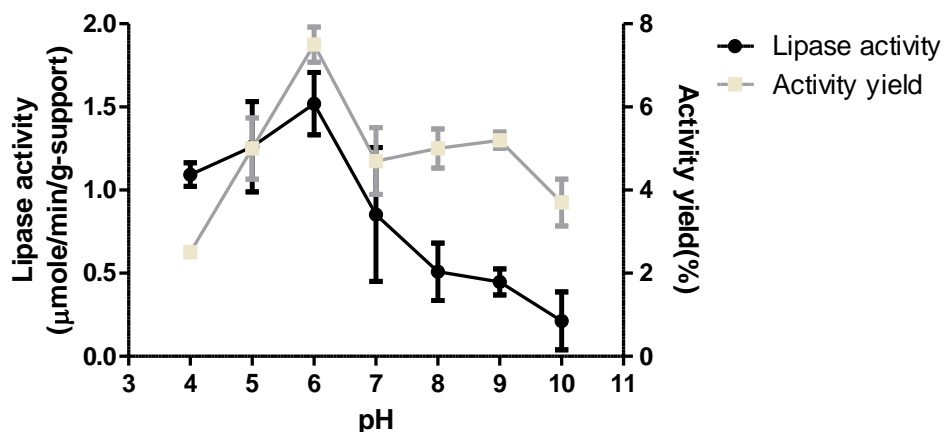
Figure 4- 2 The effect of pH on lipase activity. Crude CRL was dissolved in 3 ml 20 mM buffer solution at various pH. Then, 3 ml lipase solution (3 mg/ml) was added to 1 g of Amberlite XAD7HP and magnetically stirred for 5 hours at room temperature. Activities shown on the y-axis are the means ± SD of three individual experiments.

From the graph, it was shown that when 20 mM acetate buffer, pH 4 was used for immobilization, the lipase activity and activity yield were 1.09 μmol/min/g-support and 2.5% then gradually rose to 1.52 μmol/min/g-support and 7.5% at pH 6. However, when pH was increased to 9, the lipase activity decreased dramatically by 7 folds to 0.21 μmol/min/g-support and activity yield was 3.7%, at pH 10. Therefore, the optimal pH for immobilization was 6 and subsequently selected for the next experiment namely, effect of ionic strength.

4.2.2 Effect of ionic strength

When the optimal pH for immobilization was obtained at 6,the phosphate buffer, pH 6 at various concentrations as described in 3.5.3.2 were therefore used to study the effect of ionic strength on activity of immobilized CRL. The results were expressed as the lipase activity of immobilized lipase as shown in Figure 4-3.
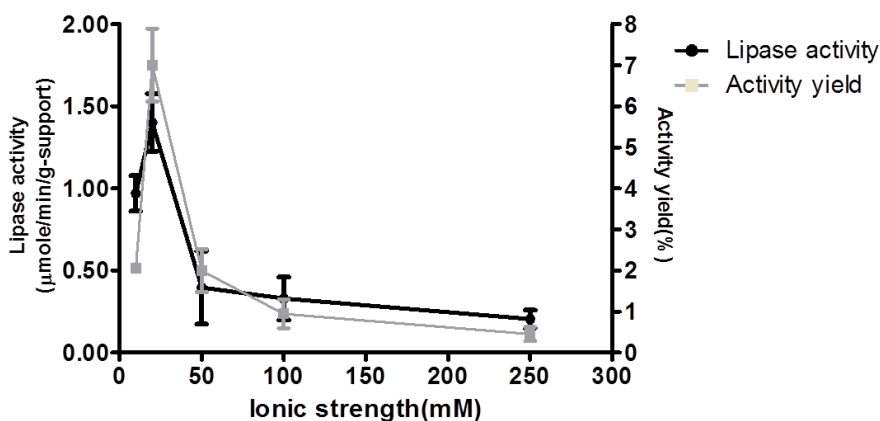
Figure 4- 3 The effect of ionic strength on lipase activity. 3 ml lipase solution (3mg/ml) containing various concentrations of phosphate buffer, pH 6.0 was added to 1 g of Amberlite XAD7HP and magnetically stirred for 5 hours at room temperature. Activities shown on the y-axis are the means ± SD of three individual experiments.

From the figure, the lipase activity and activity yield were 0.97 µmol/min/g-support and 2.05%, respectively, when 10 mM was used. Then, they significantly rose to 1.40 µmol/min/g-support and 7.00% respectively when 20 mM was used and declined dramatically by 3.5 folds when 50 mM was used for immobilization. Hence, 20 mM phosphate buffer, pH 6 was selected as the optimal concentration of buffer for immobilization of CRL to study the effect of protein loading on activity of immobilized lipase.

4.2.3 Effect of enzyme loading

The effect of enzyme loading on activity of immobilized CRL was studied by using various amounts of crude CRL in 20 mM phosphate buffer solution, pH 6 for immobilization as described in 3.5.3.3. The results were expressed as the lipase activity of immobilized lipase as shown in Figure 4-4.
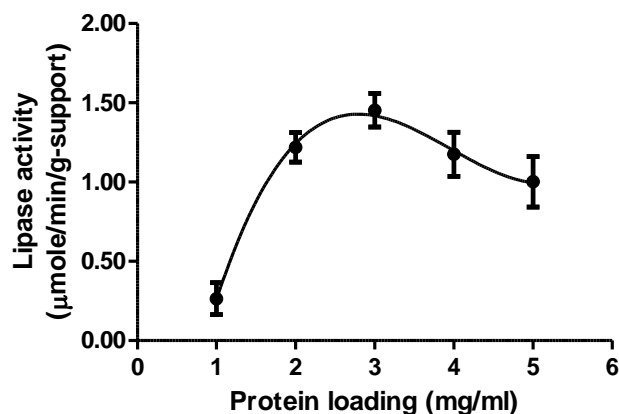
Figure 4- 4 The effect of enzyme loading on lipase activity. Lipase solution containing various quantities of protein dissolved in 20 mM phosphate buffer, pH 6.0 was added to 1 g of Amberlite XAD7HP and magnetically stirred for 5 hours at room temperature. Activities shown on the y-axis are the means ± SD of three individual experiments.

From the graph, when 1 mg/ml of CRL solution was used, the lipase activity was 0.26 µmol/min/g-support. It significantly roseto1.45 µmol/min/g-support when the quantity was increased to 3 mg/ml. However, when protein concentration was increased to 5 mg/ml, the activity of lipase was slightly decreased to 1.00 µmol/min/g-support. Therefore, the selected optimal enzyme loading for subsequent experiments was 3 mg/ml of lipase solution.

4.2.4 Effect of immobilization of time and temperature

4.2.4.1 Effect of immobilization time

After the obtained optimal conditions from the result as described above were fixed for CRL immobilization, the time parameter was investigated. The residual activities of lipase solution were checked for each time of immobilization at various temperatures as described in 3.5.3.4. The relationship of the residual activity with immobilization time at various temperatures was shown in Figure 4-5. The results were expressed as the percentage of the residual activity at 25°C.
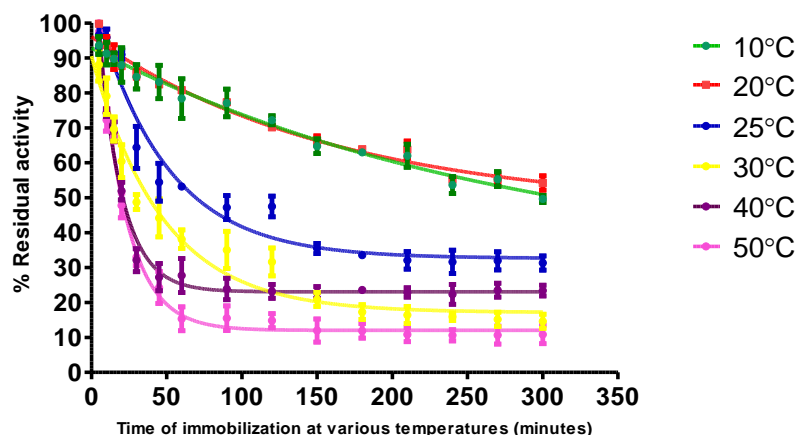
Figure 4- 5 The effect of immobilization time on the residual activity of lipase. 3 mg/ml of crude CRL solution were incubated at various temperatures. Residual activities on the y-axis are the means ± SD of three individual experiments.

When lipase was incubated at 10 and 20°C, the residual activities of lipase solution decreased to approximately 80% in the first 60 minutes. Then, they slightly declined approximately 10% after every 60 minutes and dropped to approximately 55% at 240 minutes and stayed unchanged to 300 minutes of incubation. At 25 to 50°C, the residual activities of lipase gave rather similar patterns. For 25 and 30°C, they both dramatically decreased to 53 and 38 % respectively in the first 60 minutes. They kept decreasing for the next 90 minutes to 35and 20% and then stayed unchanged to 300 minutes. At 40 and 50 °C, the activities of lipase significantly decreased from initial time to 25 and 15 % respectively within the first 60 minutes and stayed unchanged to 300 minutes.

It was observed that different temperatures gave different optimal incubation times of immobilization. Therefore, the optimal time for immobilization was 240 minutes, 150 minutes and 60 minutes at 10-20, 25-30 and 40-50 °C respectively and subsequently selected for the next experiment namely, optimal conditions for the temperature.

4.2.4.2 Effect of temperature

Next, the optimal temperature for immobilization of CRL was determined by checking the activity of immobilized lipase immobilized at various temperatures as described in 3.5.3.4. The results were expressed as the lipase activity of immobilized lipase as shown in Figure 4-6.
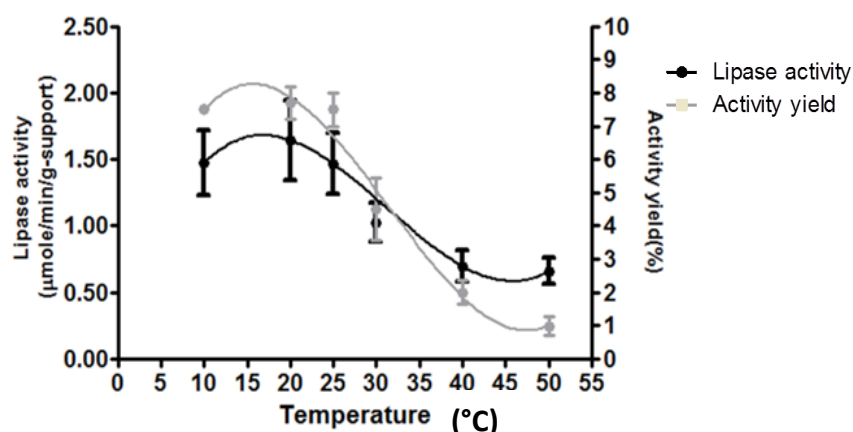
Figure 4-6 The effect of temperature on lipase activity. The immobilization was performed in 20 mM phosphate buffer, pH 6.0 with 3 mg/ml of lipase solution at specific time for each temperature. Activities on the y-axis are the means ± SD of three individual experiments.

