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OPTIMIZATION FOR IMMOBILIZATION OF LIPASE FROM POTENTIAL LIPOLYTIC MICROORGANISMS FOR BIODIESEL PRODUCTION

Miss Rungrawee Impiew

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biochemistry and Molecular Biology

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รุ่งระวี อิ่มผิว : การหาภาวะเหมาะที่สุดสำหรับการตรึงรูปไลเพสจากจุลินทรีย์ที่มีศักยภาพในการสลายไขมันเพื่อการผลิตไบโอดีเซล (OPTIMIZATION FOR IMMOBILIZATION OF LIPASE FROM POTENTIAL LIPOLYTIC MICROORGANISMS FOR BIODIESEL PRODUCTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทิฆัมพร ยงวณิชย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. วรวุฒิ จุฬาลักษณานุกูล, 102 หน้า.

ไลเพส (ไตรเอซิลกลีเซอรอล เอซิลไฮโดรเลส EC 3.1.1.3) คือเอนไซม์ที่เร่งปฏิกิริยาไฮโดรไลซิสของไตรกลีเซอไรด์ที่ชั้นระหว่างน้ำมันกับน้ำ และสามารถประยุกต์ใช้ทั่วไปในอุตสาหกรรมหลายชนิด เช่น สารซักฟอก การทำขนมปัง น้ำมันและไขมัน การทำความสะอาดพื้นผิวแข็ง การสังเคราะห์สารอินทรีย์ หนังสัตว์ กระดาษ และโดยเฉพาะอย่างยิ่งการผลิตไบโอดีเซล ซึ่งเป็นพลังงานสะอาดที่สามารถนำกลับมาใช้ใหม่ ถึงแม้ว่าไลเพสสามารถพบได้ตามธรรมชาติในสัตว์และพืชหลากหลายชนิด แต่ไลเพสจากจุลินทรีย์มีบทบาทสำคัญสำหรับการประยุกต์ใช้ในอุตสาหกรรม เนื่องจากเจริญเติบโตเร็วและให้ผลผลิตสูง อย่างไรก็ตาม ราคายังคงเป็นอุปสรรค ซึ่งการตรึงรูปสามารลดปัญหานี้ได้เนื่องจากเอนไซม์ตรึงรูปมีความเสถียรที่สูงกว่าและสามารถนำกลับมาใช้ใหม่ได้ ในงานวิจัยนี้ทดสอบเปรียบเทียบจุลินทรีย์ 4 ชนิด จากแหล่งธรรมชาติและรีคอมบิแนนท์ เพื่อเป็นแหล่งผลิตไลเพสที่มีศักยภาพ ผลที่ได้คือ เลือกรีคอมบิแนนท์ไลเพสจาก *Aureobasidium pullulans* (rAPL) ที่แสดงออกในเมทิลโลโทรฟิกยีสต์ *Pichia pastoris* เพื่อใช้ในการตรึงรูป ในการหาภาวะเหมาะสมของการเหนี่ยวนำการแสดงออกเพื่อให้ได้ผลผลิตไลเพสที่สูง พบว่าทำได้โดยการเติมเมทานอลบริสุทธิ์ร้อยละ 2 ในอาหารเลี้ยงเชื้อทุก 24 ชั่วโมง เป็นเวลา 120 ชั่วโมง ได้ค่าการทำงานของ rAPL เท่ากับ 58.14 ไมโครโมลต่อนาทีต่อมิลลิกรัมโปรตีน จากนั้นสามารถหาภาวะเหมาะสมสำหรับการตรึงรูป rAPL บนตัวค้ำจุนพอลิเมอร์ชนิดไฮโดรโฟบิก Amberlite XAD7HP ด้วยวิธีดูดซับทางกายภาพได้ โดยการนำ rAPL 15 มิลลิกรัม ในบัฟเฟอร์อะซิเตทเข้มข้น 50 มิลลิโมลาร์ ที่ความเป็นกรดด่างเท่ากับ 5 ตรึงบน Amberlite XAD7HP 1 กรัม และกวนผสมต่อเนื่องที่ 50 องศาเซลเซียส เป็นเวลา 2 ชั่วโมง ได้ค่าการทำงานของ rAPL ตรึงรูปเท่ากับ 5.67 ไมโครโมลต่อนาทีต่อกรัมตัวค้ำจุน เมื่อใช้ rAPL อิสระและตรึงรูปเร่งทรานส์เอสเทอริฟิเคชันสำหรับผลิตไบโอดีเซล โดยใช้น้ำมันปาล์มเป็นสารตั้งต้นที่ 40 องศาเซลเซียส เป็นเวลา 12 ชั่วโมง พบว่าได้ผลิตภัณฑ์จากการวิเคราะห์ด้วยโครมาโทกราฟีของเหลวสมรรถนะสูงประมาณร้อยละ 56 และ 61 ตามลำดับ rAPL ตรึงรูปบน Amberlite XAD7HP มีความเสถียรที่ 40 องศาเซลเซียส และยังคงค่าการทำงานสัมพัทธ์สำหรับทรานส์เอสเทอริฟิเคชันร้อยละ 86 หลังจากการใช้รอบที่สอง จากผลทั้งหมดแสดงให้เห็นว่าสามารถผลิตไบโอดีเซลได้โดยทรานส์เอสเทอริฟิเคชันที่เร่งด้วย rAPL ตรึงรูปบน Amberlite XAD7HP

ภาควิชา	ชีวเคมี	ลายมือชื่อนิสิต	.....
สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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RUNGRAWEE IMPIEW: OPTIMIZATION FOR IMMOBILIZATION OF LIPASE FROM POTENTIAL LIPOLYTIC MICROORGANISMS FOR BIODIESEL PRODUCTION. ADVISOR: ASSOC. PROF. TIKAMPORN YONGVANICH, CO-ADVISOR: ASSOC. PROF. WARAWUT CHULALAKSANANUKUL, Ph.D., 102 pp.

Lipases (Triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes which catalyze the hydrolysis of triglycerides at the oil water interphase and generally applicable by many industries such as detergent, baking, oil and fats, hard surface cleaning, organic synthesis, leather, paper and in particular, the production of biodiesel, a renewable green energy. Although lipases can be naturally found in various animals and plants, the microbial lipases have played more important roles for industrial applications due to their rapid growth and high production. Nevertheless, the cost remains the obstacle by which the immobilization can alleviate the problem with higher stability and reusability of the enzymes. In this work, four types of microorganisms from natural sources and the recombinants were comparatively investigated for the potential source of lipase production. Consequently, the recombinant lipase from *Aureobasidium pullulans* (rAPL) expressed in methylotrophic yeast, *Pichia pastoris* was selected for the immobilization. In order to obtain the high production of lipase, the induction of expression was optimized by the addition of 2% pure methanol in the culture every 24 hours for the period of 120 hours. The obtained activity of the rAPL was 58.14 μmole/min/mg protein. Then, the optimal conditions for the immobilization of rAPL on the hydrophobic polymeric support, Amberlite XAD7HP by physical adsorption were obtained. 15 mg of rAPL in 50 mM acetate buffer at pH 5 were immobilized on 1 g of Amberlite XAD7HP and continuously stirred at 50°C for 2 hours. The activity of the immobilized rAPL was 5.67 μmole/min/g support. When free and immobilized rAPL were used to catalyze the transesterification for the production of biodiesel using palm oil as the substrate at 40°C for 12 hours, the percentages of the obtained product from the analysis by high performance liquid chromatography were found to be approximately 56 and 61%, respectively. The immobilized rAPL on Amberlite XAD7HP was stable at 40°C and still retained 86% of relative transesterification activity after second cycle use. Overall results indicated that biodiesel production could be accomplished by transesterification catalyzed by the immobilized rAPL on Amberlite XAD7HP.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
		Co-Advisor's Signature	.....
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CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I INTRODUCTION

1.1 Statement of problem

The majority need of the energy in the world is supplied through fossil fuels such as petrochemical, coal and natural gases without the hydroelectricity and nuclear energy. Fossil fuels are non-renewable energy sources that have the essential function in the industrial economy of a developing country and used for transport of industrial and agricultural goods. However, fossil fuels generate pollutants that cause to global warming and mounting carcinogenic emission problems. In the current, the fossil fuel energy sources are finite and usage rates will be consumed shortly. Therefore, the inevitable for an alternative to fossil fuels has engendered extensive research and development of renewable biofuel resources in recent year (Babaki et al., 2016). Many alternative energy sources such as solar, wind, nuclear, hydro and biofuels such as biodiesel have been increasingly implemented worldwide. Biodiesel (fatty acid methyl ester) is the important renewable and alternative clean burning fuel for diesel engines, has gained the attention among biofuels from its similarity with conventional diesel in energy content and chemical structure (Pourzolfaghar et al., 2016). Moreover, biodiesel reduces carcinogenic compound emissions by approximately 85% compared with diesel fuel and is essentially free of sulfur metals, and polycyclic aromatic hydrocarbons (Christopher et al., 2014).

Rudolf Diesel, the inventor of the internal compression ignition engine now known as diesel engine, developed his extraordinary engine by use the vegetable oils as fuel in 1990. In a short term engine, many vegetable oils can be used directly as diesel fuel, however, the physical and chemical difference between unmodified vegetable oils and conventional diesel fuel caused against on long-term engine (Shay, 1993). Many problems associated with using vegetable oils directly in diesel engine especially in direct injection engine were encountered; these are clogging of injectors, carbon deposits, oil ring sticking and thickening or gelling of the lubricating oil. Since high viscosity was the root caused for many problems, therefore in order to reduce these problems, the converting of vegetable oils to their derivatives of ester was introduced.

Transesterification, the reaction of triglyceride (TAG) in oil with alcohol in the presence of catalyst, can produce esters of free fatty acids (FFA) such as FAME or biodiesel (Meher et al., 2006). In general, transesterification reaction can be catalyzed by acid and alkali catalysts, which give high conversion of TAG at short reaction time. However, the most disadvantage of alkali catalyst is its sensitive to FFA in oils that cause soap formation during the transesterification process. On the other hand, the acid catalyst insensitive to FFA and cause to large amount of inorganic salt that can result in the corrosion. Moreover, the use of chemical catalysts can cause many problems from high-energy consumption, recovery of byproduct, removal of catalyst and treatment of alkali wastewater (Aarthy et al., 2014). In the current, biological catalyst such as lipase can be used in transesterification. It is reported that enzymatic catalyst is insensitive to FFA and water content in the raw material (Kulkarni and Dalai, 2006). Lipase provides a solution to the aforementioned problems from its more efficiency, high selectivity, less energy consumption, less waste, easy recovery of byproduct and reusability without any separation step (Aarthy et al., 2014).

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol at an oil-water interface (Feng et al., 2013). However, they also can catalyze synthetic reactions under certain conditions such as acidolysis, alcoholysis, aminolysis, esterificaition and transesterification. The properties of lipase in both aqueous and non-aqueous media enable its use in therapeutic and medical fields, food process industries, organic chemistry, treatment of wastewater and production of biodiesel. The success of lipases in industrial applications is due to its specific properties. These depend on sources of lipase which can be naturally found in animals, plants and microorganisms. However, plant lipases are not used commercially while the animals and microbial lipases are used extensively (Vakhlu and Kour, 2006). Microbial lipases have gained special industrial attention from stability, high yields, broad substrate specificity, ease of genetic manipulation and rapid growth of microorganisms on inexpensive media. They now share about 5% of the world enzyme market after proteases and carbohydrates (Christopher et al., 2014). Both intracellular and

extracellular lipase can be produced by a widespread number of microorganisms, bacteria, yeast and fungi. In particular, lipase produced by bacteria such as *Halomonas* sp. (Gutiérrez-Arnillas et al., 2016) and *Burkholderia Ubonensis* SL-4 (Yang et al., 2016), yeast such as *Candida* sp. and *Geotrichum candidum* (De Morais Júnior et al., 2016) and fungi such as *Fusarium solani* (Jallouli et al., 2016) and belonging to the genera *Aspergillus* (Sethi et al., 2016). Recently, recombinants of lipase have received more attention. Previous research reported high activity of lipase B from *Candida antarctica* (CalB) expressed in *Pichia pastoris* in which the secretion was similar to most commercially available. This distinctly suggested that CalB was industrially promising compared with one of the most efficient production systems (Eom et al., 2013). And lipase from *Thermomyces lanuginosus* was expressed in recombinant *Pichia pastoris* system was able to convert waste cooking oils to biodiesel with 82% yield (Yan et al., 2014).

From previous studies by Biofuels by Biocatalysts Research Unit (BBBRU), the potential lipolytic microorganisms were isolated and screened from the sample of oil-contaminated soil and waste water. The obtained screened lipolytic microbes were bacterium, *Staphylococcus warneri*, unicellular yeast, *Candida rugosa* and filamentous fungus, *Fusarium solani*. To reach higher level of lipase production, lipase-production conditions were optimized and lipase activities were compared for both hydrolytic and synthetic catalysis. From this research, *Candida rugosa* lipase (CRL) exhibited the highest activity for catalyzing the biodiesel production suggesting that this microbial lipase is promising as a potential biocatalyst for production of biodiesel (Winayanuwattikun et al., 2011). In another research, the lipase gene from filamentous fungus, *Fusarium solani* was transformed and expressed in *Pichia pastoris* KM71. The characterization of *Fusarium solani* lipase (FSL) were studied and both hydrolytic and synthetic activities were investigated. The results indicated that approximately 45% of biodiesel were obtained (Thakernkarnkit, 2010).