From the results, it was found that lipase activity and activity yield were 1.47 µmol/min/g-support and 7.50% when CRL was immobilized at 10 °C and gradually rose to 1.64 µmol/min/g-support and 7.70% when immobilized temperature was increased to 20 °C. However, when immobilized temperature were at 25 and 30°C, the lipase activities and activity yield dropped and flattened out to 0.66 µmol/min/g-support and 0.98% when CRL immobilized temperature was 40 and 50°C. Therefore, it was concluded that 20°C was the optimal temperature for CRL immobilization.

From the optimal time for each temperature obtained from 4.2.4.1, the lipase activity of immobilized CRL was found to be steady at 240 minutes (4 hours). Therefore,

the optimal immobilization time of 4 hours at 20°C was selected for the study of the next parameter namely, adjuvant.

4.2.5 Effect of adjuvant

Once the optimal immobilization conditions of 20 mM phosphate buffer, pH 6, 3 mg/ml of CRL solution at 20°C for 4 hours for immobilization were obtained, the effect of adjuvants on the activity of immobilized CRL was examined by using two types of adjuvants namely, alcohols and detergents as described in 3.5.3.5.

4.2.5.1 Effect of adjuvants on activity of immobilized lipase

4.2.5.1.1 Effect of concentration

In this study, suitable concentrations of 9 adjuvants were determined. Firstly, the CRL solutions were added with various adjuvants at different concentrations as described in 3.5.3.5.1 and the reactions were carried out under the optimal conditions as described above. The results were calculated as the percentage of the activity of untreated lipase solution as shown in Figure 4-7.

(a)
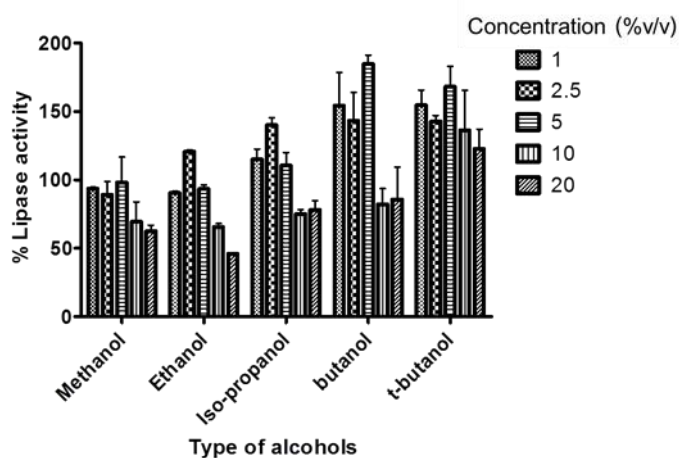
(b)
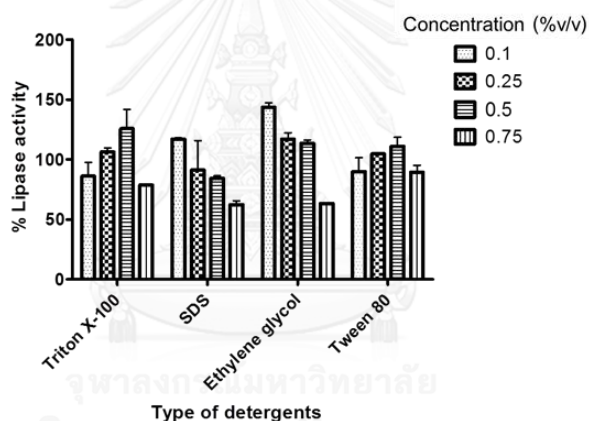
Figure 4-7 Effect of various adjuvants on the activity of CRL solution. The various concentrations of alcohols (a) and detergents (b) were added to 1 ml of 3 mg/ml of CRL and were incubated at 20°C for 4 hours. The results were calculated from the activity values of triplicate experiments and the activity of untreated CRL solution was set as 100%.

From the results in Figure 4-7, the concentrations of alcohols (a) and detergents (b) with the highest lipase activity were selected to study the effect on the activity of immobilized CRL as tabulated in Table 4-1 below.

Table4- 1 The concentrations of adjuvants with the highest activities of CRL solution.

	Adjuvant	Concentration (%v/v)
Alcohols	Methanol	5
	Ethanol	2.5
	Isopropanol	2.5
	Butanol	5
	t-butanol	5
Detergents	Triton X-100	0.5
	SDS	0.1
	Ethylene glycol	0.1
	Tween 80	0.5

4.2.5.1.2 Effect of the types

From Table 4-1, the proper concentration of each adjuvant was obtained. The effect of the type of adjuvant at highest activity on the immobilization of CRL was then investigated as described in 3.5.3.5.2. The relationship between the lipase activity with type of adjuvant was shown in Figure 4-8.
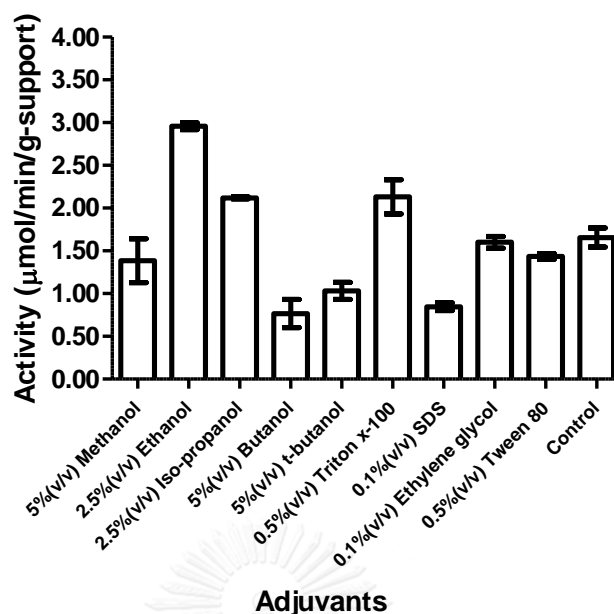
Figure4-8 The effect of the types of adjuvants on lipase activity. The selected concentration of each adjuvant was added to the enzyme solution (3 mg/ml) for 2 minutes before contact with support. The reaction mixture was magnetically stirred at 20°C for 4 hours.

From the figure, it was shown that certain alcohols enhanced the activity of CRL. It can be seen that when 2.5%(v/v) ethanol and isopropanol were used as adjuvants, the immobilized CRL displayed the highest lipase activity at 2.9 µmol/min/g-support and 2.12 µmol/min/g-support respectively. However, the addition of 5%(v/v) butanol and 5%(v/v) t-butanol gave rather low activities of 0.8 and 1.0 µmol/min/g-support respectively. When 5% methanol was used as adjuvant, lipase activity was not different from control indicating that it showed no effect to the immobilized CRL. For the effect of detergents on the CRL adsorption, the addition of 0.5%(v/v) triton X-100 gave the highest activity of 2.1µmol/min/g-support. On the other hand, the lowest immobilized lipase activity of 0.8 µmol/min/g-support was obtained in the presence of 0.1%(v/v) SDS. The effects of ethylene glycol and tween 80 were not detected.

From all these results obtained, the effects were confirmed by the catalysis of transesterification.

4.2.5.2 Effect of adjuvants on transesterification

The immobilized CRL with various adjuvants were then tested for transesterification by using the method as described in 3.5.3.5.3. The experiments were conducted to study the effect of various adjuvants under these conditions and the results were shown in Figure 4-9.
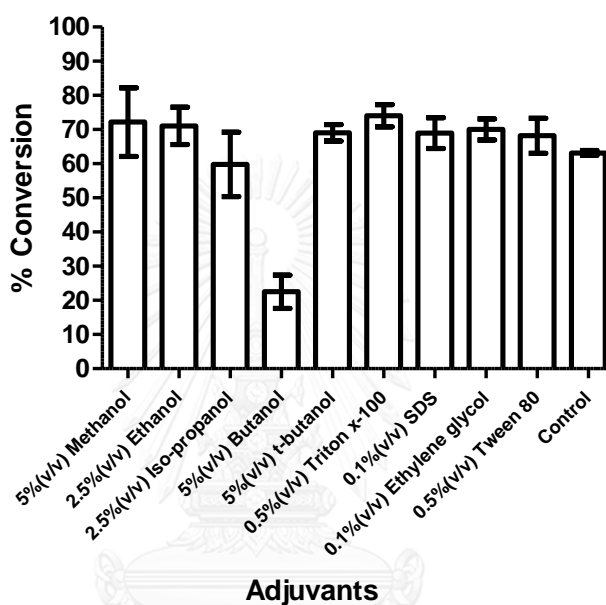
Figure4- 9 The effect of immobilized lipase with added various adjuvants on percentage of biodiesel conversion. The reactions carried out in a mixture of 0.5 g of palm oil, 3 steps addition mode of methanol, 1 to 3 mole oil to methanol and 20% (w/w of oil) immobilized lipase were magnetically stirred in a water bath at 40°C for 24 hours. Percent conversion shown in the y-axis is the means ± SD of three individual experiments.

From the figure, approximately 59-74% fatty acid methyl esters were obtained when ethanol, isopropanol, t-butanol, triton X-100, SDS, ethylene glycol and tween 80 were used. The immobilized CRL in the presence of triton X-100 gave the highest fatty acid methyl ester of 74% whereas butanol noticably gave the smallest quantity of approximately 23% among all these 9 studied adjuvants possibly due to the changes in

the polarity of lipase solution. Since no significance differences in the yield of fatty acid methyl esters were obtained, the adjuvants were not applied for the immobilization.

From overall results, the optimal conditions of CRL immobilization on Amberlite XAD7HP were summarized in Table 4-2.

Table 4- 2 Optimal conditions of lipase immobilization

pH	6
Ionic strength (mM)	20
Protein loading (mg/ml)	3
Time (hours)	4
Temperature (^{o}C)	20

4.3 Transesterification catalyzed by immobilized CRL

After the optimal conditions for immobilization of CRL from the section 4.2.1-4.2.5 were obtained, the activity of immobilized lipase was investigated for transesterification. The transesterification reactions were carried out in a mixture of 0.5 g of palm oil, 3 step additions mode of methanol, 1:3 oil:methanol ratio and 20% (w/w of oil) immobilized lipase magnetically stirred in a water bath at 40°C for 24 hours. The results revealed that approximately 50% of biodiesel were obtained.

4.4 Optimization of the transesterification catalyzed by immobilized CRL

After the optimal conditions for immobilization of CRL were obtained, the conditions of transesterification catalyzed by immobilized CRL were later optimized for biodiesel production. There were five main parameters as follows: addition mode of methanol, oil to methanol mole ratio, amount of enzyme, water content, time and temperature.