However, the major obstacle in enzymatic catalysis for the production of biodiesel is the high cost of the enzymes. One of the possible solutions to reduce the process costs is use of immobilized lipases. Immobilization reduces cost of catalysts as it can be easily

separated, reused and also confers stability to lipase towards temperature, chemicals (alcohol and glycerol) and denaturation (Guldhe et al., 2015). There are various methods for immobilization such as physical adsorption, covalent attachment, entrapment or formation of cross-linked and enzyme aggregates or whole cell biocatalysts (Nigam et al., 2014). Immobilizations of lipases have been reportedly successful by adsorption using various support matrices such as fibers, particles, by entrapping them in gel matrices and by covalent attachments. Adsorption has proved to be one of the useful techniques for improving enzymatic activity (Winayanuwattikun et al., 2011) and more favorable because it faces less mass transfer limitations compared to entrapment and cross linking (Guldhe et al., 2015). Previous research of BBBRU has reported the high activity of CRL immobilized on Amberlite XAD7HP by physical adsorption with approximately 50% yield of biodiesel from transesterification of palm oil (Winayanuwattikun et al., 2011). Moreover, 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 were studie. The results indicated that the production of biodiesel obtained was increased to approximately 74% and the immobilized CRL was stable at 40°C and still retained 45% of relative transesterification activity after three reuses (Thaipanich, 2014). Therefore, this work was concentrated on the selection of potential lipolytic microorganisms from various sources for immobilization of lipase on hydrophobic support, Amberlite XAD7HP by physical adsorption. The immobilization processes were optimized and the immobilized lipase was tested for catalysis of the biodiesel production by transesterification between palm oil and methanol.

1.2 Objective

The purpose of this work was to select the potential lipolytic microorganism and immobilize potential lipase on Amberlite XAD7HP by physical adsorption to catalyze transesterification for biodiesel production.

1.3 Scope of the investigation

1.3.1 To select the potential lipolytic microorganism from various sources

1.3.2 To optimize the conditions for the production of lipase

1.3.3 To optimize the conditions for the immobilization of lipase on Amberlite XAD7HP

1.3.4 To investigate the stability and the reusability of the immobilized lipase

1.3.5 To produce biodiesel through transesterification catalyze by immobilized lipase

1.4 Expected results

This research should provide the potential lipolytic microorganisms for lipase production and the conditions for optimal immobilization of lipase on Amberlite XAD7HP to catalyze transesterification for biodiesel production by 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, a sustainable, nontoxic, biodegradable diesel fuel substitute, can be used in current diesel car infrastructure without major engine modifications. Biodiesel is usually obtained by transesterification of fat and vegetable oils with alcohol (usually methanol or ethanol) in the presence of a catalyst, with resulting production of a fatty acid methyl ester (FAME) or fatty acid ethyl ester (FAEE) that can be used as a biofuel (Almeida et al., 2012).

2.1.1 Biodiesel production

There are four main techniques exist to reduce the potentially high viscosity of biofuels made from animal fats and vegetable oils and eliminate any operational problems from its use in a common diesel engine: direct use and blending, microemulsions (cosolvent blending), thermal cracking (pyrolysis) and transesterification (alcoholysis) (Aarthy et al., 2014).

2.1.1.1 Direct use and blending

The direct usage of animal fats and vegetable oils as biodiesel is possible. The main issues of direct use of animal fats and vegetable oils as fuel in diesel engines are their high viscosity and poor volatility. The effects of the high viscosity are a major cause of poor atomization and difficult in handling by conventional fuel injection system of compression ignition engines (Mondal et al., 2008). Blends of animal fats or vegetable oils with petrodiesel has been used to reduce the viscosity. The major advantages of the blending are the absence of technical modifications and the ease of implementation. Blending of animal fats or vegetable oils with alcohol results in a significant improvement in their physical properties (Bousbaa et al., 2012, Kumar et al., 2006, Mrad et al., 2012).

2.1.1.2 Microemulsions

Microemulsions are microstructured, thermodinamically stable mixtures of water, oil and surfactant through microemulsification. Microemulsification is a process used to formulate hybrid diesel fuels by solubilisation of vegetable oil/alcohol mixtures through the addition of amphiphiles. Alcohols such as CH_3OH or ethanol (CH_3CH_2OH) have limited solubility in nonpolar animal fats and vegetable oils. Therefore, amphiphilic compounds such as phospholipids, sorbitansesquiolate (Span83), n-butanol, 2-octanol, carboxylate surfactants and the like, are added to increase solubility, dilute the oil, and reduce viscosity. An equilibrium dispersion of optically isotropic fluid microstructures with an average diameter less than one quarter the wavelength of visible light that spontaneously form upon the addition of amphiphiles to a mixture of microemulsion (Schwab et al., 1983).

2.1.1.3 Pyrolysis

Pyrolysis or cracking involves the thermal decomposition of organic material to smaller molecules through the application of heat without the addition of supplemental air or oxygen. Thermal cracking of triglyceride materials (animal fats and vegetable oils) represent an alternative method of producing renewable bio-based products suitable for use in fuel and chemical applications. Considerable investigations have been done on the pyrolysis of different varieties of vegetable oils to produce chemicals and diesel-like fuel (Alencar et al., 1983, Omar and Robinson, 2014). Animal fat pyrolysis has received much less interest as compared to vegetable oils. Disadvantages of this process include the equipment for thermal cracking and pyrolysis is expensive for modest throughputs. In addition, while the products are chemically similar to petroleum-derived gasoline containing sulfur which makes it less eco-friendly (Ma and Hanna, 1999).

2.1.1.4 Transesterification

Transesterification is the most commonly used method to reduce viscosity of animal fats and vegetable oils. It generates products commonly known as biodiesel (alkyl esters of fats or oils) (Gerpen, 2005). This biodiesel production technique has been reported to have particular advantages over other processes as follow: reaction

conditions are mild, the process is environmentally friendly and a wide variety of feedstocks can be processed through this technique. Transesterification of triglycerides is the reversible reactions and proceeds essentially by mixing the reactants to produce fatty acid alkyl esters and glycerol. There are diglycerides and monoglycerides as the intermediates. However, the presence of a catalyst (a strong acid or base) accelerates the conversion (Figure 2-1) (Meher et al., 2006).

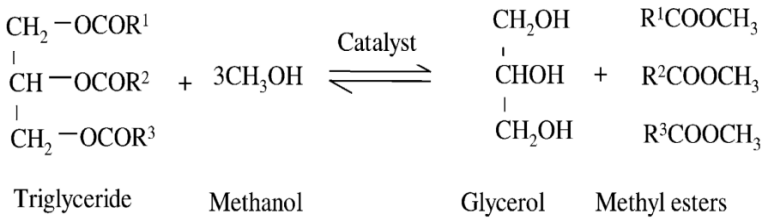


Figure 2-1 Equation for transesterification of triglycerides (Adewale et al., 2015)

2.1.1.4.1 Acid catalyst

The acid-catalyzed transesterification of vegetable oils, carboxylic acids can be formed by reaction of the carbocation with water present in the reaction mixture. This suggests that an acid-catalyzed transesterification should be carried out in the absence of water, in order to avoid the competitive formation of carboxylic acids, which reduce the yield of alkyl esters. These catalysts give very high yields in alkyl esters, but the reactions are slow, requiring typically high temperatures to reach complete conversion. However, the performance of the acid catalyst is not strongly affected by the presence of free fatty acids in the feedstock. Thus, a great advantage with acid catalysts is that they can directly produce biodiesel from low-cost feedstocks, generally associated with high free fatty acid concentrations (Ejikeme et al., 2010).

2.1.1.4.2 Base catalyst

The base-catalyzed transesterification of vegetable oils are reported to proceed faster than the acid-catalyzed reactions and result in good yields of the esters (Freedman et al., 1986). Because of this and the fact that the alkaline catalysts are less corrosive than acidic compounds, industrial processes usually favour base catalysts. However, even if

water-free alcohol/oil mixture is used, some water is produced in the system by the reaction of the hydroxide and the alcohol. The presence of water gives rise to hydrolysis of some of the produced ester, with consequent soap formation (Freedman et al., 1984). This undesirable saponification reaction reduces the ester yields and considerably makes difficult the recovery of the glycerol due to the formation of emulsions, increase in viscosity and greatly increased product separation cost (Ejikeme et al., 2010).

2.1.1.4.3 Enzymatic catalyst

Lipases are able to catalyze transesterification reactions, which allows the production of biodiesel from cheaper raw materials with high free fatty acid and water content (Fjerbaek et al., 2009). Lipase-catalyzed biodiesel production is high selectivity, high specificity, and mild conditions technology. Despite the many advantages, the application of lipase in biodiesel synthesis has some obstacles, such as low biocatalyst productivity (due to low reaction rate and low stability), and the high cost of enzymes which can be addressed by replacement of immobilized lipase (Gog et al., 2012). Recently, it has been found that immobilized lipase can be used in transesterification reaction. The use of immobilized lipases as biocatalysts for biodiesel synthesis can produce very high purity FAME with lower or no downstream process. Immobilized lipases are easy to recover by relatively simple operations such as filtration or centrifugation, making it possible its reuse or to carry out continuous processes while, at the same time, a glycerol, byproduct with high purity can be easily recovered without requiring any complex separation process (Aguieiras et al., 2015).

2.2 Lipase

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are a class of enzymes that preferentially act on the carboxylic ester bonds at the interface between lipid and water in heterogeneous systems (Tran et al., 2013). They carry out the hydrolysis of triglycerides to glycerol and fatty acids. These enzymes are proven to be excellent biocatalysts for performing various reactions such as hydrolysis, esterification and transesterification (Li et al., 2013).

2.2.1 Source of lipase

Lipases is ubiquitous in nature and produced by animals, plants and microorganisms. Due to their ease of manipulation, their unlimited supply, and their particular catalytic properties, microbial lipases occupy the prominent position as industrially important biocatalysts. Indeed, according to the list of enzymes compiled by the Association of Manufactures and Formulators of Enzymes Products (AMFEP) and updated in May 2015, the main sources of lipases used in industrial processes are fungi, followed by yeasts, bacteria and animals. The animal lipases used in industrial processes include pancreatic and pregastric lipases. Pancreatic lipases and pregastric lipases purified from calf, goat and lamb gullets are used in the acceleration of cheese ripening and flavour generation, as well as in the lipolysis of butter, fat and cream (El-Hofi et al., 2011). Within cereal seeds, the lipases from rice, wheat, barley, oat, and maize have been tested for their use as biocatalysts, and most of these are resistant to high temperatures and alkali conditions (Borrelli and Trono, 2015).

2.2.2 Lipase producing microorganisms

Commercially available enzymes are derived from animals, plants and microorganisms. The microbial lipases are more stable and cover a greater variety of catalytic activities than lipases derived from animal and plant sources (Jemli et al., 2016). Moreover, microorganisms can rapidly grow in inexpensive media, thus reaching high yields and representing an always available source, as their growth is not affected by seasonal fluctuations. They have great adaptative capabilities even in inhospitable environments, such as in the waters of the deep-seas, the Dead Sea, the Antarctic, the alkaline lakes, hydrothermal and volcanic vents and contaminated soils (Lefevre et al., 2009). Lipases from yeasts and fungi are the most relevant for commercial applications followed by lipase from bacteria. Lipases producing microorganisms have been screened and isolated from various sources such as lipase producing bacterium was isolated from oil-contaminated soil samples and identified taxonomically as *Stenotrophomonas*

maltophilia (Li et al., 2013), the fungal was isolated from water samples of the Caspian Sea (the north of Iran) and identified as *Cladosporium langeronii* (Sadati et al., 2015).

Genetic engineering nowadays represents the most efficient approach to generate a "tailor-made" enzyme as recombinant lipases for a given application (Bornscheuer, 2008, Bornscheuer, 2013). For example, the *P. pastoris* strain transformed with lipase gene from *Rhizopus oryzae* (Li et al., 2011).

2.2.3 Properties of lipase

The lipases structure is an α/β hydrolase fold, and it is common also to many other hydrolases, such as esterases, proteases, dehalogenases, epoxide hydrolases, and peroxidases (Carr and Ollis, 2009). The canonical α/β hydrolase fold is characterized by the presence of a central β-pleated sheet that contains eight parallel β-strands with the exception of β2 strand which is antiparallel with respect to the others. The β3 to β8 strands are connected by α helices which pack on the two sides of the central β sheet (Anobom et al., 2014). The active site of the lipases consists of a highly conserved catalytic triad: one nucleophilic residue, one catalytic acidic residue and one histidine, the nucleophilic residue is always a serine which is located in the so-called "nucleophilic elbow" and identified by the highly conserved pentapeptide GXSXG. The catalytic acidic residue is located after the β6 or β7 strand of the central β-sheet, and it is hydrogen-bonded to the catalytic histidine that is located in a loop after the β8 strand of the α/β hydrolase fold (Jaeger et al., 1999). Another important feature of the lipases is the oxyanion hole, which is the "pocket" that promotes the stabilization of the negatively charged intermediate generated during the ester-bond hydrolysis (Pleiss et al., 2000). In addition, a lid structure usually found covering the top of the active site, can move from closed to open conformation when these lipases absorb to the lipid–water interface (Xu et al., 2012), thus making the catalytic site accessible to the substrate and the solvent (Secundo et al., 2006). Lipases from *Bacillus subtilis, Bacillus pumilis* and *Bacillus licheniformis* lack the lid domain, and therefore have low molecular weights and do not show interfacial activation (Arpigny and Jaeger, 1999, Hjorth et al., 1993). In contrast, the lipase from *Pseudomonas* sp. MIS38, which belongs to Family I.3, has two lids: lid1 that

represents the widely known lid of the bacterial lipases, and lid2 that is unique to this *Pseudomonas* sp. MIS38 lipase and other Family I.3 lipases (Angkawidjaja et al., 2007).

The structure of lipases from different sources show the different specificity of lipases and the other factors affecting the specificity include structure of the substrate and factors affecting binding of the enzyme to the substrate. Types of specificity are as follows. I. Substrate: different rates of lipolysis of triglyceride, diglyceride, and monoglyceride by the same enzyme II. Positional: (a) primary esters; (b) secondary esters; and (c) all three esters or nonspecific hydrolysis. III. Fatty acid, preference for similar fatty acids. IV. Stereospecificity: faster hydrolysis of one primary*sn* ester as compared to the other. V. Combinations of I-IV.