4.4.1 Effect of addition mode of methanol

The effect of addition mode of methanol was studied as the method in 3.5.6.1, the results were shown in Figure 4-10.
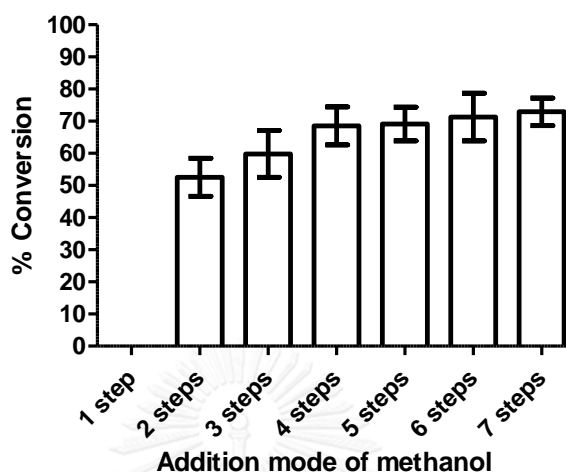
Figure 4- 10 The effect of addition mode on percentage of biodiesel conversion. The conditions were 20% (w/w of oil) enzyme, 1:3 mole ratio of oil to methanol divided to the various content for addition steps and no water in the reaction. The reactions were carried out at 40°C for 24 hours continuously stirred by magnetic stirrer. The results were average values of triplicate experiments.

From the figure, when three moles of methanol were totally added all at once, no biodiesel was detected. However, the yield of fatty acid methyl ester dramatically rose to approximately 52 and 63%, for two and three step of methanol feeding respectively. The conversion then gradually rose to approximately 68% with four and five step of methanol feedings and remained steady at approximately 72% of biodiesel when six to seven steps were conducted. Therefore, the optimal addition mode of methanol was four steps and selected to further study the effect of oil to methanol mole ratio.

4.4.2 Effect of oil to methanol mole ratio

With the fixed optimal 4 feeding steps of methanol, the effect of oil to methanol mole ratio was examined from one to nine by using the method as described

in 3.5.6.2. The results of the effect of oil to methanol mole ratio were shown in Figure 4-11.
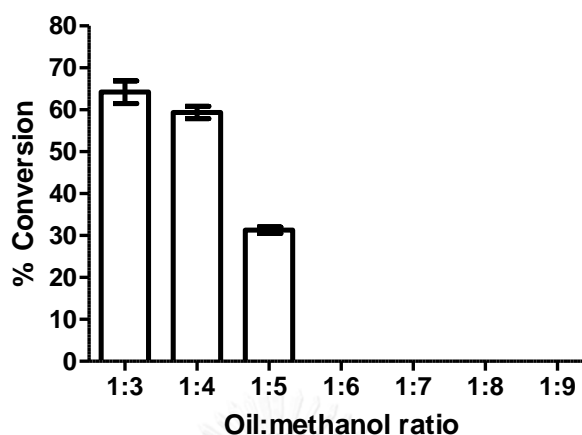
Figure 4-11 The effect of oil to methanol mole ratio on percentage of conversion to biodiesel. The conditions were 20% (w/w of oil) enzyme, the various moles of methanol were divided for four steps addition in the reaction with no water. The reactions were carried out at 40°C for 24 hours continuously stirred by magnetic stirrer. The results were average values of triplicate experiments.

From the figure, the ratio of methanol to oil at three gave the highest conversion of fatty acid methyl esters, approximately 64%. When the ratio of methanol to oil was 4, the yield of biodiesel decreased to approximately 59% but it obviously dropped nearly 50 % to approximately 31% when the ratio was equal to five. Finally, when the ratio of methanol was raised to six, no biodiesel was obtained. Thus, the ratio at three was optimal for the production of biodiesel. From these results, the optimal conditions obtained were the ratio of oil to methanol was one to three with four feeding steps. Then, these 2 conditions were used for the effect of enzyme loading for transesterification.

4.4.3 Effect of amount of enzyme

From the result in 4.4.2, the optimal oil to methanol mole ratio was obtained. Afterwards, the suitable content of the immobilized CRL for biodiesel

production was studied by using the method in 3.5.6.3. The results of the effect of enzyme loading were shown in Figure 4-12.
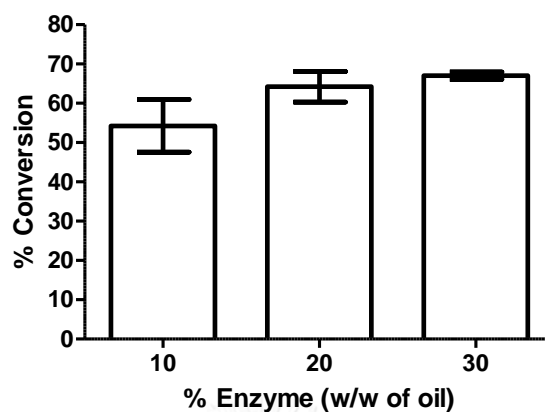
Figure 4- 12 The effect of amount of enzyme on percentage of conversion to biodiesel. The conditions were the various content (% w/w of oil) of immobilized enzyme, the ratio of oil to methanol was one to three with no water. The reactions were carried out at 40°C for 24 hours continuously stirred by magnetic stirrer. The results were average values of triplicate experiments.

From the figure, it was found that approximately 54% of biodiesel were obtained when 10% (w/w of oil) immobilized CRL was used in the reaction. Next, the fatty acid methyl esters increased to approximately 64 % when 20% (w/w of oil) was used and at approximately 67% when enzyme loading was 30% (w/w of oil). Therefore, the selected optimal enzyme loading for subsequent experiment was 20% (w/w of oil). From all these results, the optimal conditions for transesterification were 20% (w/w of oil) immobilized CRL, the ratio of oil to methanol was one to three and four addition steps of methanol to further study the effect of water content on transesterification.

4.4.4 Effect of water content

With the fixed optimal conditions obtained from 4.4.3, the water content for transesterification was examined by using the method as described in 3.5.6.4. The results of the effect of water content on biodiesel yield were shown in Figure 4-13.

Figure 4- 13 The effect of water content on percentage of conversion to biodiesel. The conditions were 20% (w/w of oil) of immobilized enzyme, the ratio of oil to methanol was one to three, four steps of methanol and added with the various contents of water. The reactions were carried out at 40°C for 24 hours continuously stirred by magnetic stirrer. The results were average values of triplicate experiments.

From the figure, the results obtained from the different quantities of water were found to be indifferent that approximately 62-71% of fatty acid methyl esters were obtained when 0-7% (v/v of oil) of water were added in the reaction. The results indicated that different water content did not display any positive effect on the production of biodiesel by transesterification. The water wasn't necessary in the production. Therefore, the following optimal conditions for transesterification of 20% (w/w of oil) enzyme, the ratio of oil to methanol of one to three, four addition steps of methanol were used to further study the effect of reaction time and temperature.

4.4.5 Effect of reaction time and temperature

The effects of reaction time and temperature were studied with the obtained optimal conditions from above by using the method as described in 3.5.6.5. The reactions were carried out at various temperatures for 24 hours during which the samples were taken at 3, 6, 9, 12, and 24 hours as shown in Figure 4-14.

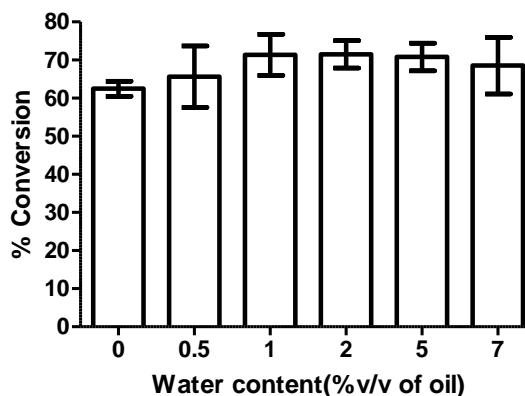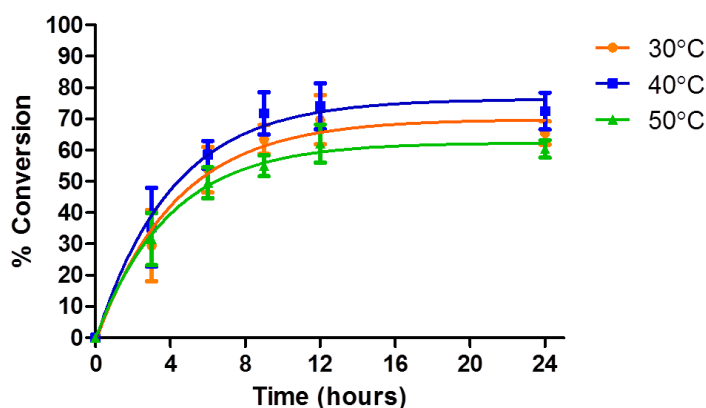Figure 4- 14 The effect of reaction time and temperature on percentage of conversion
to biodiesel. The conditions were 20% (w/w of oil) of immobilized enzyme,
the ratio of oil to methanol was one to three and four steps of methanol with
no water. The reactions were carried out at various temperatures for 24
hours continuously stirred by magnetic stirrer. The results were average
values of triplicate experiments.

From the results, when the reactions were carried out at 30 to 50°C from 0 to 24
hours, the patterns of conversion were similarly obtained in all three conditions. The
highest yield of biodiesel, approximately 74%, was detected at 40 °C. From the figure, it
can be seen that in the first four hours, the yields of fatty acid methyl esters from all three
conditions increased to approximately 30 to 35 %. The biodiesel kept increasing for the
next eight hours to approximately 69, 74 and 62% at 30, 40 and 50°C respectively. At 24
hours, the productions remained indifferent from 12 hours. Experimental results showed
that maximal fatty acid methyl ester was obtained when temperature increased to 40°C
and started to decrease when temperature subsequently rose to 50°C since
denaturation tends to increase under high temperature.

From the above, the 5 conditions for the optimized transestericiations were as
follows: four addition steps of methanol, the ratio of oil to methanol was one to three,
20% (w/w of oil) of immobilized CRL on Amberlite XAD7HP with no water at 40°C for 12
hours. Under these optimal conditions, approximately 74% of biodiesel were obtained.

4.5 Stability of immobilized lipase

4.5.1 Thermal stability for transesterification

The optimal immobilization conditions of 3 mg/ml of CRL in 20 mM phosphate buffer, pH 6 for 240 minutes at 20°C were applied using the method described in 3.5.7.1. Then, the thermal stability of immobilized CRL for transesterification was investigated. Since the optimal temperature for the transesterificaion was found to be 40°C, this experiment was performed by incubating 5 mg of immobilized CRL at 40°C for 24 hours. Afterwards, the incubated immobilized enzymes were periodically sampled every 3 hours. The percentages of residual activities relative to the untreated control obtained were shown in Figure4-15.
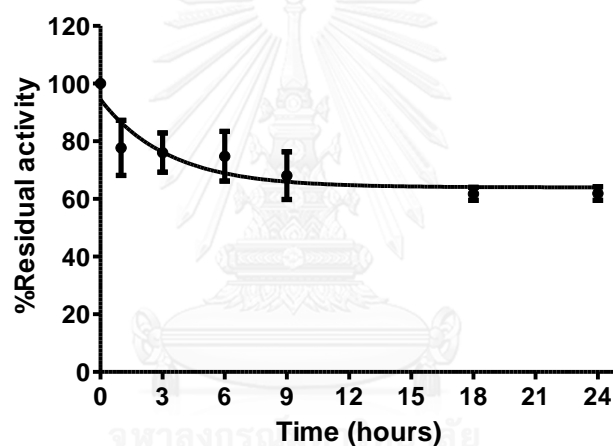
Figure 4- 15 The thermal stability of immobilized CRL. 5 mg of immobilized lipase were incubated at 40°C for 1, 3, 6, 9, 18 and 24 hours. The results were the average values of triplicate experiments.

It was found that the residual activities gradually decreased from 100 to about 68% within 9 hours and stayed unchanged until 24 hours. The results indicated that immobilization helps preserve the enzyme structure from thermal inactivation.

Then, the half life ($t_{1/2}$) of the immobilized enzyme was calculated as shown in Appendix D. The results were expressed as the percentages of relative of the residual activity and half life time as shown in Figure 4-16. It was shown that half life of immobilized CRL at 40°C was 25.38 hours.
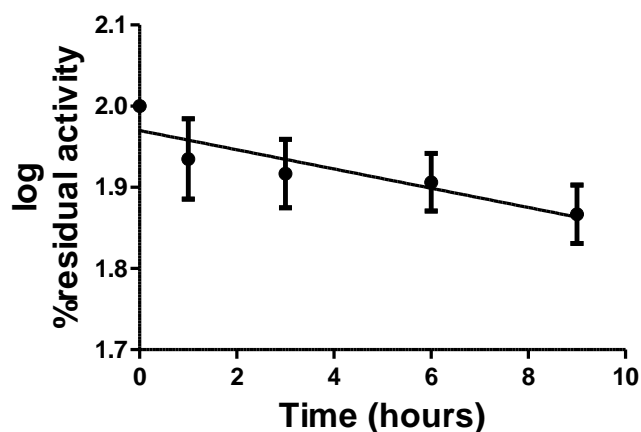
Figure 4- 16 Half life time ($t_{1/2}$) of immobilized CRL. 5 mg of immobilized lipase were incubated at 40°C. The results were average values of triplicate experiments.

4.5.2 Repeated use of the immobilized CRL for transesterification

The optimal conditions applied in this reusability study were 0.5 g of palm oil, 4 steps addition mode of methanol, 1 to 3 of oil to methanol mole ratio, 20% (w/w of oil) of immobilized CRL, continuously stirred in a water bath at 40°C for 12 hours. Under these conditions, approximately 74% of fatty acid methyl ester were obtained (Figure 4-14).

The immobilized CRL were then comparatively tested for the effect of washing with water, hexane and t-butanol. The immobilized CRL were rinsed with water, t-butanol and hexane after each batch to select the optimal washing solution and to remove glycerol and oil in carriers. The immobilized CRL were then dried in the desiccators and later used in the next batch reaction composed of new substrates. The results of each batch for the production of fatty acid methyl ester contents were graphically shown in Figure 4-17. The obtained conversion for each cycle of reuse was compared relative to the first use which was set at 100% for all treatments.
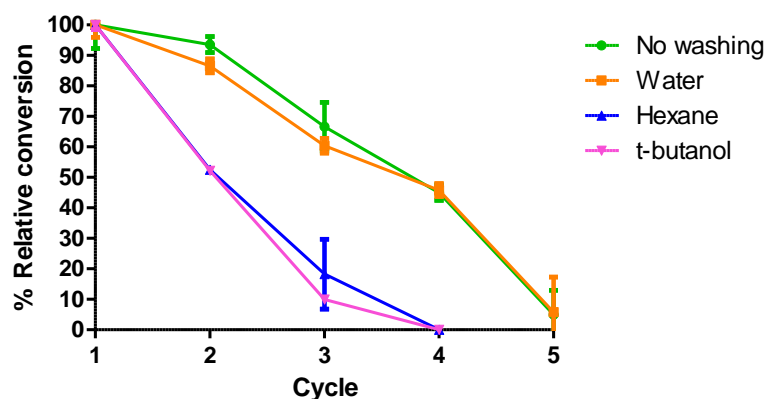
Figure 4- 17 Operational stability of immobilized CRL catalysis for transesterification. The reactions were carried out in a mixture of 0.5 g of palm oil, 4 steps addition mode of methanol, 1 to 3 of oil to methanol mole ratio, 20% (w/w of oil) immobilized lipase and continuously stirred at 40 °C for 12 hours. The lipase was transferred into the same system for a new cycle after completion of former reaction in 12 hours.

From the results of washing with water, it was found that both the yields of fatty acid methyl esters from unwashed and washed CRL gradually decreased to 94 and 86 % in the 2nd cycle (first reuse) and continually dropped to 67 and 60 % respectively in the 3rd cycle. After the 4th cycle, it can be seen that both immobilized CRL could similarly still retain approximately 45% of the initial activities and finally decreased to approximately 5% in the 5th cycle. On the contrary, when the immobilized CRL were washed with hexane and t-butanol, the conversion to fatty acid methyl esters dramatically decreased to 50 % after the 2nd cycle and approximately only 10-18% were retained after the 3rd cycle and finally lost all of the activities after the 4th cycle. Hence, the unwashed immobilized CRL was applied for transesterification.

4.6 Continuous operation

After the optimal conditions for transesterification of immobilized CRL from section 4.4.1-4.4.5 were obtained, the continuous production of biodiesel was investigated using the packed bed reactor (PBR). In the continuous process, the

substrate mixture containing 1 to 1 mole ratio of oil to methanol was fed from the substrate tank into the reactor at the constant rate of 0.2 ml/min. The enzyme loading was kept as one gram. Reactor temperature was maintained at 50 °C and the eluent was collected every 2 hours at the outlet provided at the top of the reactor. Later, the biodiesel conversion at each interval was analyzed by HPLC as shown in Figure 4-18.
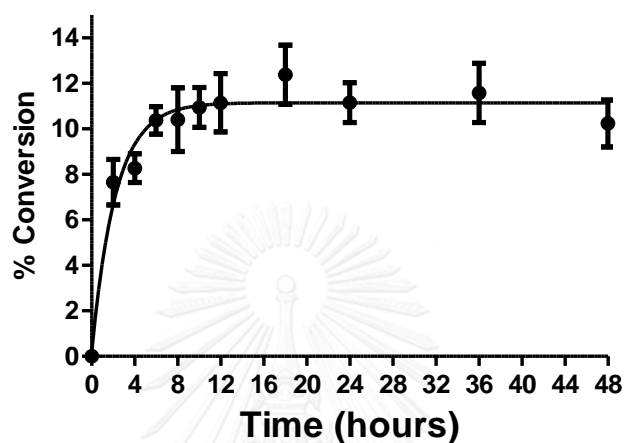
Figure 4- 18 Biodiesel conversion obtained from continuous PBR. The reaction mixture contained 1 to 1mole ratio of palm oil and methanol, 1 to 4 of immobilized CRL. The column diameter containing the glass beads was 1.6 cm with the length of 8 cm and the flow rate was set at 0.2 ml/min. The experiment was conducted at 50°C for 48 hours. Percent conversions shown on the y-axis are the means ± SD of three individual experiments (Suptaweewut, 2007).

From the figure, when the mixture of palm oil and methanol was fed into the reactor system, the percent conversion of fatty acid methyl esters rose from 0 to approximately 8 % in the first 4 hours. Then, the biodiesel increased to 10.4% for the next four hours and remained rather steady until 48 hours. Since the yield of biodiesel in batch reaction was approximately 74%, it was found that the production from PBR was 7 folds lower. This may have been the results from glycerol layer which might affect the mass transfer of immobilized CRL.

CHAPTER V DISCUSSION

5.1 CRL preparations

Although lipases are ubiquitous enzymes found in animals, plants and microorganisms, lipases of microbial origin are more stable and available in bulk at lower cost compared to lipases of other origins. From previous report, three different types of microorganisms; bacteria *Staphylococcus warneri*, unicellular yeast *Candida rugosa* and filamentous fungus *Fusarium solani*, were examined to select the most promising lipase for industrial application. The result showed that *Candida rugosa* lipase (CRL) had the highest transesterification activities (Winayanuwattikun et al, 2011). Hence, initially, in this work, lipase from both wild type and UV radiated mutant of *Candida rugosa* were comparatively studied. Both were cultured at 30°C, 200 rpm for 7 days. The growth curves were obtained by measuring the absorbance at 600 nm and lipase production was determined by lipase activity as shown in Figure 4-1. It was found that similar growth patterns were obtained. For the first 12 hours, both samples were still in the lag phase. Then the growth was drastically increased into logarithmic phase within a day and later stayed unchanged at stationary phase. Unfortunately, the obtained lipase activities from both were very low suggesting that the samples were not suitable for further studies. This can be explained from the facts that the mutants were obtained from UV irradiation. The genome might have been repaired and reversed to its normal physical and functional states. As a consequence, the commercial CRL was finally used for further studies in this work.

5.2 Immobilization of CRL

Lipases may exist in two different structural forms, the closed one where a polypeptide chain (lid or flat) isolates the active center from the medium, and the open form where this lid moves and the active center is exposed. This equilibrium is shifted towards the open form in the presence of hydrophobic surfaces where the lipase becomes adsorbed by the large hydrophobic pocket around their active center and the

internal face of the lid. Moreover, lipases may become adsorbed to other hydrophobic surfaces following a similar mechanism (Scheme1): droplets of oils, hydrophobic proteins, or on the surface of hydrophobic supports. The immobilization of lipases by their interfacial activation on hydrophobic supports may be suitable and simple method (Zivkovic et al, 2015).
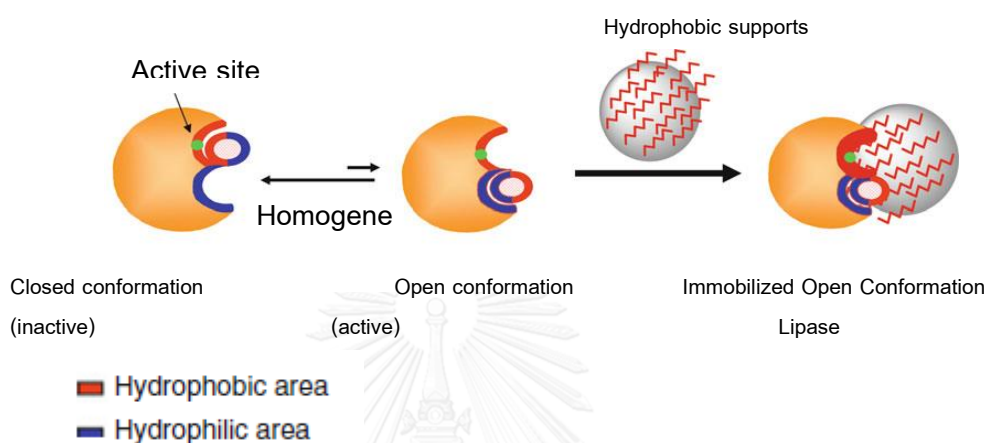
Scheme 1. Immobilization of lipases on hydrophobic surfaces (Palomo et al, 2013)