2.2.4 Enzymatic reaction of lipase

Lipases have the natural catalytic function of hydrolyzing fatty acid ester bonds in water and are the most commonly used in organic synthesis. Under natural conditions such as in the presence of excess water, lipases act at the organic–aqueous interface to catalyze the hydrolysis of carboxylate ester bonds and release free fatty acids (FFAs) and organic alcohols. As the equilibrium between the forward (hydrolysis) and reverse (esterification) reactions are controlled by the water activity (a_w) of the reaction mixture under limiting water conditions, the esterification can occur. Under low a_w, the different transesterification reactions can also be achieved. The term of transesterification refers to the exchange of groups between an ester and an alcohol (alcoholysis), an ester and an acid (acidolysis), an ester and an amine (aminolysis) or two esters (interesterification) as show in Figure 2-2 (Borrelli and Trono, 2015).

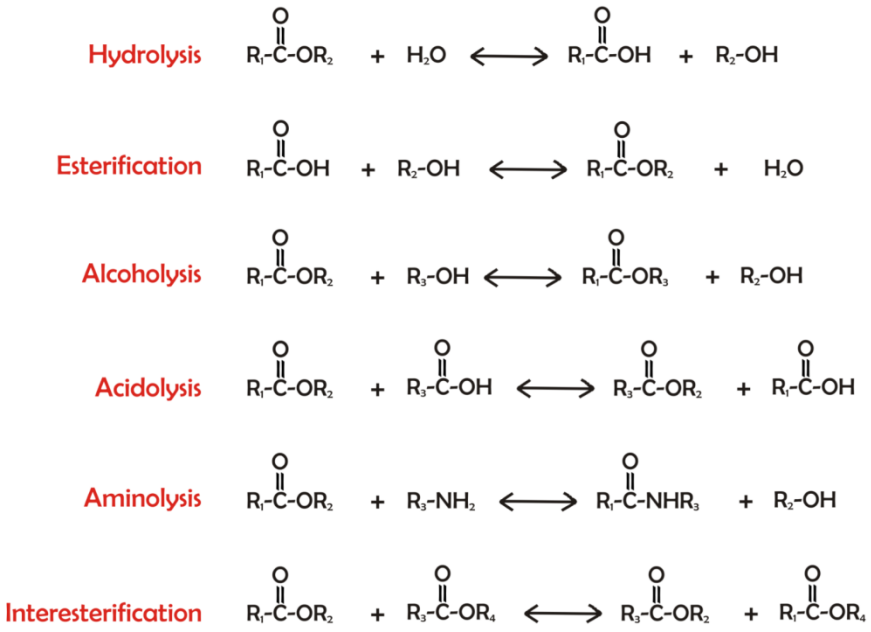


Figure 2-2 Reactions catalyzed by lipase (Borrelli and Trono, 2015)

2.2.5 Applications of lipase

Microbial lipases constitute an important group of biotechnologically valuable enzymes due to versatility of their applied properties and ease of mass production. Microbial lipases were probably attractive applications in industries due to its wide enzymatic properties (Ray, 2012). There are some of the industrial applications such as the detergent, fat and oil, biodegradable polymer, food, pharmaceutical, biosensor and also the biodiesel production (Borrelli and Trono, 2015).

2.3 Immobilization of lipase

In recent years, production of biodiesel using immobilized lipases has attracted great interest. An immobilized enzyme is defined as the enzyme is physically confined to a certain defined region while it is retaining its most catalytic activity (Jegannathan et al., 2008). For the application in biodiesel production, immobilized lipases have much more advantages than free lipases such as easy recovery and reuse, higher adaptability for continuous operation, less effluent problems, greater pH and thermal stability, and higher tolerance to reactants and products (Ozturk, 2001). In contrast, the current immobilized

lipases still have several drawbacks for industrial applications including loss of enzymatic activity during immobilization, high cost of the carriers, low stability in oil-water systems and requirement of novel reactors for well mixing and maximizing oil to biodiesel conversion (Zhao et al., 2015).

2.3.1 Method of enzyme immobilizations

Various techniques have been developed for lipase immobilization as reviewed in recently published papers (Figure 2-3). Generally, these techniques can be classified into three types: carrier bonding, cross-linking and entrapment. Depending on the type of interactions between enzymes and carriers, these techniques can be further classified into irreversible and reversible immobilization techniques (Brena et al., 2013). The methods for irreversible immobilization of lipases are covalent bonding, entrapment and cross-linking. Enzymes are attached to supporting materials, they cannot be detached without destroying either the biological activity of the enzyme or the support. On the other hand, physical adsorption and various non-covalent bonding such as affinity bonding and chelation bonding are reversible immobilization. Enzymes can be detached from the support under gentle conditions. Each immobilization technique has its own advantages and some disadvantages for lipase immobilization (Zhao et al., 2015).

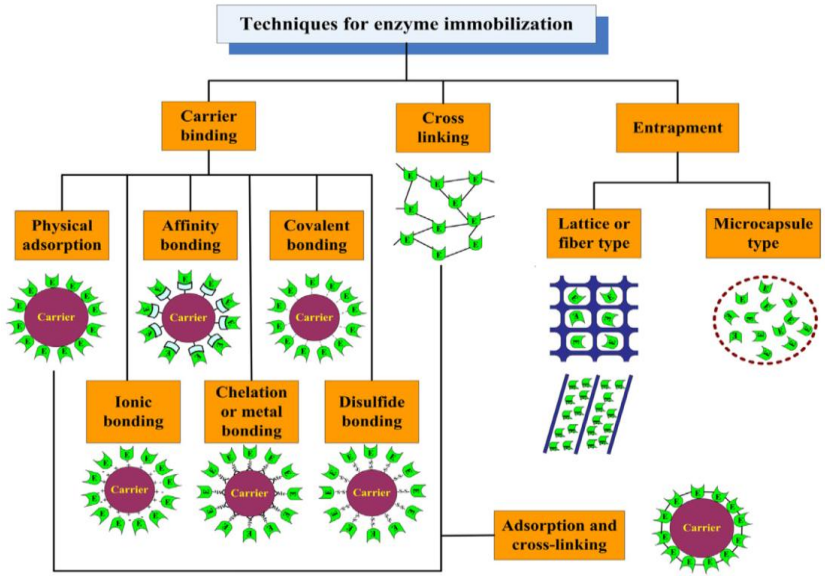


Figure 2-3 Various techniques for enzyme immobilization (Zhao et al., 2015)

2.3.1.1 Physical adsorption

Adsorption is a commonly used method to immobilize lipases including non-specific physical adsorption, bio-specific adsorption, affinity adsorption, electrostatic interaction and hydrophobic interaction. Compared with other immobilization techniques, adsorption immobilization have many advantage such as mild conditions and easy operation, relatively low cost of carrier materials and immobilization procedure, no requirement of chemical additives during adsorption, easy regeneration of carriers for recycling and high lipase activity recovery (Zhang et al., 2012). For the immobilization by physical adsorption, the enzyme is adsorbed through non-specific forces such as van der Waals forces, hydrogen bonds and hydrophobic interactions. Lipase can be derived from different sources such as *Candida antarctica* (Watanabe et al., 2000), *Candida* sp. 99–125 (Lu et al., 2010) and *Pseudomonas fluorescence* (Salis et al., 2008). The commonly used support materials are polymer resins such as polystyrene, polypropylene and polyacrylate. These polymers are cheap to obtain. Both carrier properties and immobilization conditions have significant influences on the immobilization efficiency. Typically, porous support is advantageous since lipases can be adsorbed at both the outer surface and within the pores of supporting material (Ozturk, 2001). The hydrophobicity of the carrier has been found to show significant influence on the lipase activity after immobilization. Relative activity of lipase is increased when the enzyme is selectively adsorbed on hydrophobic supports, because lipases can recognize the surfaces similarly to those of their natural substrates and they suffer interfacial activation during immobilization (Fernandez-Lafuente et al., 1998). However, the stability of immobilized lipase obtained from physical adsorption method still needs to be further improved. The activity of Immobilized lipase will be decreased continually with recycling batches. This is the main reason of relatively weak interaction and stripping lipase off the carrier during the transesterification process. The production of an extracellular lipase from *Geotrichum candidum* and its immobilization on eco-friendly and cheap polyhydroxybutylate (PHB) particles by physical adsorption were reported by Ramos et al. They found that the immobilization provided a slight thermal stabilization and a greater

stabilization on alkaline pH when compared to the free enzyme. Furthermore, the high stability can be obtained in six consecutives cycles of reaction. It is indicated that the immobilized lipase of *G. candidum* on PHB could be applied for esters synthesis of industrial interest (Ramos et al., 2015).

2.3.1.2 Ionic bonding or Covalent bonding

In the immobilization process by ionic bonding, the enzymes are bound through salt linkages. The carriers typically contain ion-exchange residues such as polysaccharides and synthetic polymers. This method is simple and reversible but it is difficult to find conditions which the enzyme remains both strongly bound and fully active. Compared with covalent bonding method, ionic bonding can be conducted under much milder condition. Therefore, the ionic binding method causes little changes in the conformation and the active site of the lipase retaining lipase activity. However, the binding forces between enzymes and carriers are less strong than covalent binding and leakage of enzyme from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH (Ozturk, 2001).

Covalent binding is the immobilization process of enzymes by the chemical reaction between the active amino acid residues outside the active catalytic and binding site of the enzyme towards the active groups of the carrier (Stoytcheva et al., 2011). The groups usually used in the covalent binding are thiol and amine groups of enzymes (Brena et al., 2013). Various carriers have been employed for covalent binding including silica gel, chitosan, and magnetic particles. Since the binding force between lipase and carrier is strong, the immobilized lipase shows high stability during transesterification with almost no lipase leaching. This type of immobilized lipase is also resistant to extreme conditions. However, the condition of preparation for covalent immobilization is rigorous with use of some toxic coupling reagents, and the enzyme might lose its activity during the immobilized process (Zhao et al., 2015). For the previous research, Mendes et al. compared the immobilizations of *Penicillium camembertii* lipase by different methods. They found that covalent immobilization on epoxy-SiO_2-PVA in organic medium seemed to be the most promising method for immobilization of lipase (Mendes et al., 2011).

2.3.1.3 Cross linking

Immobilization of lipase by cross-linking is the process to immobilize the enzyme by the formation of intermolecular cross-linkages. It can be achieved by the addition bi- or multi- functional cross-linking reagents such as glutaraldehyde (Ozturk, 2001). This immobilization technique usually involves joining enzymes to each other to form a three dimensional structure. Lipase can be directly immobilized from fermentation broth and recovered as cross-linked enzyme aggregates (CLEAs). The formed CLEAs have significantly high stability in aqueous solutions within a broad range of pH and temperature values (Lai et al., 2012). This immobilization method is very simple and inexpensive. In particular, there are no costs associated with a solid support. The application of this method for the production of biodiesel has been studied by You et al.. Lipase from *Burkholderia cepacia* was immobilized on modified attapulgite by cross-link reaction for biodiesel production with jatropha oil as feed stock. This immobilized lipase seemed to be the promising biocatalyst for biodiesel production and conversion of 92% has been obtained (You et al., 2013). However, the disadvantages of this method are usually performed under relatively harsh conditions such as using cross-linking reagents that can change the conformation of lipases and potentially lead to significant loss of activity. Other disadvantages associated with cross-linking immobilization are low immobilization yields and absence of desirable mechanical properties (Zhao et al., 2015).

2.3.1.4 Entrapment

Entrapment immobilization is the capture of enzymes within a polymeric network or microcapsules of polymers that allows the substrate and products to pass through but retains the enzyme (Brena et al., 2013). After entrapment, lipase proteins are not attached to the polymeric matrix or capsule, but their diffusion is constrained. Compared with physically adsorbed lipases, entrapment-mobilized lipases are more stable. Entrapment immobilization is relatively simpler to perform than covalent bonding while the activity of lipases is maintained. The use of these methods is limited by mass transfer limitations through membranes or gels. When entrapped lipases are used for biodiesel production,

the conversion rate is relatively low. In addition, the entrapped lipases also show relatively low stability. The recent work by Jegannathan et al. showed that *Burkholderia cepacia* lipase can be encapsulated into κ-carrageenan. The encapsulated lipase retained 72.3% of its original activity after 6 cycles of hydrolysis of *p*-NPP (Jegannathan et al., 2009).

CHAPTER III MATERIALS AND METHODS

3.1 Lipase sources

The two natural sources of microorganisms in this study were the microorganisms screening from the Chao Phraya River and the bacteria from the hot springs of Chaesorn National Park Wildlife and Plant Conservation Department, Lampang, Thailand. The other two samples of microorganisms were the recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* and recombinant *Pichia pastoris* containing lipase gene of *Fusarium solani* kindly provided from Biofuels by Biocatalysts Research unit, Faculty of Science, Chulalongkorn University, Thailand.

3.2 Equipments

Apollo Silica 5U column	(Shimadzu, Japan)
Autoclave	(Ta Chang Medical instrument, Taiwan)
Balance	(Satorius, Germany)
Desiccator	(Sigma-Aldrich, USA)
Digital balance	(Mettler Toledo, USA)
Evaporative Light Scattering Detector	(Shimadzu, Japan)
High Performance Liquid Chromatography (HPLC)	(Shimadzu, Japan)
Incubator shaker	(Kuhner shaker, Switzerland)
Laminar flow	(Thermo electron corporation, USA)
Lyophilizer	(Labconco, USA)
Lyophilize chamber	(Labconco, USA)
Magnetic bar	(Lio Lab Limited Partnership)
Magnetic stirrer	(KIKA Labortechnik, Malaysia)
Microcentrifuge	(HettichZentrifugen, Germany)
Microplate reader spectrophotometer	(AnthosZenyth200, USA)
pH meter	(Model 250, Dever Instrument)
Refrigerated centrifuge	(Heto, Denmark)

Refrigerated incubator shaker	(New Brunswick Scientific Co., Ltd, China)
UV-VIS spectrophotometer	(Thermo scientific, UK)
Vacuum pump	(Scientific industries, USA)
Whatman No.1	(Whatman, UK)
Water bath	(T.S. Instrument, Thailand)

3.3 Chemicals

Amberlite XAD7HP	(Sigma, USA)
Biotin	(Sigma, USA)
Bovine serum albumin (BSA)	(Merck, Germany)
Bradford's reagent	(Biorad, USA)
Dextrose	(Himedia, India)
Dipotassium hydrogen phosphate	(Scharlau, Spain)
Eicosane	(Aldrich, Gernany)
Ethanol	(Lab scan, Thailand)
Formic acid	(Lab scan, Thailand)
Glacial acetic acid	(Lab scan, Thailand)
Glycerol	(Sigma, USA)
Hexane	(Lab scan, Thailand)
Isopropanol	(Lab scan, Thailand)
Methanol	(Lab scan, Thailand)
Palm oil	(Morakot industry, Thailand)
Peptone	(Himedia, India)
p–nitrophenyl laurate	(Sigma, USA)
p–nitrophenol	(Fluka, Switzerland)
Potassium dihydrogen phosphate	(Merck, Germany)
Sodium acetate anhydrous	(Sigma, USA)
Sodium carbonate	(Merck, Germany)
Yeast extract	(Sigma, USA)
Yeast Nitrogen Base (YNB)	(Difco, USA)

3.4 Data analysis

Graph analysis program (Graph Pad Prism 6)

HPLC data analysis program (LC solution software)

3.5 Research methodology

3.5.1 Selection of potential lipolytic microorganisms

The potential lipolytic microorganism was selected by comparison of the highest lipase activity.

3.5.1.1 Natural microorganisms

3.5.1.1.1 Chao Phraya River

3.5.1.1.1.1 Bacteria isolation

The water sample from Chao Phraya River was serial diluted according to standard techniques. It was spreaded on nutrient agar (NA) for bacteria isolation and then incubated at 37°C for 16-18 hours. A colony was picked and then streaked on NA and incubated at 37°C for 16-18 hours to obtain a single colony. Preliminary screening of lipolytic bacteria was carried out on NA supplemented with 0.001% (w/v) rhodamine B and 1% (w/v) palm oil (Kouker and Jaeger, 1987). A single colony of lipolytic bacteria were inoculated into 5 ml of nutrient broth (NB) and incubated at 37°C, 250 rpm for 16-18 hours. Then, 0.5 ml of culture was transferred into 50 ml of liquid production media that containing 0.4% glucose, 0.6% $NH_4(SO_4)_2$, 0.1% KH_2PO_4, 0.05% $MgSO_4$ and 3% palm oil and later grown at 37°C, 250 rpm for 24 hours. Then, the cultured was sampled to preliminarily measure lipase activity. The supernatant were collected by centrifugation at 10,000g, 4°C for 5 minutes to determine the lipase activity by lipase activity assay as described in section 3.5.6.1 and the protein content was determined by Bradford method as described in section 3.5.6.2.

3.5.1.1.1.2 Yeast isolation

The water sample from Chao Phraya River was serial diluted according to standard techniques. It was spreaded on yeast peptone dextrose agar (YPD) for yeast

isolation and then incubated at 30°C for 48 hours. A colony was picked and then streaked on YPD agar and incubated at 30°C for 48 hours to obtain a single colony. Preliminary screening of lipolytic yeast was carried out on YPD agar supplemented with 0.001% (w/v) rhodamine B and 1% (w/v) palm oil (Kouker and Jaeger, 1987). A single colony of lipolytic yeast were inoculated into 5 ml of YPD and incubated at 30°C, 250 rpm for 48 hours. Then, 0.5 ml of culture was transferred into 50 ml of liquid production media that containing 0.4% glucose, 0.6% $NH_4(SO_4)_2$, 0.1% KH_2PO_4, 0.05% $MgSO_4$ and 3% palm oil and later grown at 30°C, 250 rpm for 72 hours. Then, the cultured was sampled to preliminarily measure lipase activity. The supernatant were collected by centrifugation at 10,000g, 4°C for 5 minutes to determine the lipase activity by lipase activity assay as described in section 3.5.6.1 and the protein content was determined by Bradford method as described in section 3.5.6.2.

3.5.1.1.2 Hot springs at Chaesorn National Park Wildlife and Plant Conservation Department

The sample of bacteria from the hot springs of Chaesorn National Park Wildlife and Plant Conservation Department, Lampang, Thailand was kindly provided from Biofuels by Biocatalysts Research unit as culture stock and maintained in glycerol at -80°C. It was initially streaked on nutrient agar (NA) and incubated at 37°C for 16-18 hours. A single colony was inoculated into 5 ml of nutrient broth (NB) and incubated at 37°C, 250 rpm for 16-18 hours. Then, 0.5 ml of culture was transferred into 50 ml of liquid production media that contained 0.4% glucose, 0.6% $NH_4(SO_4)_2$, 0.1% KH_2PO_4, 0.05% $MgSO_4$ and 3% palm oil and later grown at 37°C, 250 rpm. After that, the culture was sampled at 0, 2, 4, 6, 8, 10, 12, 14, 18, 24, 36, 48 and 60 hours to determine the growth rate. The supernatants were collected at intervals by centrifugation at 10,000g 4°C for 5 minute to determine the lipase production by lipase activity assay as described in section 3.5.6.1 and the protein content were determined by Bradford method as described in section 3.5.6.2.

3.5.1.2 Recombinant *Pichia pastoris*

The samples of *P. pastoris* containing lipase gene inserted in pPICZαA vector were kindly provided from Biofuels by Biocatalysts Research unit as culture stock and maintained in glycerol at -80°C. They were initially streaked on YPD agar and incubated at 30°C for 72 hours. A single colony was precultured in 3 ml of YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) at 30°C with shaking at 250 rpm for 24 hours. The culture was transferred into 25 ml BMGY (Buffered Glycerol-complex Medium) (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6, 1.34% (w/v) YNB, 4 x 10^{-5}% (w/v) biotin, 1% (v/v) glycerol) at OD_{600} of 0.05-0.08 and incubated at 30°C with shaking at 250 rpm for 16-18 hours. Then, the cell pellets were collected by centrifugation at 5,700 rpm for 3 minute at room temperature and resuspended in 50 ml of BMMY (Buffered Methanol-complex Medium) (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6, 1.34% (w/v) YNB, 4 x 10^{-5}% (w/v) biotin, 0.5% (v/v) methanol) at OD_{600} of 1. They were then incubated at 30°C with shaking at 250 rpm for 7 days. Methanol was added to a final concentration of 2% (v/v) to optimize the expression every 24 hours for 7 days. The cultures were sampled everyday to determine the growth rate. The supernatants were collected at intervals by centrifugation at 10,000g 4°C for 5 minute to determine the lipase production by lipase activity assay as described in section 3.5.6.1 and the protein content were determined by Bradford method as described in section 3.5.6.2.

3.5.2 Optimization of the lipase gene expression in *Pichia pastoris*

The important factors that affect the expression of lipase gene in *P. pastoris* are cultured time and methanol concentration.

3.5.2.1 Effect of time and methanol concentration

The recombinant *P. pastoris* was initially streaked on YPD agar and incubated at 30°C for 72 hours. A single colony was precultured in 3 ml of YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) at 30°C with shaking at 250 rpm for 24 hours. The culture was transferred into 25 ml BMGY (Buffered Glycerol-complex Medium) (1%

(w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6, 1.34% (w/v) YNB, 4 x 10^{-5}% (w/v) biotin, 1% (v/v) glycerol) at OD_{600} of 0.05-0.08 and incubated at 30°C with shaking at 250 rpm for 16-18 hours. Then, the cell pellets were collected by centrifugation at 5,700 rpm for 3 minute at room temperature and resuspended in 50 ml of BMMY (Buffered Methanol-complex Medium) (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6, 1.34% (w/v) YNB, 4 x 10-5% (w/v) biotin, 0.5% (v/v) methanol) at OD_{600} of 1. They were then incubated at 30°C with shaking at 250 rpm for 5 days. Methanol was added to a final concentration of 0.5, 1, 2 and 3% (v/v) to optimize the expression every 24 hours for 7 days. The culture was sampled everyday to determine the growth rate. The supernatants were collected at intervals by centrifugation at 10,000g 4°C for 5 minutes to determine the lipase activity as described in section 3.5.6.1 and the protein content were determined by Bradford method as described in section 3.5.6.2.

3.5.3 Preparation of rAPL

The rAPL solution was prepared with the optimal condition obtained as described in section 3.5.2.1. The supernatant was frozen at -80°C and concentrated by lyophilization for 24 hours. The solution was centrifuged at 3000 rpm, 4°C for 3 minutes. After centrifugation, solution was collected and the protein content was determined by 3.5.6.2.

3.5.4 Immobilization of rAPL

3.5.4.1 Preparation of supports

The supports, Amberlite XAD7HP, were prepared by suspending 1 g of support powder in 3 ml methanol. The suspension was kept stirred at 350 rpm at room temperature. After 30 minutes, methanol was removed from the reaction. The supports were then washed 3 times with 20 mM phosphate buffer, pH 6 and kept stirred at 350 rpm at room temperature for 30 minutes. After that, the supports were dried in desiccator before used for the immobilization.

3.5.4.2 Immobilization of enzyme

The rAPL solution was prepared as described in 3.5.3. The supernatant with protein content 9 mg was brought in contact with 1 g of support and stirred at 350 rpm for 4 hours. The solution was separated from pellets and washed with 3 ml of 20 mM phosphate buffer, pH 6 for 5 minutes until no enzyme was detected. Then, the immobilized lipases were dried in desiccators at room temperature. 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 filtrates and washing solutions after immobilization. The Immobilized rAPL was assayed for activity as described in section 3.5.6.1.

3.5.5 Optimization of lipase 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 and temperature.

3.5.5.1 Effect of pH

The effect of pH on the immobilization of rAPL was studied at different pH values of buffer ranging from 4 and 9. The stock of buffer solution was prepared as 1 M buffer solution. All these buffer solutions were diluted to 20 mM before adjusting the protein content of rAPL solution for immobilization. The immobilized rAPL activity was assayed by the method described in section 3.5.6.1. The result was expressed as the immobilization efficiency.

3.5.5.2 Effect of ionic strength

After the optimal pH was obtained, the following concentrations of buffer were prepared: 10, 20, 50, 100, 250 and 500 mM from the stock solution to adjust the protein content of rAPL solution for immobilization. The immobilized rAPL activity was assayed by the method described in section 3.5.6.1. The result was expressed as the immobilization efficiency.

3.5.5.3 Effect of protein loading

The effect of protein loading on the immobilization lipase was performed by adjusting protein loading values from 3, 6, 9, 15, 21 and 27 mg. The amount of proteins in lipase solution was prepared in the proper buffer solution from section 3.5.5.1 and the ionic strength from section 3.5.5.2. The immobilized rAPL activity was assayed by the method described in section 3.5.6.1. The result was expressed as immobilization efficiency.

3.5.5.4 Effect of time and temperature

After the optimal pH, ionic strength and enzyme loading were obtained as described in section 3.5.5.1-3.5.5.3, 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 6 hours at various temperatures namely, 10, 20, 25, 30, 40, 50 and 60°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 3.5.6.1. The result was expressed as immobilization efficiency.

3.5.6 Immobilization efficiency

3.5.6.1 Lipase activity assay

Activities of the free and immobilized rAPL were assayed using 25 mM of *p*-nitrophenyl laurate (*p*-NPL) in ethanol as substrate. The reaction was started by adding the 30 µl of substrate into the prepared 470 µl of immobilized lipase or 125 µl of free lipase in the presence of 50 mM phosphate buffer, pH 7 and mixed for 5 minutes at 37°C. Then, 500 µl of 0.25 M Na_2CO_3 was added followed by centrifugation at 14,000 rpm for 5 minutes to terminate the reaction. The increase in the absorbance at 410 nm produced by the release of *p*-nitrophenol in the enzymatic hydrolysis of *p*-NPL was spectrophotometrically

measured. One unit (U) of lipase activity was defined as the amount of lipase that can convert 1 µmol/min of *p*-NPL to *p*-nitrophenol under the mentioned conditions (Marini et al., 2012). The calculation was shown in Appendix D.

3.5.6.2 Protein determination

The protein content was determined by Bradford method. The reaction mixture of 5 µl of sample with 300 µl of Bradford reagent in 96 well plates was incubated at room temperature for 5 minutes and later measured for the absorbance at 595 nm. Standard curve was prepared to determine concentration of protein using bovine serum albumin (BSA) at the concentration of 0.1-0.6 mg/ml (Bradford, 1976). The calculation was shown in Appendix C.

3.5.7 Transesterification catalyzed by free and immobilized rAPL

After the optimal conditions for immobilization of rAPL were obtained by the method described in section 3.5.5, the capability to catalyze transesterification of free and the immobilized rAPL was determined. Transesterification reactions were carried out in 20 ml screw-capped vials containing 0.5 g of palm oil and 20% (w/w of oil) of the immobilized rAPL or equal activity of free rAPL and later added with 3: 1 mole ratio of methanol using 4 steps addition mode of methanol at 0, 2, 4 and 6 hours. The reaction mixture was magnetically stirred in water bath at 40°C for 12 hours. After the reaction complete, the samples were taken and analyzed by HPLC as described in section 3.5.9.

3.5.8 Stability of immobilized rAPL

3.5.8.1 Thermal stability

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

3.5.8.2 Repeated use of the immobilized rAPL for transesterification

The reusability of immobilized rAPL was measured by repeated use in transesterification reaction under the conditions as follows: 0.5 g of palm oil, 20% (w/w of oil) of immobilized rAPL, 1: 3 oil: methanol ratio and the mixture was magnetically stirred in a water bath for 12 hours. After the reaction complete, the enzyme was dried in desiccator at room temperature and later used in the next batch with the fresh substrates. The residual activity was shown as relative conversion. The conversion obtained in the first batch was set at 100.