Obviously, the characteristics of immobilized enzyme preparations are governed by the properties of both the enzyme and the carrier material. Numerous supports for the immobilization of lipases have been used. There are varieties of support materials such as chitosan (Feresti & Ferresira, 2007), silica (Blanco et al, 2004; Blanco et al, 2007) and acrylic resin (Talukdera et al, 2006). The choice of supports is often limited by some other factors related to their structure such as the specific surface area, pore shape and particle size (Ghiaci et al, 2009). The supports will allow only the immobilization of small enzymes within the pores. In contrast, the larger enzymes can only be adsorbed on the external surfaces of the particles. In 2006, Yang *et al* studied the immobilization of lipase on macroporous resin and concluded that the increase of the resin pore diameter will increase the degree of immobilization (Yang et al, 2006). For instance, the internal surface may not be fully used to adsorb enzyme molecules even when pore sizes are wide enough (Blanco et al, 2007). Another key factor is the

specific surface area. There is probably significant contribution of the micropore regions in the immobilization of the enzyme and the external surface area of the support is also accessible to the enzyme molecules. The support can affect the partitioning of substrates, products, and water in the reaction mixture and thereby can influence the catalytic properties of the enzyme (Palomo et al, 2002). From previous research of Biofuels by Biocatalysts, the immobilized lipase from potential lipolytic microbes were studied to catalyze biodiesel production using palm oil as feed stock. The CRL were immobilized on seven types of commercial hydrophobic supports with pore diameters of around 10-60 nm namely Amberlite XAD 2, Amberlite XAD 4, Amberlite XAD 7 HP, Amberlite XAD 16, Amberlite XAD 761, Sepabeads EC-BU and Sepabeads EC-OD .The results indicated that dramatic differences existed in the activity of lipases supported on different materials (Winayanuwattikun et al, 2011).

In this study, Amberlite XAD7HP was selected as the carrier materials for immobilization of CRL. They are highly porous macroreticular aliphatic crosslink polymers for enzyme immobilization with low compressibility and high resistance to microbial attack. The immobilization by physical adsorption on hydrophobic support, Amberlite XAD7HP is suitable to stabilize this enzyme. From the literature, immobilized lipase from *Pseudomonas* sp AK by adsorption on Amberlite XAD7HP demonstrated that adsorption on Amberlite XAD 7 significantly stabilizes lipase from *Pseudomonas* sp. AK. The stabilities of free and immobilized lipase were tested in hexane, heptane, and isooctane at 32 °C for 30 h. The free lipase exhibited less than 5% hydrolytic activity whereas the immobilized lipase was found to be stable even after 30 h of incubation, maintaining 100% of the synthetic activity in all of the organic solvents tested (Lo & Ibrahim, 2005). 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.

5.3 Optimization of immobilization

Previous studies have shown that many factors such as properties of enzyme molecule, type of support and the ratio of enzyme and support can affect the enzyme activities in immobilization process. In this work, Amberlite XAD7HP was used as the carrier materials for immobilization of CRL. The effect of following factors were investigated namely; pH, ionic strength, protein loading, time, temperature, and various adjuvants.

5.3.1 Effect of pH

The optimal 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 maximal 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 CRL is quite stable in acidic environment and the optimal pH values 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 CRL can be slightly higher and lower than the free enzyme (Blanco et al, 2004). 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 proteins.

5.3.2 Effect of ionic strength

The effect of ionic strength on activity of immobilized CRL was shown in Figure 4-3. From this study, various concentrations of phosphate buffer at pH 6 were tested from 10 to 300mM. From the results, it was found that the activity of lipase significantly rose in the presence of 10 mM phosphate buffer, pH 6 and kept rising to 44 % until the highest activity was reached in the presence of 20 mM. This means that 20 mM of buffer effectively represents the suitable concentration for CRL to attach on Amberlite XAD 7HP. At low ionic strength (5-20 mM phosphate buffer), lipase molecules may absorb on the hydrophobic areas surrounding the active center resulting in stabilized open form of the lipase (Mateo et al, 2007). When the ionic strength was elevated from 20 to 300 mM, lipase activity declined dramatically by 3.5 folds. This was consistent to the previous studies that adsorption of lipases was decreased when the ionic strength increased (Lafuente et al, 1998). Moreover, in 2010, Suthasinee Putha studied the effect of ionic strength on activity of immobilized CRL by adsorption on Amberlite XAD 761. The result indicated that 20 mM phosphate buffer at pH 6 also gave highest lipase activity (Putha, 2010). The same optimal ionic strength obtained for CRL immobilized on Amberlite XAD 7HP and Amberlite XAD 761 possibly resulted from the same Amberlite family. They both contain hydrophobic surfaces with macroreticular aliphatic crosslinked polymer and crossing phenol-formaldehyde polycondensate resin. However, the lipase activity immobilized on Amberlite XAD 7HP was approximately 2.3 folds higher. This might be partially attributed to their larger surface and higher adsorption capacity of the Amberlite XAD 7HP, approximately 380 m^2/g of surface area compared to 250 m^2/g of surface area of the Amberlite XAD 761.

5.3.3 Effect of protein loading

The amount of enzyme loaded on the surface has a large effect on the performance of biocatalytic surface. In this study, different amount of CRL were immobilized on the support by varying the protein loading of enzyme solution from 1 to 5 mg/ml. The effect of protein loading on activity of immobilized CRL was shown in Figure

4-4. The results showed that the activity of immobilized CRL increased with an increase in protein loading of CRL solution (less than 3 mg/ml). This indicated that there were enough spaces in the support for CRL molecules to be accommodated. When it was 3 mg/ml, lipase activity reached the maximum. Thus, 3 mg/ml was the optimum protein loading of CRL solution for immobilization. When protein loading increased up to 4 and 5 mg/ml, the activity of immobilized CRL decreased slightly probably due to excessive adsorption of CRL. It could be explained that binding site on the surface areas of the support are limited and the enzyme molecules need enough space for catalyzing the reaction of the substrate. For protein loading amount higher than 3 mg/ml, the multilayer adsorption might have occurred and possibly inhibited access to enzyme active sites. This result agrees with those obtained in the study conducted by Egwim *et al*. They found that the hydrolytic activity of the immobilized enzyme increased as more lipase was loaded onto the support until it reaches a particular threshold where reduction in activity was observed (Egwim et al, 2012). Therefore, 3 mg/ml of CRL solution was selected as the optimal enzyme loading for subsequent experiment.

5.3.4 Effect of immobilization of time and temperature

5.3.4.1 Effect of immobilization time

The effect of time and temperature were simultaneously studied. The lipase molecule in the bulk phase must be transported to the support surface. Even in well stirred systems, there exists a stagnant layer closed to the surface that must be penetrated by diffusion. Thus, the amount of soluble protein was rapidly decreased with the increment of the immobilization time. The relationship of the residual activity with immobilization time at various temperatures from 10 to 50°C was shown in Figure 4-5. When lipase was incubated at 10 and 20°C, the residual activities of lipase solution decreased 20% in the first 60 minutes indicating that CRL was adsorbed on the surfaces and distributed throughout the pores of the support. For the next 180 minutes, residual activities gradually decreased approximately 10% every 60 minutes and later dropped to 55% at 240 minutes. These results showed that the adsorption rate slowed down

probably due to the aggregation of CRL molecules at the surface and pore mouth of the support in the earlier stage of adsorption. After 240 minutes, residual activity stayed unchanged until 300 minutes. It is considered that adsorption was close to saturation. At low temperature, the enzyme possibly requires longer interaction time to permit the correct alignment of groups located in the already immobilized, and partially rigidified enzyme, and the rigid surface of the support (Mateo et al, 2007). At 25 to 50 °C, the residual activities of lipase gave rather similar patterns. At 25 and 30 °C, residual activities of lipase dramatically decreased in the first 60 minutes and then slightly decreased to 150 minutes. It could be explained that the adsorption of CRL occurred rapidly in the first 60 minutes and maximal loading was reached around 150 minutes. When temperatures were increased to 40 and 50 °C, the residual activities of lipase significantly decreased from initial time to 60 minutes and stayed unchanged to 300 minutes. This means that the immobilization was found to proceed very fast because it was almost complete within 60 minutes.

From these results, it could be seen that higher temperature usually renders shorter time for the reaction to completion than low temperature. It was however obvious that enzyme denaturation causing the loss of activity is inevitable. Thus, the activity of the enzyme immobilized at various temperatures were further studied. As a consequence, the optimal immobilization time has to compromise 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.

5.3.4.2 Effect of temperature

The optimal temperature for immobilization was determined by measuring the activity of immobilized lipase at various temperatures from 10 to 50 °C. The results were shown in Figure 4-6. From the results, the catalytic site of enzyme at 10°C is not highly flexible leading to a decrease in lipase activity. When temperature was increased to 20 °C, lipase activity reached the maximum. This result was similar to the immobilization studied by Chang *et al*. The immobilization temperature of CRL on

celite was varied from 0 to 20°C. They found that the highest activity was obtained at 20°Cbecausethe functional groups of amino acids at the catalytic site show higher flexibility than low temperature (Chang et al, 2007). Although enzyme molecule at 20°C slowly are transferred to the support but lipases tend to keep their original structure. However, when the immobilization were conducted at 25 and 30 °C, lipase activity dropped because some lipase molecules started to denature and finally lipase activity flattened out to nearly zero when CRL immobilized temperature was 40 and 50°C.The result confirmed that the activity of immobilized CRL decreased from heat inactivation before immobilization. Thus, 20 °C was suitable for enzyme immobilization in this study.

From the optimal immobilization time obtained in section 4.2.4.1, the activity of immobilized lipase was found to be steady at 20°C for 240 minutes of incubation period. Therefore, the optimal immobilization time and temperature for subsequent experiment were 4 hours at 20°C.

5.3.6 Effect of adjuvants

5.3.6.1 Effect of adjuvants on activity of immobilized lipase

5.3.6.1.1 Effect of concentration.

Once the optimal conditions of 20 mM phosphate buffer, pH 6, 3 mg/ml of CRL solution for immobilization at $20^{\circ}C$ for 4 hours were obtained, the effect of adjuvants on the activity of immobilized CRL was examined by using two categories of adjuvant namely; alcohol and detergents. There have been the previous studies reporting that the alcohols may affect the protein-protein association or 3D structure of the enzyme (Gao et al, 2010) while the detergents may activate and stabilize the open active form of lipase molecules (Mateo et al, 2007). Both the concentration and the type of the adjuvants were thus also investigated.

Firstly, the proper concentration of each adjuvant was screened by adding in CRL solution and the reactions were carried out under the optimal conditions as described above. The effects of concentrations from 1 to 5% of 5 types of

alcohols on the activity of CRL were shown in Figure 4-7(a). From the results, it was found that the concentrations of 2.5% of ethanol, Isopropanol and 5% methanol, butanol, t-butanol gave highest activities. The effects of concentrations from 0.1 to 0.75% of 4 types of detergents on the activity of CRL were shown in Figure 4-7(b).The results showed that the concentrations of 0.1% of SDS, ethylene glycol and 0.5% of triton X-100, tween 80 gave highest activities. Therefore, the suitable concentration of each adjuvant was later used for immobilization.

5.3.6.1.2 Effect of the types

The selected concentration of each adjuvant was used for immobilization and the reactions were carried out under the optimal conditions as described above. The relationship between the lipase activity and adjuvant was shown in Figure 4-8. From the results, when 5% methanol was used as adjuvant, lipase activity was not different from control indicating that it had no effect to the immobilized CRL. Nevertheless, when 2.5% (v/v) ethanol and isopropanol were used, the immobilized CRL displayed the highest lipase activity. It could be explained that the surface tension between the enzyme solution and the hydrophobic supports decreases which then facilitates the diffusion of lipase molecules into the inside of pores and makes more use of the inner surface (Gao et al, 2010). The phenomenon has also been observed by other researchers. Blanco *et al*. studied the effect of ethanol treatment on the activity of *Candida antarctica* B immobilized on a hydrophobic support. It was found that the presence of 10% (v/v) ethanol decreases the hydrophobicity of the channels and the access of the enzyme seems to be significantly improved (Blanco et al, 2007). In the same year, Talukder *et al*. reported an improved method of lipase immobilization by adding isopropanol into the lipase solution in the adsorption process and they found that the isopropanol treatment had the significant effects on the activities of several lipases, especially on *Rhizopus oryzae* lipase, whose activity was increased 2.5 folds compared to the untreated (Talukder et al, 2007). However, the addition of 5% (v/v) butanol and t-butanol gave rather low activities. In 2010, Gao *et al* studied influence of alcohol treatment on the activity of lipase immobilized on methyl-modified silica aerogels. They

found that the addition of 10%(v/v) butanol and t-butanol have the negative effect of their loading amount. The effect of alcohols on lipase loading is more complicated than the change of microenvironment of the hydrophobic support. Though the enzyme solution can infiltrate the pore and make more use of its inner surface, the interactions between lipase molecule, the outer of which is more hydrophobic and the support may be weakened. It might be easier for water molecules to drag lipases back into the aqueous phase. Besides, some alcohols may cause rearrangement of the secondary structure of lipase and may stretch or compress the molecule, so as to promote or prevent its accessibility into the pores of the immobilization carrier (Gao et al, 2010).

For the detergents, they present several effects on CRL immobilization. From the results, it can be seen that the addition of 0.5%(v/v) triton X-100 gave the highest activity. This was consistent to the previous studies that the effects of triton X-100 may be in part an increase in the proportion of active open forms of lipases (Salameh & Wiegel, 2010). On the other hand, the lowest immobilized lipase activity was obtained in the presence of 0.1%(v/v) SDS. The partial inhibition of the activity can be ascribed to the formation of lipase-anionic surfactant complexes that affects negatively on the lipase activity (Niyonzima & More, 2015). The hydrophobic tail of the SDS may interact with the active center of the lipase and possibly behaves as a competitive inhibitor (Lorente et al, 2006). In 2014, Souzaa *et al* studied the effect of detergents on lipase stability. They found that the *Analipus japonicus* LAB01 lipase was sensitive to the anionic surfactant SDS, which completely inhibited the lipase hydrolytic activity (Souzaa et al, 2013). Besides, there were other two detergents namely ethylene glycol and tween 80 which showed no effects indicating no influence on the CRL adsorption. Although detergents could really favor the shifting of the open-closed forms of lipase towards the open forms but it also presents some negative effects by behaving as an inhibitor or deactivating agent.

From overall results of the effect of adjuvants, the addition of alcohols especially ethanol appeared more positive for the CRL immobilization than the detergents. However, the effect of adjuvants on lipase immobilization is rather complex

(Gao et al, 2010; Salameh & Wiegel, 2010). It is generally believed that lipase activity depends on the exposure of the active center. Considering the hydrophobic amino residues existing around the active center, the protein hydrophobicity analysis might be a possible method to get a more comprehensive understanding on the relationship between lipase conformation and its activity. In fact, the hydrophobic interaction is the most important force that maintains the 3-D structure of lipases and is a significant factor that affects the protein stability, conformation and function (Gao et al, 2010). Since hydrolytic and esterification activities in lipase may not be positively correlated (Sanderval & Marty, 2007), these results were also confirmed by using the immobilized CRL with the presence of various adjuvants to catalyze transesterification using palm oil as substrate.

5.3.6.1.3 Effect of adjuvants on transesterification

Adjuvants may present several effects on immobilized CRL. From the result of hydrolysis activity as described above, ethanol, isopropanol and triton X-100 gave higher lipase activity than control whereas the addition of t-butanol and SDS gave rather low activities. In addition, methanol, ethylene glycol and tween 80 were used as adjuvants, lipase activities were not different from control. So, the effects of adjuvants were confirmed by the catalysis of transesterification. The immobilized CRL with various adjuvants were tested for transesterification and the results were shown in Figure 4-9. From the results, butanol noticably gave the smallest quantity among all these 9 studied adjuvants. The addition of butanol may result in the reduction of lipase loading due to the changes in the polarity of lipase solution. When ethanol, isopropanol, t-butanol, triton X-100, SDS, ethylene glycol and tween 80 were used, no significance differences in the fatty acid methyl esters were obtained. It could be seen that the obtained results from transesterification and hydrolysis were not positively correlated. From this, it could be explained that the catalytic properties of CRL may be more preferable for hydrolysis than transesterification since lipase is generally categorized as hydrolases which catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids (Hung et al, 2003). Furthermore, the ping pong bi-bi mechanism of lipase catalysis in hydrolysis and

transesterification were different. Hydrolysis represents one step reaction since the second substrate in the reaction is water in which it could be negligible while transesterification requires two steps of the reaction because the second substrate is alcohol. The adjuvants may therefore improve the binding of enzyme and substrate with no change of limiting step for the reaction. Consistent to this study, the hydrolytic activities by the addition of 0.5%(v/v) triton X-100 in CRL were similarly reported to improve significantly after the immobilization on Amberlite XAD 761 while transesterification activity was not (Putha, 2010). In order to reduce cost of operation, no adjuvant was added for the immobilization of CRL on Amberlite XAD 7HP.

5.4 Transesterification catalyzed by immobilized CRL

After the optimal conditions for immobilization of CRL were obtained, the transesterification was investigated using palm oil as the substrate for biodiesel production. The reactions were conducted as described in section 3.5.5. The yield was quantitated by HPLC and approximately 50% were obtained. To increase the yield of biodiesel, the optimal conditions of transesterification catalyzed by immobilized lipase were therefore investigated.

5.5 Optimization of the transesterification catalyzed by immobilized CRL

In this work, the effects of various factors on transesterification catalyzed by CRL immobilized on Amberlite XAD7HP were studied. There were six main variables as follows: addition mode of methanol, mole ratio of oil to methanol, enzyme loading, water content and the reaction time and temperature.

5.5.1 Effect of addition mode of methanol

The effect of addition mode of methanol has been previously 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, the methanol was therefore divided

and step wisely added to maintain the methanol content at the desired appropriate level. The effect of addition mode of methanol from one to seven steps was studied. From Figure 4-10, when three moles of methanol were totally added all at once, no biodiesel was detected indicating that the immobilized CRL must have been severely denatured. However, the yield of fatty acid methyl ester rose to approximately 52 and 63% when two and three step additions of methanol were performed respectively. These results clearly showed that the stepwise addition of methanol is more effective than one step indicating that CRL was better protected from inactivation. Later, the conversion then gradually rose to approximately 68% with four and five step of methanol feedings and remained steady at approximately 72% when six to seven steps were conducted. In order to reduce time and man power, four feeding step of methanol was selected as the optimal addition mode to further study the effect of the ratio of methanol and oil for the production of biodiesel.

5.5.2 Effect of oil to methanol mole ratio

Although the high mole ratio of methanol results in greater methyl ester conversion in shorter time but it can also inactivate the enzyme. So, the suitable ratio of methanol to oil was examined at different ratios from 1:3 to 1:9 and the results were shown in Figure 4-11. From the results, the ratio of oil to methanol of one to three gave the highest conversion of fatty acid methyl ester at 64% which was quite similar to the obtained yield of biodiesel when the ratio of oil to methanol increased to 1:4. This indicated that the one to three ratio of oil to methanol was suitable for the production of biodiesel. When the ratio of oil to methanol increased to 1:5, the yield of biodiesel obviously dropped nearly 50%. From these results, it could be explained that the immobilized CRL probably were inactivated when the ratios of methanol are high. Besides, the higher concentration of methanol may distort the essential water layer that stabilize the immobilized enzyme leading to their denaturation (Kose et al, 2002; Yeow & Shamel, 2013). Finally, when the ratio of oil to methanol was increased from 1:6 to 1:9, no biodiesel was obtained. Obviously, excess more methanol in the reaction system inhibits enzyme activity. In addition, the mole excess of methanol over oil results in the

insolubility in the reaction mixture from the result of emulsion formation (Chen et al, 2011). Thus, the ratio at one to three was optimal for the production of biodiesel. From these results, the optimal ratio of oil to methanol was one to three with four addition steps which was later used for further studies of the remained variables.

5.5.3 Effect of amount of enzyme

After the 4 addition steps of methanol and optimal mole ratio of 1 to 3 were obtained, the content of the immobilized CRL from 10 to 30% (w/w) of oil for the high conversion of biodiesel yield was studied and the results were shown in Figure 4-12. From the results, with the increasing lipase quantity from 10 to 20 % (w/w) of oil, there was an increase in the fatty acid methyl ester yield. However, it was found that the highest biodiesel of 64% was obtained when the CRL loading was 20 % (w/w) of oil. From this, it could be explained that there were more abundancy of activated sites and sufficiency of mass content in 20% immobilized CRL, the biodiesel yield was consequently higher. The further increase of the enzyme to 30 %(w/w) of oil did not exert any more effect on the yield of biodiesel probably due to aggregation of catalysts and thereafter causing the diffusion problems of the substrates and products. The similar phenomenon was also observed from the previous studies by Pirajan and Giraldo. When the enzyme loading was increased, there was a sudden surge in the formation of biodiesel and followed by a slower rate at higher enzyme loadings (Pirajan & Giraldo, 2011).Therefore, the selected optimal enzyme loading for subsequent experiment was 20%(w/w) of oil. From all these results, the optimal conditions for transesterification of 20%(w/w) of oil enzyme, the ratio of oil to methanol was one to three and four addition steps of methanol were applied to study the effect of water content on transesterification.