3.5.9 Analysis of the FAME

The samples were taken from the reaction mixture for biodiesel content analysis using HPLC. The biodiesel samples were quantified by centrifuge at 10,000 rpm for 15 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 was described in Appendix E (Winayanuwattikun et al., 2008).

CHAPTER IV RESULTS

4.1 Selection of potential lipolytic microorganisms

The selection of potential lipolytic microorganisms from various sources for the immobilization was studied.

4.1.1 Natural microorganisms

The lipolytic microorganisms from the natural sources were screened, namely the Chao Phraya River that is near Bangchak Petroleum Public Company Limited and from the hot springs at Chaesorn National Park Wildlife and Plant Conservation Department, Lampang, Thailand.

4.1.1.1 Chao Phraya River

A total of 12 microorganisms, including 7 bacteria and 5 yeasts were isolated from the water sample in Chao Phraya River using the method as described in 3.5.1.1.1. Then, They were subsequently screened for potential lipase production in solid medium plates containing palm oil with rhodamine B as indicator. A total of three isolates, including 2 bacterial and 1 yeast isolates showed hydrolysis on the palm oil plates as shown in Figure 4-1. From the results, it indicates the presence of lipase acting on long chain triglyceride.

In order to select the potential lipase producer, the selected 3 isolates were cultured in liquid medium by using palm oil as lipase producer as described in 3.5.1.1.1. After centrifugation of the culture media, lipase activity in the supernatants was determined by using *p*-nitrophenyl laurate as substrate and the liberated *p*-nitrophenol was measured at 410 nm as shown in Figure 4-2.

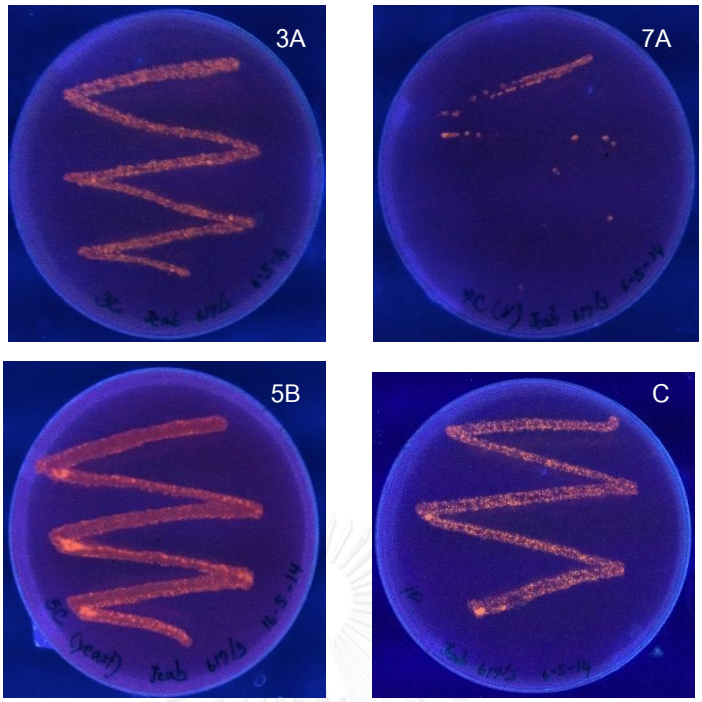


Figure 4-1 Potential lipolytic microorganisms. (A) bacteria, (B) yeast and (C) positive control, yeast; *Candida rugosa* grown on agar containing rhodamine B and palm oil.

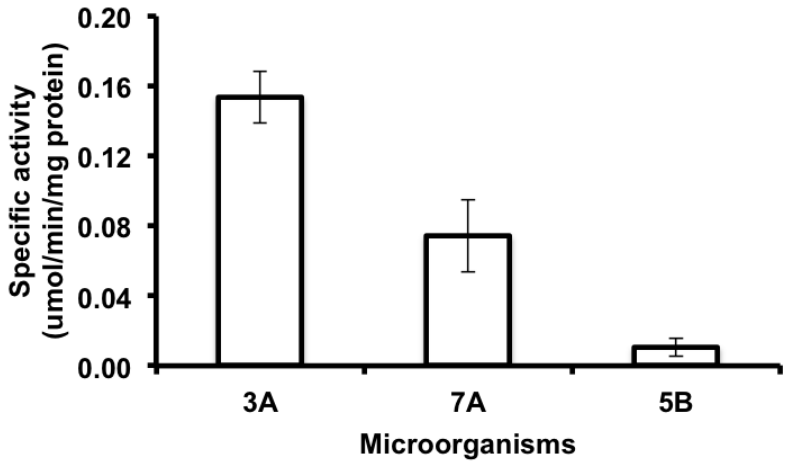


Figure 4-2 Specific activities of lipases from potential lipolytic microorganisms. (A) bacteria, (B) yeast were cultured in lipase producing medium for 24 hours and 72 hours, respectively. Specific activities shown on the y-axis are the means ± SD of three individual experiments.

From the graph, lipolytic bacterium namely, 3A and 7A were cultured in lipase producing medium containing palm oil as inducer at 37°C, 250 rpm for 24 hours. Then, the specific activities were measured and they were 0.15 ± 0.01 and 0.07 ± 0.02 μmol/min/mg protein, respectively. For lipolytic yeast namely 5B was cultured at 30°C, 250 rpm for 72 hours. The obtained specific activity was 0.01 μmol/min/mg protein. Unfortunately, the obtained specific activities from all microorganisms were very low. As a consequence, the next experiments were performed to investigate the lipolytic activities of the obtained potential lipolytic microorganisms and then the lipolytic activities were compared to select the best potential lipolytic microorganisms.

4.1.1.2 Hot springs at Chaesorn National Park Wildlife and Plant Conservation Department

The lipolytic bacteria from hot springs at Chaesorn National Park Wildlife and Plant Conservation Department kindly provided from Biofuels by Biocatalysts Research unit, Faculty of Science, Chulalongkorn University. The lipolytic bacteria was cultured in lipase producing media containing palm oil as inducer at 37°C, 250 rpm for 60 hours to optimize conditions for lipase production as described in 3.5.1.1.2. Cell growths and lipolytic activities were determined by monitoring the absorbances at 600 nm and lipase activity assay, respectively. Typical growth and lipase producing curve are shown in Figure 4-3. From the results, it can be seen that the highest lipase production was found at the beginning of the stationary phase. The obtained highest lipase activity was 3.07 ± 0.02 μmol/min/ml at after 18 hours.

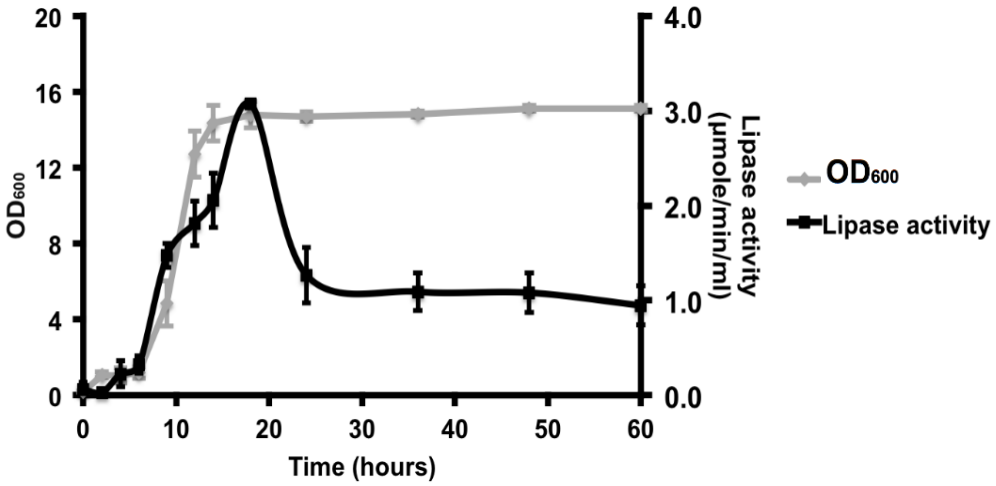


Figure 4-3 Growth curve and lipase activities of bacteria from Chaesorn National Park Wildlife and Plant Conservation Department, Thailand. Lipase activities measured in the growth medium at intervals using *p*-nitrophenyl laurate as substrate. The data shown on the y-axis are the means ± SD of three individual experiments.

4.1.2 Recombinant *Pichia pastoris*

The lipolytic microorganisms namely, recombinant *Pichia pastoris* containing lipase gene of *Fusarium solani* (Wongwatanapaiboon et al., 2016b) and recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* (Wongwatanapaiboon et al., 2016a) kindly provided from Biofuels by Biocatalysts Research unit, Faculty of Science, Chulalongkorn University. They were cultured at 30°C, 250 rpm for 168 hours in BMMY medium by using 2% (v/v) methanol as lipase producer as the method described in 3.5.1.2. The cultures were sampled at intervals to determine the relationship between growth rate and lipase production. The growth rates were obtained by measuring the absorbance at 600 nm and lipase production was determined by lipase activity assay using *p*-nitrophenyl laurate as substrate. Typical growth and lipase producing curves are shown in Figure 4-4 and 4-5. From the results, it can be seen that the highest lipase production from *Pichia pastoris* containing lipase gene of *Fusarium solani* and *Aureobasidium pullulans* were 3.65 ± 0.36 μmol/min/ml at after 96 hours and 16.74 ± 2.97 μmol/min/ml at after 120 hours, respectively.

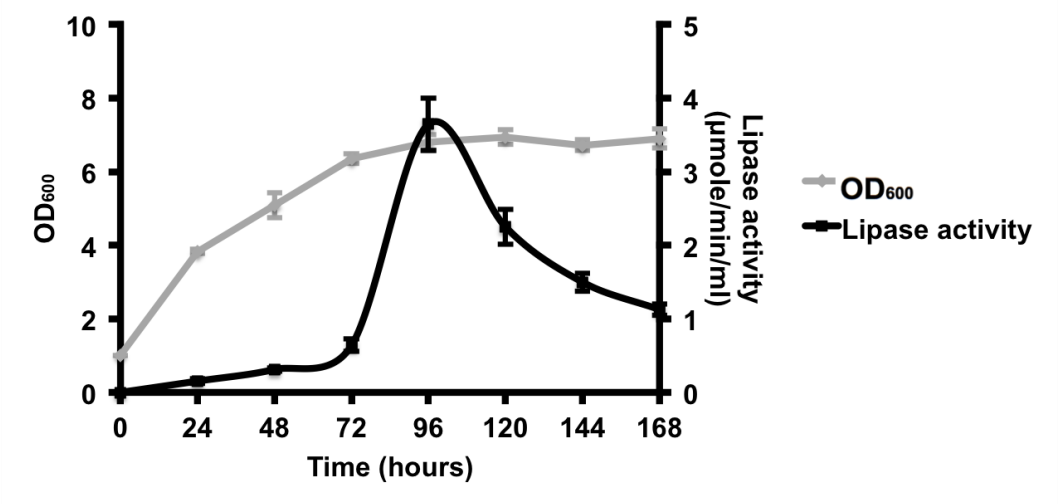


Figure 4-4 Growth curve and lipolytic activity of recombinant *Pichia pastoris* containing lipase gene from *Fusarium solani*. Lipase activities measured in the growth medium at intervals using *p*-nitrophenyl laurate as substrate. The data shown on the y-axis are the means ± SD of three individual experiments.

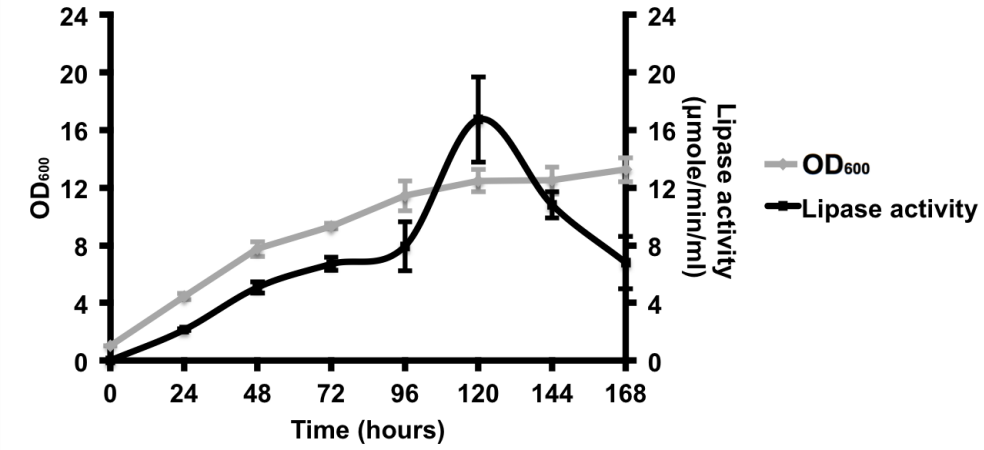


Figure 4-5 Growth curve and lipolytic activity of recombinant *Pichia pastoris* containing lipase gene from *Aureobasidium pullulans*. Lipase activities measured in the growth medium at intervals using *p*-nitrophenyl laurate as substrate. The data shown on the y-axis are the means ± SD of three individual experiments.

Table 4-1 The specific activities of lipase from potential lipolytic microorganisms.

Microorganism	Specific activity (μmol/min/mg protein)
Bacteria from Chaesorn National Park Wildlife and Plant Conservation Department, Thailand	7.29 ± 0.95
Recombinant *Pichia pastoris* containing lipase gene of *Fusarium solani*	13.12 ± 1.50
Recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans*	58.14 ± 5.24

The comparative results of all studied microorganisms as shown in Table 4-1. The protein concentration in the supernatants at optimal cultured time was measured. Then, the specific activities of lipase were calculated. The lipase from recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* exhibited the highest specific activity of lipase, suggesting that this microbial lipase is promising as a potential lipolytic microorganism and was selected for studied in the next experiment.