5.5.4 Effect of water content

Since catalysis of lipase requires the presence of oil-water interface therefore the high interfacial areas were needed and can be obtained by the addition of water (You et al, 2013). However, the excessive amount of water might lead to some unintended side

reactions such as hydrolysis. As a consequence, the water contents from 0 to 7 (%v/v of oil) were examined to compromise the minimizing hydrolysis and maximizing synthetic activities for the transesterification. The results of the effect of water content on biodiesel yield were shown in Figure 4-13. With the fixed optimal conditions obtained from above, the yields of fatty acid methyl esters were found to be indifferent between 62-71% when various contents of water were added in the reactions. The similar result was reported in the previous studies of CRL immobilized on Amberlite XAD 761. It was found that biodiesel yield were also not significantly different when various contents of water were present (Putha, 2010). This indicated that the water content in the reaction mixture might have been sufficient for lipase catalysis. Therefore, the following optimal conditions for transesterification; 20% (w/w of oil) enzyme, the ratio of oil to methanol of one to three, four addition steps of methanol with no addition of water were used to further study the effect of reaction time and temperature.

5.5.5 Effect of reaction 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. Thus, the effect of reaction time and temperature on lipase catalyzed transesterification for biodiesel production was studied using the obtained optimal conditions as described above and the results were shown in Figure 4-14. From the results, the biodiesel increased when temperature increased from 30 to 40 °C. At 40°C, fatty acid methyl esters reached the maximum at 74%. This could be explained that higher reaction temperature can decrease the viscosities of oils and result in an increased reaction rate. Similarly, the effect of temperature on transesterification of palm oil catalyzed by immobilized CRL on Amberlite XAD 761 was studied and 40°C gave highest biodiesel production at 72%. Furthermore, Pinyaphong *et al*. studied the methanolysis of fish oil derived from the discarded parts of fish using immobilized *Carica papaya* lipase at various reaction temperatures from 30 to 60°C. It was found that

the highest biodiesel was obtained at 40 °C (Pinyaphong et al, 2011). Therefore, 40 °C can effectively represent the suitable temperature for biodiesel production for various lipases. However, at 50°C, the yield of fatty acid methyl ester was found to be 62%, 12% lower than 40 °C, indicating that immobilized lipase were likely inactivated from the heating.

From the results of all temperatures, the highest yield of fatty acid methyl ester was obtained at 12 hours and remained steady till 24 hours of reaction time. These results indicated that increase in the reaction time did not have the effect on the production of biodiesel. The phenomenon have also been observed by other researchers. In 2013, You *et al.* studied the reaction time ranges from 0 to 36 hours on methanolysis of jatropha oil. It was found that the methyl ester conversion was practically constant over reaction time ranges between 24 and 36 hours indicating that optimal reaction time could be 24 hours (You et al, 2013).

From the above 5 conditions, the optimal conditions for transesterification were as follows: 20% (w/w of oil) enzyme, the ratio of oil to methanol was one to three, four addition steps of methanol with no addition of water at 40°C for 12 hours. From these obtained optimal conditions, the 74% of fatty acid methyl ester were obtained which was 24% higher than before the optimization. This indicated that the CRL immobilized on Amberlite XAD7HP could produce biodiesel and show promising potential for the industrial applications.

5.6 Stability of immobilized lipase

5.6.1 Thermal stability for transesterification

The resistance of immobilized lipase to temperature is an important potential advantage for practical applications of this enzyme. Since the optimal temperature for transesterification was found to be 40°C, this experiment was performed by incubating 5 mg of immobilized CRL at 40°C for 24 hours. Afterwards, the incubated immobilized CRL were periodically sampled every 3 hours. The percentages of residual activities relative to the untreated control obtained were shown in Figure 4-15. The results

revealed that the percentages of residual activity at 40°C significantly decreased from 100 to 68% within 9 hours and stayed unchanged until 24 hours. Thermal inactivation resulted in approximately 32 % loss of the activities. According to Hung *et al,* they reported that the free lipase could remain stable only up to 39°C (Hung et al, 2003). These results therefore clearly showed that thermal stability of lipase was enhanced by the immobilization. When the half life time ($t_{1/2}$) of immobilized enzyme was investigated, the results were expressed as the percentages of relative of the residual activity and half life time as shown in Figure 4-16. It was shown that the half life of the immobilized CRL at 40°C was 25.38 hours whereas the soluble free lipase was 3.03 hours (Miranda et al, 2011). Evidently, the immobilization on Amberlite XAD7HP has considerably increased the thermal stability of lipase approximately 8.4 folds higher than the free CRL. This perhaps is due to lipase located inside the macroporous pores which offer a good protection against alterations. When an immobilization of CRL by adsorption on Amberlite XAD 761 was studied, the half life was about 21.08 hours, approximately 4 hours less than XAD7HP at 40°C (Putha, 2010). This might be from the fact that the pore diameter of Amberlite XAD 7HP was approximately 0.75 folds smaller. As a result, this might increase the thermal stability of the immobilized CRL. The similar phenomenon was also observed in the previous studies that immobilization inside the narrow pores of support is expected to improve thermal stability of lipase (Zivkovic et al, 2015).

5.6.2 Repeated use of the immobilized CRL for transesterification

It has been demonstrated that the cost of lipase accounts for a large part in overall biodiesel production. One of the main advantages of an immobilized lipase is that it can be used repeatedly over an extended period of time. The byproduct, glycerol is the main problem of reusable immobilized CRL in transesterification as it can deactivate enzymes, particularly in continuous and repeated batch processes. The glycerol molecules can be adsorbed on the surface of these carriers thereby forming the hydrophilic coating resulting in the difficult accessibility of the enzyme molecules to substrates. To investigate the stability of the immobilized lipase, the optimal conditions for transesterification were conducted to obtain the production of biodiesel and

repeated every 12 hours. Under these conditions, approximately 74 % of FAME content were obtained. After completion of each cycle of the reaction for 12 hours, the immobilized CRL was rinsed with water, hexane and t-butanol in comparison. The purpose was to select the best washing solution for the removal of glycerol and oil from the carrier. The immobilized CRL were then dried in the desiccators and later used in the next batch reaction composed of new substrates. The results of each batch for the production of fatty acid methyl ester contents were graphically shown in Figure 4-17. The obtained conversion for each cycle of reuse was compared relative to the first use which was set at 100% for all treatments. From the results of washing with water, it was found that both the yields of fatty acid methyl esters from unwashed and washed CRL gradually decreased to 94 and 86 % in the 2nd cycle (first reuse) and continually dropped to 67 and 60 % respectively in the 3rd cycle. Although both the yields of fatty acid methyl esters from unwashed and washed enzymes decreased in the 2nd and 3rd cycle but unwashed enzyme gave higher biodiesel than washing with water. It could be explained that leaching of lipase from supporting surface was the main problem in the reaction progress and washing process. After the 4th cycle, it can be seen that both immobilized CRL could similarly still retain approximately 45% of the initial activities. These results suggested that immobilized CRL on Amberlite XAD 7HP could be reactivated and reused even after losing some of it activity. However, the yields of fatty acid methyl esters from unwashed and washed enzymes decreased to approximately 5% of its initial activity in the 5th cycle. These results strongly confirmed that the activity loss of immobilized lipase results from desorption of lipase from the support and conformational changes from repeated use. The frequent treatments would undeniably denature their molecular structure (Winayanuwattikuna et al, 2014). So, unwashed immobilized CRL on Amberlite XAD 7HP presents the advantage that it can be reused for three cycles and retained 45% of its initial activities while unwashed immobilized CRL on Amberlite XAD 761 was stable and retained 30% relative transesterification activity after two cycles. These results imply a strong interaction between the CRL and Amberlite XAD 7HP and thereby suggest more potentiality of this support. On the

contrary, when the immobilized CRL were washed with hexane and t-butanol, the conversion to fatty acid methyl esters dramatically decreased to 50 % after the 2nd cycle and finally lost all of the activities after the 4th cycle. Similarly, immobilization CRL on Amberlite XAD761 were also washed with t-butanol and hexane. The result showed that the conversion to fatty acid methyl esters rapidly decreased to approximately 20% after the 2nd cycle and lost all of its activity after 3rd cycle. It appears that the immobilized CRL was desorbed from carriers and inactivated by t-butanol and hexane.

5.7 Continuous operation

On a laboratory scale, the most commonly used systems are batch operation. The advantage of this operation is easy handling. However, batch mode has a low throughput due to the need to empty, clean and reload the reactor before a new batch can start. The low productivity disadvantage of the batch can be eliminated by using a continuous packed bed reactor (PBR) where the enzyme is retained in the reactor (Christopher et al, 2014). The recycling method is advantageous as it allows the substrate solution to be passed through the column at a desired velocity. They also allow the reuse of CRL without separation in comparison to stirred batch reactor. In this work, the PBR was therefore investigated in comparison with the batch operation. The results of fatty acid methyl ester contents from continuous PBR were graphically shown in Figure 4-18. The results showed that when the mixture containing 1 to 1 mole ratio of palm oil and methanol was fed at the constant rate of 0.2 ml/min into the reactor with one gram of immobilized CRL and the reactor was heated by 50°C circulatory water bath, the percent conversion of fatty acid methyl esters rose from 0 to approximately 8 % in the first 4 hours. It was observed that the fatty acid methyl ester content increased along with the increase in reaction time due to no mass transfer limitation. In the next 4 hours, the biodiesel increased to 10.4%. During biodiesel production, glycerol forms hydrophilic phase and creates a film layer around the immobilized enzyme. Glycerol can affect the mass transfer of immobilized enzyme thereby decrease the conversion rate. After 8 hours, fatty acid methyl esters remained rather steady until 48 hours. This might

have been that some immobilized CRL were blocked by glycerol. Large molecules such as triglycerides cannot easily diffuse through the pores to reach the enzyme. In 2007, Suwimon Suptaweewut studied biodiesel production from vegetable oil catalyzed by Novozyme 435 in PBR using the same conditions as described above. The results showed that 11.7% of fatty acid methyl esters were obtained (Suptaweewut, 2007). It is reasonable to conclude that the glycerol creates mass transfer limitation resulting in negative impact on lipase activity and stability. The strategy has been developed to reduce glycerol effect by using organic solvents such as t-butanol. Li *et al* reported that t-butanol can increase solubility of glycerol that prevents lipase inhibition (Li et al, 2006). Since the yield of biodiesel in batch reaction was approximately 74%, it was then 7 folds higher production than that was obtained from PBR. Some factors should be considered for PBR in order to improve biodiesel production. Firstly, a lower flow rate should be considered in order to obtain sufficiently longer retention time for the higher yield. Moreover, the conversion of biodiesel increased with increasing amount of lipase. The higher quantity of enzyme will not result in higher cost since it can be reused many times in the reactor (Chulalaksananukul et al, 2002). The addition of organic solvent such as hexane also should provide significant effect on biodiesel production. The presence of hexane in specified amounts may have improved the solubility of methanol in the reaction mixture so that lipase can still maintain high activity even despite the methanol presence in the system (Garlapati et al, 2013).

CONCLUSION

In this research, lipase from *Candida rugosa* (CRL) was successfully immobilized on Amberlite XAD7HP by physical adsorption. The optimal conditions for the immobilization were obtained as pH 6, 20 mM ionic strength, 3 mg/ml of CRL at 20°C for 4 hours. When the transesterification catalyzed by immobilized CRL was carried out for the production of biodiesel, the yield of 74% were achieved under the conditions of four steps addition mode of methanol, 1 to 3 mole ratio of oil:methanol, 20% (w/w) of oil for CRL loading at 40°C for 12 hours. The obtained immobilized CRL was found to be stable at 40°C and could be reused for three cycles with the half life of approximately 25 hours. In addition, the continuous packed bed reactor (PBR) system was also investigated. Approximately 10% of biodiesel were obtained under the conditions of 1 to 1mole ratio of methanol to palm oil, flow rate at 0.2 ml/min, 1 gram of CRL with 1 to 4 ratio of amount of enzyme to glass beads (weight by weight) at 50°C for 48 hours. Thus, the immobilized CRL on Amberlite XAD7HP can efficiently catalyze the transesterification for the production of biodiesel in batch operation. Nevertheless, the improvement of the yield for PBR requires further and thorough investigations.

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APPENDIXS

APPENDIX A

Properties of Amberlite XAD7HP
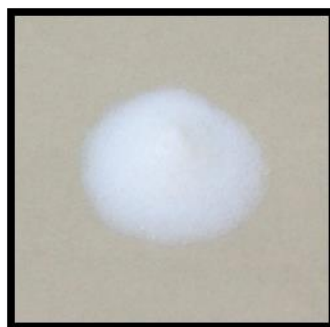
Figure A- 1 Amberlite XAD7HP

TableA- 1 Details of Amberlite XAD7HP

Matrix:	Macroreticular aliphatic crosslinked polymer
Physical form:	White translucent beads
Moisture holding capacity[1] :	61 - 69%
Shipping weight:	655 g/L
Specific gravity:	1.06 to 1.08
Particle size	
Harmonic mean size:	0.56 to 0.71 mm
Uniformity coefficient:	$\leq$ 2.0
Fines content:	< 0.300 mm: 7.0% max.
Coarse beads:	> 1.180mm: 8.0% max.
Porosity[2] :	$\geq$0.5 ml/ml
Surface area[2] :	$\geq$380 m^2/g
Average pore diameter:	450 Å

[1] Contractual value

[2] Values based on statistical quality control (SQC)

APPENDIX B

Media and Hydrolysis assay

1. Preparation for media

1.1 Yeast Malt Extract

Yeast extract	3 g
Malt extract	5 g
Peptone	5 g
Glucose	10 g

All components were dissolved in 1000 ml distilled water and then steriled at 121 °C, 15 lb/in^2 for 15 min.

1.2 Yeast Malt Extract Agar

Yeast extract	3 g
Malt extract	5 g
Peptone	5 g
Glucose	10 g
Agar	15 g

All components were dissolved in 1000 ml distilled water and then steriled at 121 °C, 15 lb/in^2 for 15 min.

1.3 Liquid production media

Yeast extract	5 g
$MgSO_4.7H_2O$	0.5 g
KH_2PO_4	5 g
$NaNO_3$	1 g
Palm oil	3%(v/v)

All components were dissolved in 1000 ml distilled water and then steriled at 121 °C, 15 lb/in^2 for 15 min

2. Preparation of solutions for hydrolysis assay

2.1 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

2.2 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 $4^{\circ}C$.

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).

TableC- 1 Composition for standard BSA

BSA	Reagent volume (µl)	
(mg)	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.
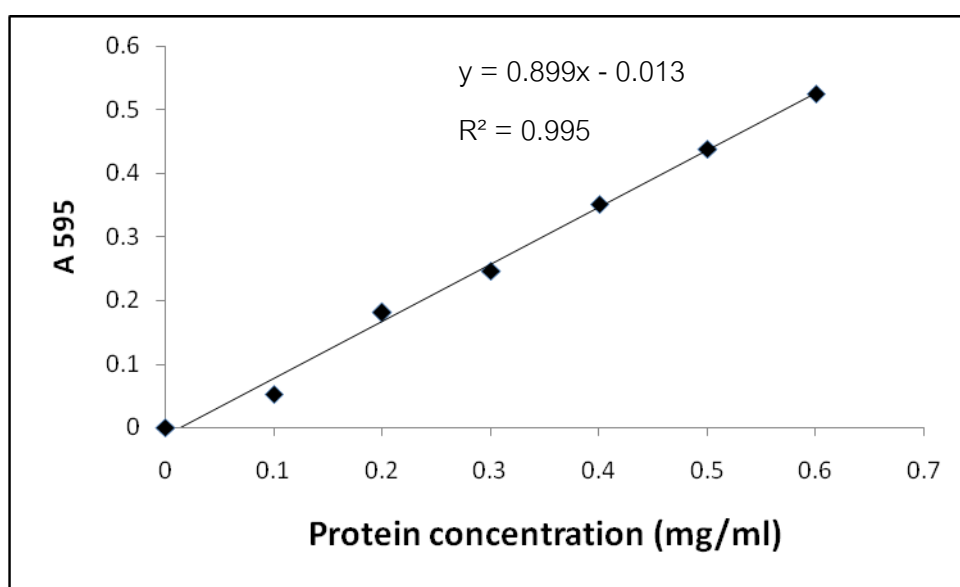

Figure C- 1 Calibration curve for protein determination by Bradford's method

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:

$$p \quad = \quad \frac{C_iV - (C_fV_f + C_wV_w)}{m_s}$$

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

C_i = Initial protein concentration (mg/ml)

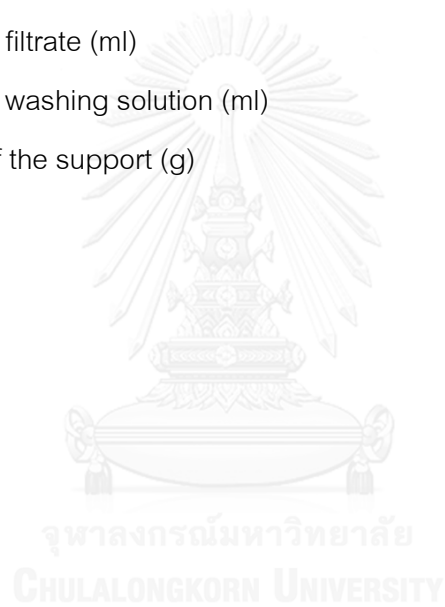C_f = Protein concentration of filtrate (mg/ml)

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

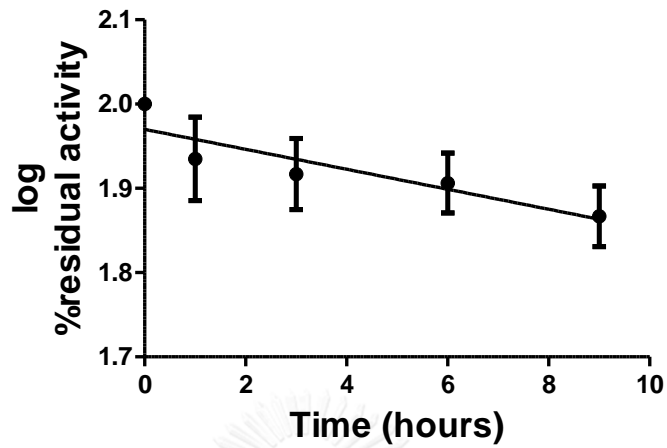Thermal stability was calculated according to equation (1) and (2).

Figure D- 1 Half life time ($t_{1/2}$) of immobilized CRL. 5 mg of immobilized lipase were

incubated at 40°C. The results were average values of triplicate

experiments.

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

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

Where

A_{in} = the hydrolytic activity at given time

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

k_d = thermal deactivation constant

t = the incubation time

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

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

untreated immobilized enzyme

APPENDIX E

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.
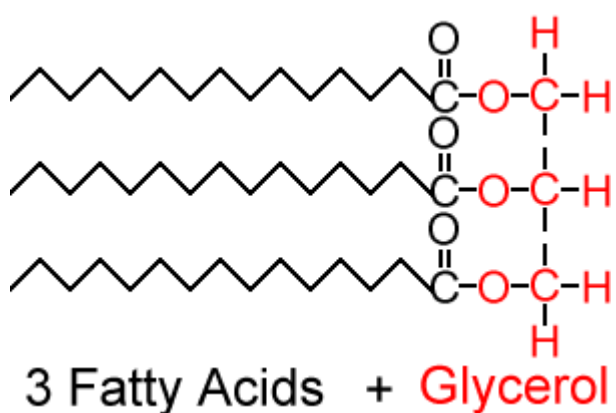
FigureE- 1 Molecular structure of triglyceride

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

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

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)

TableE- 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;

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

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

= 226.823

MW_{TG} = (3 × 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.
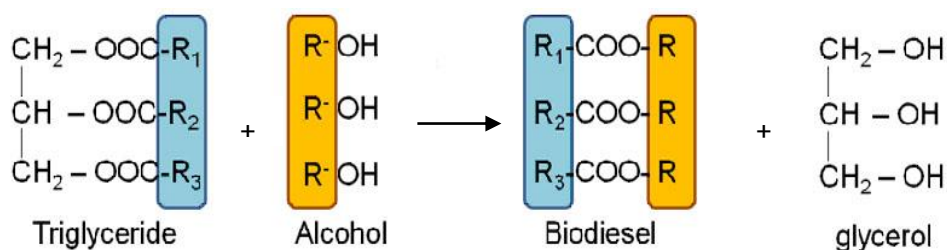
FigureE- 2 Transesterification of palm oil and methanol

So, 0.5 g of palm oil $= 0.5/853.47$

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

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

$= 1.76 \times 10^{-3}$ 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 $D = \dfrac{M}{V}$

D = Density of methanol (0.792 g/ml)

M = Mass of methanol (0.056 g)

V = volume of methanol (ml)

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

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

3. % conversion from HPLC analysis

All FAME obtained by HPLC can be calculated as follows;

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

FAME = Concentration of methyl ester

FFA = Concentration of free fatty acid

TAG = Concentration of triglyceride

DAG = Concentration of diglyceride

MAG = Concentration of monoglyceride

VITA

Miss Wasinee Thaipanich was born on September 13, 1987 in Kamphangphet, Thailand. She graduated with the Bachelor Degree of Science from the Department of Biotechnology, Faculty of Science and Technology, Thammasat University in 2009. Later in 2011, 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 June of 2015. During her studies, part of her work was presented as poster presentation at Burapa University International Conference 2013 Global Change: Human and Social Impacts on the topic of "Production of biodiesel from transesterification catalyzed by Candida rugosa lipase immobilized on Amberlite XAD7HP" at Jomtien Palm Beach Hotel & Resort, Pattata, Thailand on 4-5 July, 2013.