4.2 Optimization of the lipase gene expression in *Pichia pastoris*

When the best potential lipolytic microorganism, namely recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* was obtained, the optimal cultured conditions were studied to accomplish high lipase production. This recombinant *Pichia pastoris* was cultured in BMMY using methanol as lipase inducer. Therefore, the effect of time and methanol concentration was optimized as described in 3.5.2.

4.2.1 Effect of time and methanol concentration

The recombinant *Pichia pastoris* was cultured in BMMY medium at 30°C, 250 rpm. Methanol was added to a various final concentrations every 24 hours for 7 days. The supernatants were then sampled every day for the assay of lipase activities. The results were revealed as the lipase activity at various times as shown in Figure 4-6.

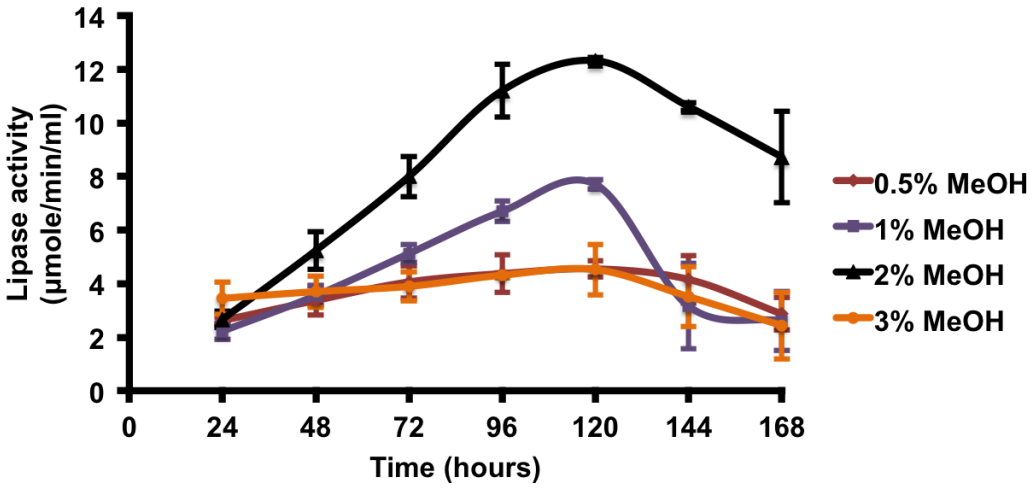


Figure 4-6 The optimal methanol concentration for the induction of lipase production in recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans*. Methanol was added to a final concentration of 0.5, 1, 2 and 3% (v/v) to optimize the expression every 24 hours for 7 days. Lipase activities measured in the growth medium at intervals using *p*-nitrophenyl laurate as substrate. The data shown on the y-axis are the means ± SD of three individual experiments.

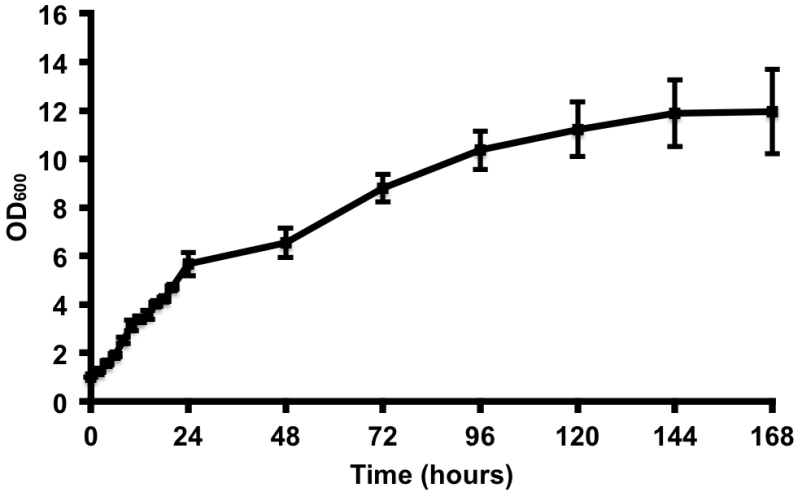


Figure 4-7 Growth curves of recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* in BMMY medium with 2% (v/v) of methanol. The data shown on the y-axis are the means ± SD of three individual experiments.

From the graph, it can be concluded that the highest lipase activity was 12.33 ± 0.11 μmol/min/ml when 2% (v/v) of methanol was added every 24 hours for 5 days after cultured in BMMY medium. From Figure 4-7 also illustrated the growth curve of the recombinants revealing the obtained highest lipase production at the stationary phase approximately 5 days.

4.3 Preparation of rAPL

When the conditions for induction of lipase production from recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* were obtained, the enzyme solution was prepared with the optimal condition as described in 3.5.3. Then, the prepared rAPL solution was immobilized on hydrophobic support, Amberlite XAD7HP by physical adsorption as described in 3.5.4.

4.4 Optimization of lipase immobilization

The optimal conditions for the immobilization of rAPL on Amberlite XAD7HP were studied. To accomplish high activity of the immobilized rAPL depends on many factors, for instance pH, ionic strength, enzyme loading, time and temperature. In this research, the effects of these factors for immobilization were investigated.

4.4.1 Effect of pH

The lipase activities at various pH were studied. The results were shown in Figure 4-8.

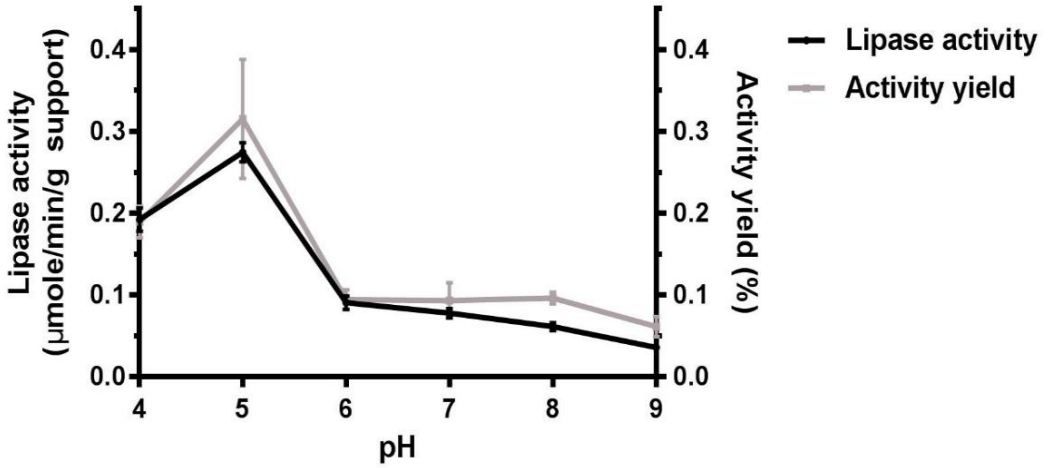


Figure 4-8 The effect of pH on the lipase activity. rAPL solution with 9 mg of protein content containing 20 mM buffer solution at various pH was mixed with 1 g of Amberlite XAD7HP. Then magnetically stirred for 4 hours at 20°C. Activities shown on the y-axis are the means ± SD of three individual experiments.

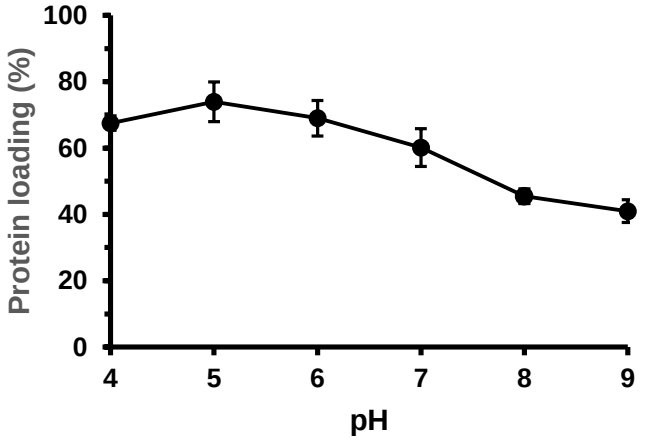


Figure 4-9 The effect of pH on the protein loading. rAPL solution with 9 mg of protein content containing 20 mM buffer solution at various pH was mixed with 1 g of Amberlite XAD7HP. Then magnetically stirred for 4 hours at 20°C. Protein loading shown on the y-axis are the means ± SD of three individual experiments.

From the results, it was shown that when the buffer of the immobilization system was 20 mM acetate buffer, pH 4, the lipase activity was 0.19 ± 0.01 μmol/min/g support and the protein loading was 67%. Then the activity significantly rose to 0.28 ± 0.01 μmol/min/g support and the protein loading increase to 77% at pH 5. Nevertheless, when pH was increased to 6, the lipase activity dramatically decreased by 3 folds to 0.09 ± 0.01 μmol/min/g support then slightly decreased to 0.04 μmol/min/g support at pH 9 while the protein loading continually decreased to 41% at pH 9. Therefore, the optimal pH for rAPL immobilization was 5 and subsequently selected for the next experiment.

4.4.2 Effect of ionic strength

When the optimal pH for rAPL immobilization was obtained at 5, the acetate buffer, pH 5 at various concentrations as described in 3.5.5.2 were used to study the effect of ionic strength on activity of immobilized rAPL. The results were revealed as the lipase activity of immobilized lipase as shown in Figure 4-10.

From the graph, the lipase activity was 0.40 ± 0.04 μmol/min/g support and the protein loading was 78% when 10 mM was used. Then, they significantly rose by 1.5 fold to 0.56 ± 0.01 μmol/min/g support when the concentration was increased to 50 mM and dramatically decreased by 3.5 fold to 0.16 ± 0.04 μmol/min/g support when 250 mM was used. Then slightly decreased to 0.04 ± 0.02 μmol/min/g support at 500 mM. The protein loading slightly rose 81% when the concentration was increased to 50 mM and slightly decreased to 78% when 100 mM was used and stayed unchanged until 500 mM. Therefore, the optimal ionic strength for rAPL immobilization was 50 mM and subsequently selected for the next experiment.

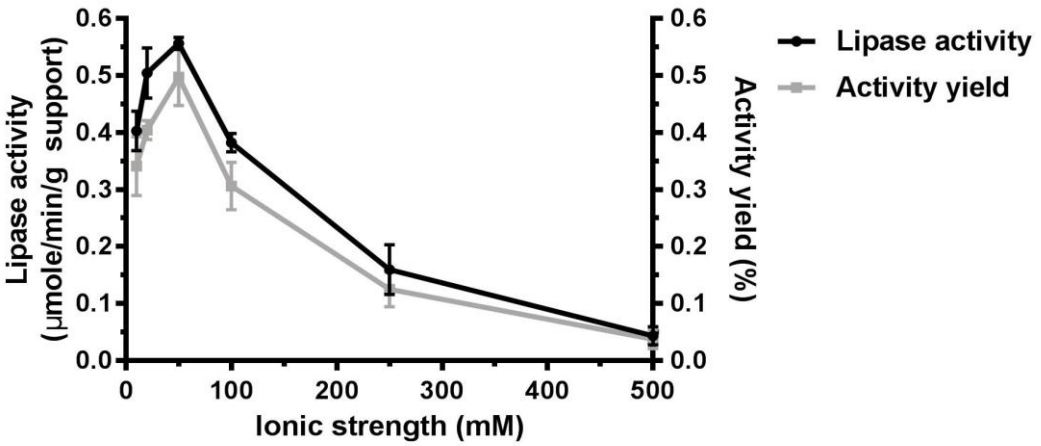


Figure 4-10 The effect of ionic strength on lipase activity. rAPL solution with 9 mg of protein content containing various concentrations of acetate buffer, pH 5 was mixed with 1 g of Amberlite XAD7HP. Then magnetically stirred for 4 hours at 20°C. Activities shown on the y-axis are the means ± SD of three individual experiments.

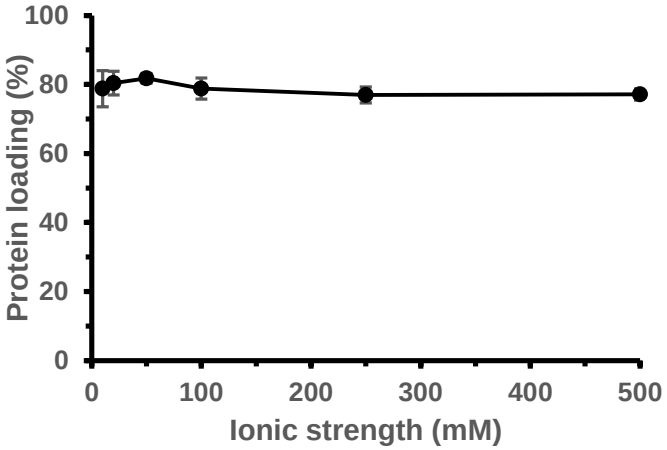


Figure 4-11 The effect of ionic strength on the protein loading. rAPL solution with 9 mg of protein content containing various concentrations of acetate buffer, pH 5 was mixed with 1 g of Amberlite XAD7HP. Then magnetically stirred for 4 hours at 20°C. Protein loading shown on the y-axis are the means ± SD of three individual experiments.

4.4.3 Effect of enzyme loading

When the optimal pH and ionic strength for rAPL immobilization was obtained, the effect of enzyme loading on activity of immobilized rAPL was studied by using various protein contents of rAPL containing 50 mM acetate buffer solution, pH 5 for immobilization as described in 3.5.5.3. The results were revealed as the lipase activity of immobilized lipase as shown in Figure 4-12.

From the graph, when rAPL solution containing 3 mg of protein content was used, the lipase activity was 0.29 ± 0.04 μmol/min/g support and the protein loading was 72%. Then, the activity significantly rose to 0.71 ± 0.02 μmol/min/g support and the protein loading slightly rose to 78% when the protein content was increased to 15 mg. However, when the protein content was increased to 27 mg, the lipase activity only slightly increased to 0.89 ± 0.08 μmol/min/g support while the protein loading slightly decreased to 74%. Therefore, in order to reduce the cost for further applications, the selected optimal enzyme loading for subsequent experiments was 15 mg protein content of rAPL solution.

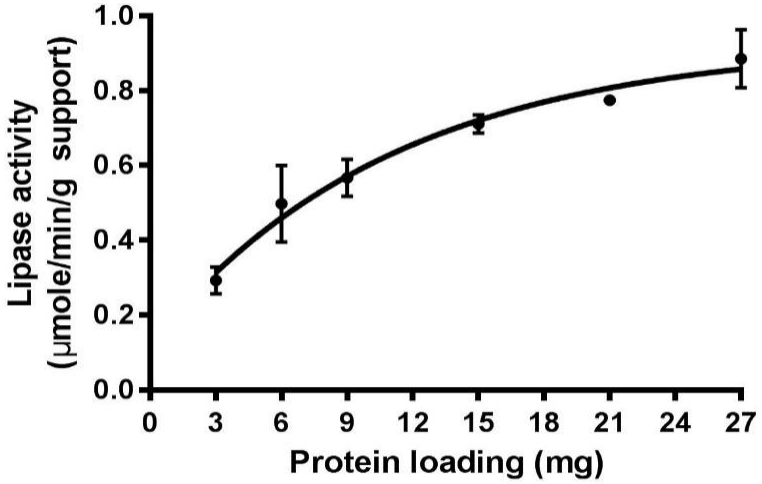


Figure 4-12 The effect of enzyme loading on lipase activity. rAPL solution containing various contents of protein in 50 mM acetate buffer, pH 5 was added to 1 g of Amberlite XAD7HP. Then, magnetically stirred for 4 hours at 20°C. Activities shown on the y-axis are the means ± SD of three individual experiments.

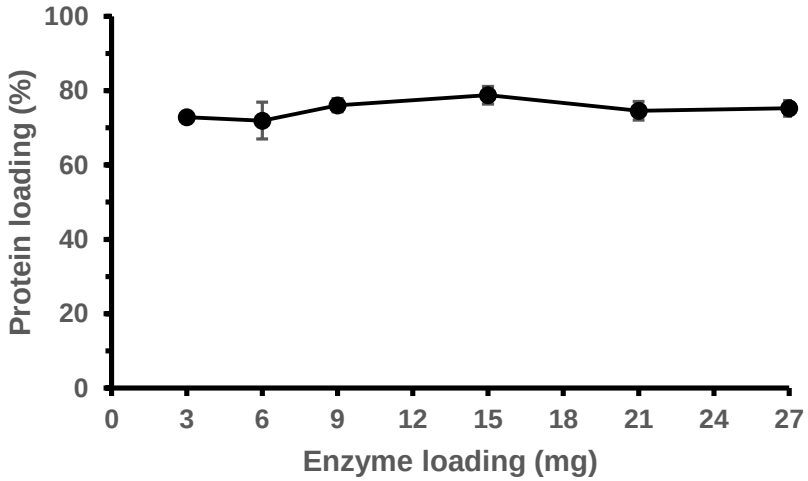


Figure 4-13 The effect of enzyme loading on the protein loading. rAPL solution containing various contents of protein in 50 mM acetate buffer, pH 5 was added to 1 g of Amberlite XAD7HP. Then magnetically stirred for 4 hours at 20°C. Protein loading shown on the y-axis are the means ± SD of three individual experiments.

4.4.4 Effect of time and temperature

4.4.4.1 Effect of immobilization time

When the optimal conditions from the results as described above were obtained and fixed for rAPL immobilization, the effect of immobilization time was investigated. The residual activities of rAPL solution were checked for each time of immobilization at various temperatures as described in 3.5.5.4. The relationship of the residual activity with immobilization time at various temperatures was revealed in Figure 4-14. The results were shown as the percentage of the residual activity at 20°C.

From the graph, when the lipase was incubated at 10 to 40°C, the residual activities of lipase gave rather similar patterns. There were the residual activities of lipase solution decreased to approximately 50-60% in the first 90 minutes. Then, at 10 to 25°C the residual activities of lipase slightly decreased approximately 10% at 150 minutes and stayed unchanged to 360 minutes of incubation. On the contrary, at 30 and 40°C, the residual activities of lipase slightly decreased approximately 10% at 120 minutes and stayed unchanged to 360 minutes of incubation. Nevertheless, for 50 and 60°C, the residual activities of lipase significantly decreased from initial time to 25 and 10%, respectively within the first 120 minutes and stayed unchanged to 360 minutes.

From the results, it revealed that different temperatures gave different optimal incubation times of immobilization. Therefore, the optimal time for rAPL immobilization was 150 and 120 minutes at 10 to 25 and 30 to 60°C, respectively and subsequently selected for the next experiment.

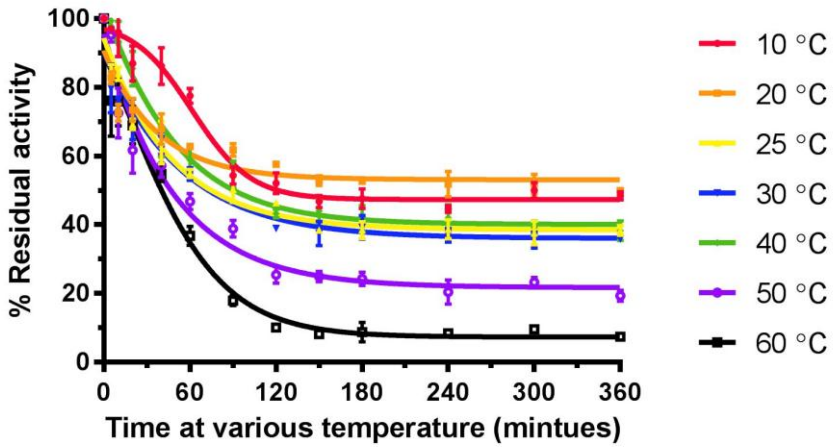


Figure 4-14 The effect of immobilization time on the residual activity of lipase. rAPL solution was incubated at various temperatures. Residual activities shown on the y-axis are the means ± SD of three individual experiments.

4.4.4.2 Effect of temperature

The effect of temperature on lipase activity of immobilized rAPL was studied by checking the activity of immobilized lipase at various temperatures as described in 3.5.5.4. The results were revealed as the lipase activity of immobilized lipase as shown in Figure 4-15.

From the graph, it found that the lipase activity of immobilized rAPL was 0.65 ± 0.08 μmol/min/g support when rAPL was immobilized at 10°C and gradually rose to 1.56 ± 0.10 μmol/min/g support when immobilized temperature was increased to 30°C. Then, the lipase activity significantly rose by 3.6 fold to 5.67 ± 0.67 μmol/min/g support when immobilized temperature was increased to 50°C. However, when immobilized temperature was 60°C, the lipase activity decreased to 4.57 ± 0.83 μmol/min/g support. While the protein loading was 50 % when immobilized temperature was 10°C and gradually rose to 87% when the immobilized temperature was increased to 60°C. Therefore, it was concluded that 50°C was the optimal temperature for rAPL immobilization.

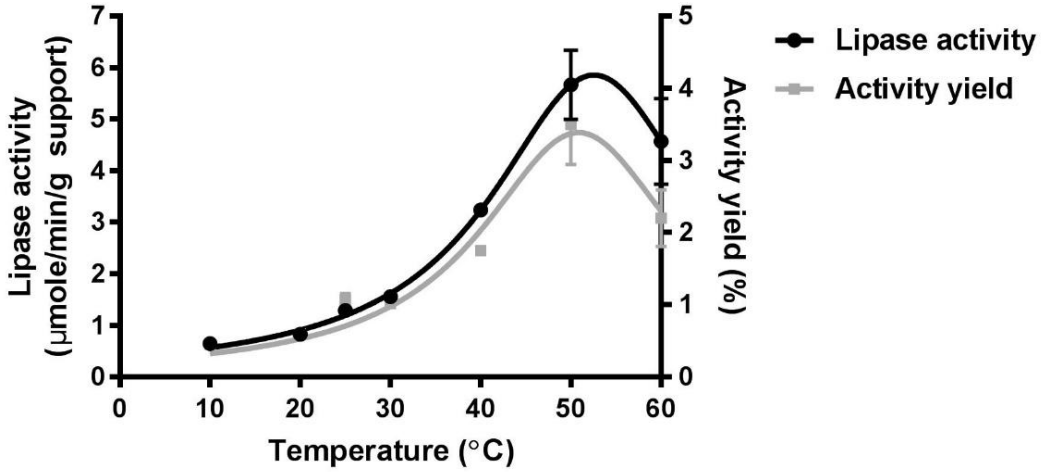


Figure 4-15 The effect of temperature on lipase activity. The rAPL immobilization was performed in 50 mM acetate buffer, pH 5 with rAPL solution containing 15 mg protein content at specific time for each temperature. Activities shown on the y-axis are the means ± SD of three individual experiments.

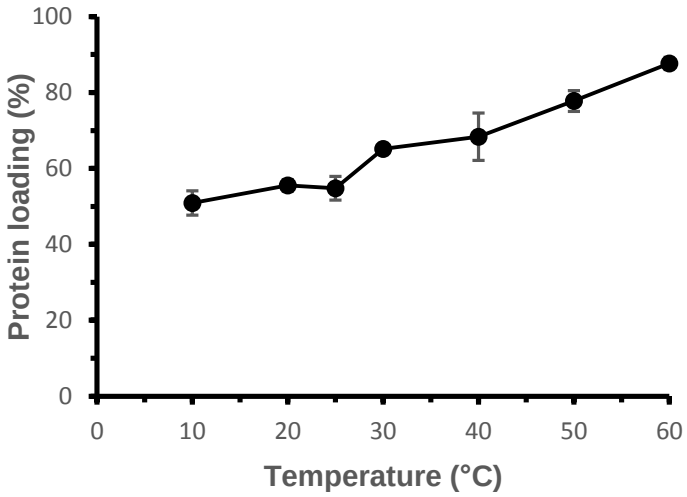


Figure 4-16 The effect of temperature on the protein loading. The rAPL immobilization was performed in 50 mM acetate buffer, pH 5 with rAPL solution containing 15 mg protein content at specific time for each temperature. Protein loading shown on the y-axis are the means ± SD of three individual experiments.

4.5 Transesterification catalyzed by free and immobilized rAPL

After the optimal conditions for immobilization of rAPL were obtained, the capability to catalyze transesterification of free and the immobilized rAPL was determined. The transesterification reactions were carried out in a mixture of 0.5 g of palm oil, 4 step additions mode of methanol, 1: 3 oil: methanol ratio and 20% (w/w of oil) immobilized lipase or equal activity of free rAPL magnetically stirred in a water bath at 40°C for 12 hours. The results revealed that the obtained biodiesel yields from transesterification reactions catalyze by free and immobilized rAPL were 56 and 61%, respectively.

4.6 Stability of immobilized rAPL

4.6.1 Thermal stability for transesterification

The optimal immobilization conditions of 15 mg protein content of rAPL in 50 mM acetate buffer, pH 5 for 120 minutes at 50°C were applied using the method described in 3.5.8.1. Afterwards, the thermal stability of immobilized rAPL for transesterification was studied. Since the optimal temperature for transesterification was found to be 40°C, the experiment was performed by incubating 5 mg of immobilized rAPL at 40°C for 24 hours. The incubated immobilized rAPL was periodically sampled. The percentages of residual activities relative to the untreated control obtained were shown in Figure 4-17.

From the graph, the initial activities was set 100% at 5.78 μmol/min/g support and gradually decreased to approximately 62% within 4 hours and stayed unchanged until 24 hours. The results indicated that immobilization helps preserve the enzyme structure from thermal inactivation.

The half life ($t_{1/2}$) of immobilized rAPL was calculated as shown in Appendix D. The results were revealed as the percentages of relative value of the residual activity and half life time as shown in Figure 4-18. The results shown that half life time of immobilized rAPL at 40°C was 5.78 hours.

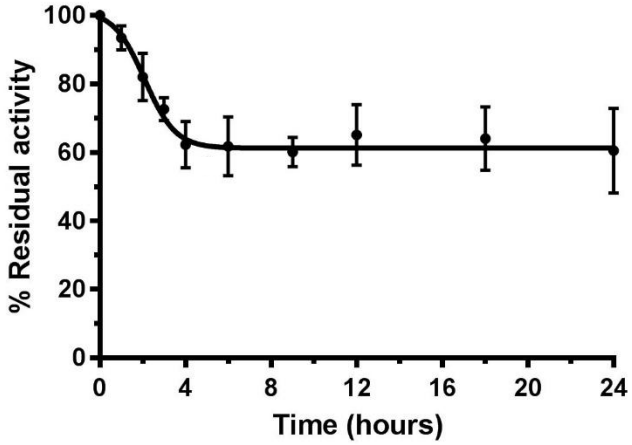


Figure 4-17 The thermal stability of immobilized rAPL. The immobilized rAPL were incubated at 40°C for 1, 2, 3, 4, 5, 6, 9, 12, 18 and 24 hours. The results are the means ± SD of three individual experiments.

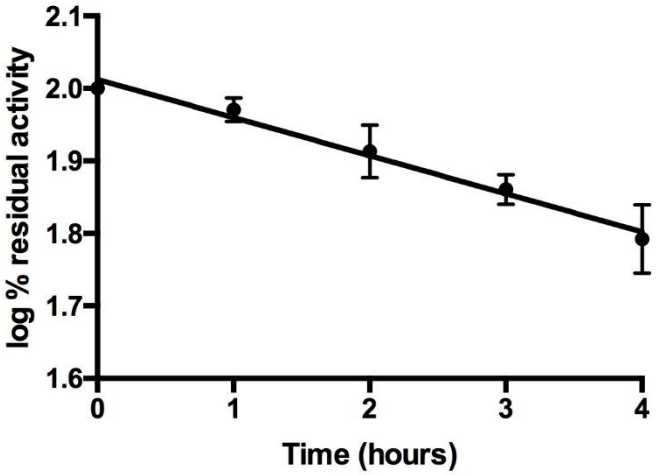


Figure 4-18 Half life time ($t_{1/2}$) of immobilized rAPL. The immobilized rAPL were incubated at 40°C. The results are the means ± SD of three individual experiments.

4.6.2 Repeated use of the immobilized rAPL for transesterification

The reusability of immobilized rAPL was investigated by using the method that described in 3.5.8.2. The results of each batch for the production of fatty acid methyl ester content were shown in Figure 4-19. The residual activity was shown as relative conversion. The conversion obtained in the first batch was set at 100%.

From the results, it was found that the yield of fatty acid methyl ester gradually decreased to 86% in the 2nd cycle (first reuse). Then, it dramatically dropped to 23% in the 3rd cycle and finally decreased to approximately 9% in the 4th cycle.

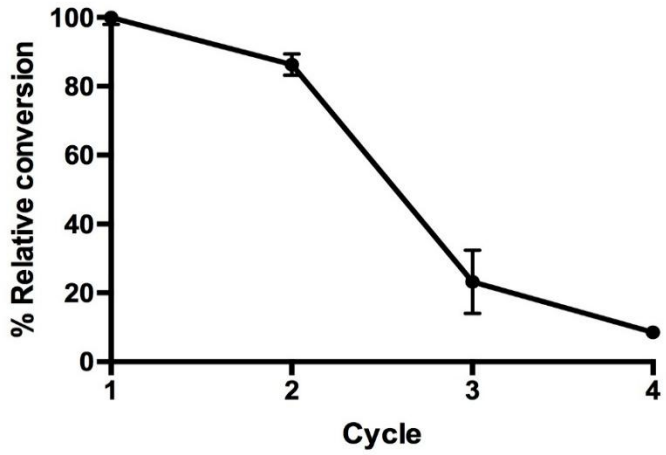


Figure 4-19 Operational stability of immobilized rAPL catalysis for transesterification. The reactions were carried out in a mixture of 0.5 g of palm oil, 4 step additions mode of methanol, 1: 3 oil: methanol ratio and 20% (w/w of oil) immobilized lipase magnetically stirred in a water bath at 40°C for 12 hours. The immobilized rAPL was transferred into the same system for a new cycle after completion of reaction in 12 hours. The results are the means ± SD of three individual experiments.

CHAPTER V DISCUSSION

5.1 Selection of potential lipolytic microorganisms

Lipase (triacylglycerol ester hydrolases) are enzymes widely found in biological systems that catalyze the hydrolysis of triacylglycerol to glycerol and free fatty acids. However, they are able to catalyze synthetic reactions under certain conditions such as acidolysis, alcoholysis, aminolysis, esterification and transesterification (Aarthy et al., 2014). In many industries, lipases are one of the most industrial biocatalysts, especially microbial lipases from their high stability, broad substrate specificity, possible high yields, ease of genetic manipulation and rapid growth of microorganisms on inexpensive media (Hasan et al., 2006). The mode of action for lipases in substrate transesterifications to biodiesel depends on their origin and specific properties. The overall structure of lipases as a structure with the active serine placed in a loop called catalytic elbow. The activation which is often necessary for lipase is the movement of the lid. The structural properties of lipase from different sources might be the reason for showing different activity on different oil substrates. Therefore, the need to optimize the process is based on the selected enzymes and substrates for biodiesel production. Based on their substrate specificity, lipases can be divided into three types: 1,3-specific, fatty acid-specific, and non-specific lipases. 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. Initially, the best potential lipolytic microorganism was selected from comparison of natural microorganisms and recombinants *Pichia pastoris* then the selected microorganism was used for further studies in this work.

5.1.1 Natural microorganisms

Microbial lipases have been studied extensively. Lipase producing microorganisms are widely distributed in nature and diversified in their properties. They 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. From previous reports, lipolytic microorganism namely *Staphylococcus hominis* was isolated from oil contaminated soil at Salem District, Tamil Nadu, India that effectively showed production of lipase (Marimuthu, 2013). Moreover, a thermophilic bacteria of genus *Geobacillus* was isolated from a hot spring located in Gilgit, Northern Areas of Pakistan could produce thermophilic lipase (Tayyab et al., 2011). In this study, microorganisms from water in Chao Phraya River and lipolytic bacteria from hot springs at Chaesorn National Park Wildlife and Plant Conservation Department were investigated for the ability of lipase production.

5.1.1.1 Chao Phraya River

The lipolytic microorganisms were isolated from the expected oil rich water in Chao Phraya River. For the assumption, some isolates might be having the activity towards the target fatty acids. In this work, a Rhodamine B (RhB) plate assay with palm oil was used for the identification of microbial lipases. The interaction of hydrolyzed substrates with RhB resulting in the formation of orange fluorescent halos around microbial colonies visible upon UV irradiation (Olusesan et al., 2009). The advantages of using RhB are insensitivity to pH changes and no inhibition of growth or change its physiological properties (Kouker and Jaeger, 1987). A total of three types of microorganisms including two bacterial and one yeast isolates showed capabilities to a hydrolyze palm oil when cultivated on agar plates as shown in Figure 4-1. The lipase activities of three selected isolates were determined by the hydrolysis of *p*-nitrophenyl laurate and *p*-nitrophenol was measured to monitor the activity as shown in Figure 4-2. Unfortunately, all of microorganisms showed the very low specific activity of lipase probably due to the low accumulation of oil in the sample that ineffectively induce the production of lipase and the different specificity to oil substrate of lipase from each microorganism

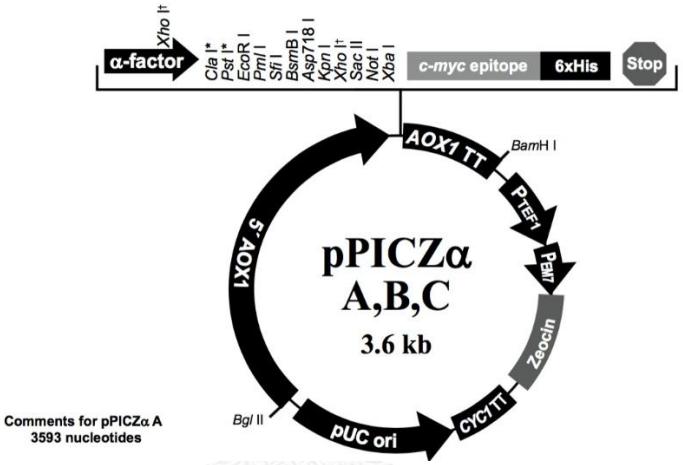
5.1.1.2 Hot springs at Chaesorn National Park Wildlife and Plant Conservation Department

The lipolytic bacteria from hot springs were optimized for lipase production at 37°C, 250 rpm for 60 hours. The growth curves were obtained by measuring the absorbance at 600 nm and lipase production was determined by lipase activity assay as shown in Figure 4-3. It can be seen that the growth pattern was still in lag phase for the first 6 hours. Then, the growth was immediately increased into logarithmic phase within 15 hours and afterward remained constant at stationary phase. From the result, the obtained highest lipase activity was 3.07 ± 0.02 μmol/min/ml at the beginning of the stationary phase after 18 hours.

5.1.2 Recombinant *Pichia pastoris*

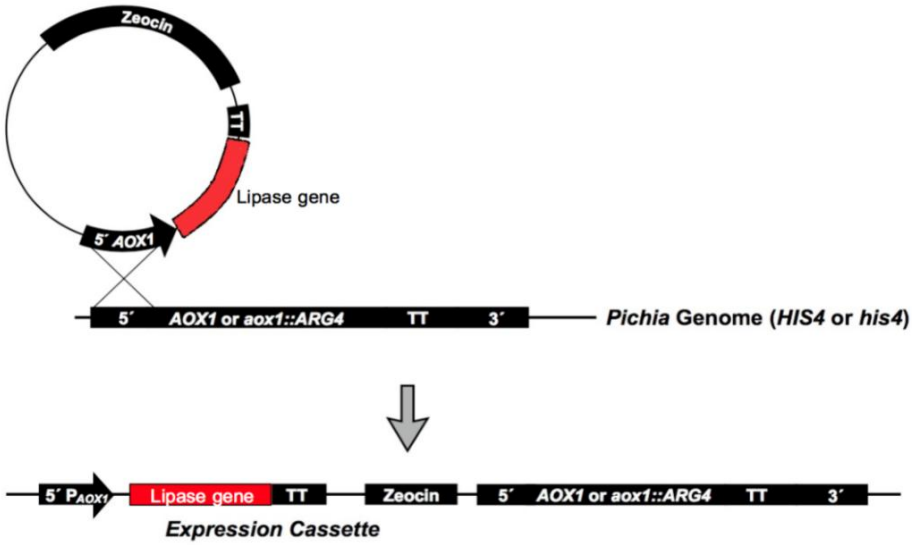
The microbial lipases are much more suitable for industrial application. However, there are many drawback of working with native microorganisms. Any biocatalytic application using a crude lipase preparation bears the risk of irreproducible results and unwanted side effects caused by unrelated enzymes. Thus, the recombinant DNA technology was used to produce recombinant lipases on a host microorganisms. The two general categories of expression systems are prokaryotic as *Escherichia coli* and eukaryotic systems as *Saccharomyces cerevisiae* and *Pichia pastoris* (Valero, 2012). Normally, yeasts are greatly acceptable for the pharmaceutical proteins production. In contrast, *E. coli* has toxic cell wall pyrogens and mammalians cells may contain oncogenic or viral DNA (Rai and Padh, 2001). Historically, *Saccharomyces cerevisiae* has been used as a host of recombinant protein production. Nevertheless, it was found to have limitations: low product yields, poor plasmid stability, difficulties in scaling-up production, hyperglycosylation, and low secretion capacities while *Pichia pastoris* combines the abilities of growing on minimal medium at very high cell densities (higher than 100 gDCW/L) and of secreting the recombinant proteins to the media. In addition *Pichia pastoris* secretes only few own proteins to the extracellular medium and no extracellular enzyme with lipase or esterase activity (Mattanovich et al., 2009). Thus, recombinant lipases are not going to contaminate with *Pichia pastoris* lipases. Therefore,

Pichia pastoris has been shown as the most promising host system (Valero, 2012). In this study, *Pichia pastoris* was used as the expression system for recombinant lipases production. The pPICZαA was used as expression vector that contain the methanol-induced alcohol oxidase (AOX1) promoter for regulates the expression of lipase gene and contain the α-factor secretion signal for secretion of recombinant protein. Moreover, it contains a zeocin resistance gene for recombinant *Pichia pastoris* selection as shown in Scheme 1 (Weidner et al., 2010).



Scheme 1 The features of the pPICZαA expression vector (Invitrogen, 2010)

From the Scheme 2, the lipase gene was ligated into multiple cloning sites of pPICZαA vector and then the recombinant vector was transformed into *Pichia pastoris*. The recombinant vector was integrated with *Pichia pastoris* genome via homologous recombination between the transforming DNA and regions of homology within the genome (Invitrogen, 2010).



Scheme 2 The recombination and integration of lipase gene in *Pichia pastoris* (Invitrogen, 2010)

The *Pichia pastoris* expression system uses the methanol-induced alcohol oxidase (AOX1) promoter, which control the gene that codes for the expression of alcohol oxidase. The alcohol oxidase is the enzyme that catalyzes the first step in the methanol metabolism. Thus, the methanol was used as carbon source and inducer of recombinant lipase production in this system (Cregg et al., 2000). The capability for lipase production of two interested recombinant *Pichia pastoris*, the recombinant *Pichia pastoris* containing lipase gene of *Fusarium solani* and the recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* were determined. They were cultured at 30°C, 250 rpm for 168 hours in BMMY (Buffered Methanol-complex Medium) by using 2% (v/v) methanol as lipase producer. The growth curves were obtained by measuring the absorbance at 600 nm and lipase production was determined by lipase activity assay as shown in Figure 4-4 and 4-5. It can be seen that the growth curve of recombinant *Pichia pastoris* containing lipase gene of *Fusarium solani* was still in logarithmic phase by 96 hours and afterward remained constant at stationary phase. From the result, the obtained highest lipase activity was 3.65 ± 0.36 μmol/min/ml at the beginning of the stationary phase after 96 hours. In part of the recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans*, the growth curve was still logarithmic phase until 120 hours and afterward remained constant at stationary phase. From the result, the obtained highest

lipase activity was 16.74 ± 2.97 μmol/min/ml at the beginning of the stationary phase after 120 hours.

The potential lipolytic microorganism was selected by comparison of lipase specific activity from three microorganism namely, the bacteria from Chaesorn National Park Wildlife and Plant Conservation Department, Thailand, the recombinant *Pichia pastoris* containing lipase gene of *Fusarium solani* and the recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* as shown in Table 4-1. The results showed that the lipase from recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* exhibited the highest specific activity suggesting that this microbial lipase is promising as a potential lipolytic microorganism and was selected for the next experiment namely, optimization of the lipase gene expression.

5.2 Optimization of the lipase gene expression

The optimization of lipase gene expression in the recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* was studied. The optimal culture conditions was determined of high lipase production. The effects of lipase gene expression, time and methanol concentration were investigated.

5.2.1 Effect of time and methanol concentration

The optimal time and methanol concentration for lipase production were investigated with various culture conditions. The recombinant *Pichia pastoris* was cultured in BMMY at 30°C, 250 rpm. Methanol was added to a various final concentrations (0.5 to 3% (v/v)) every 24 hours for 7 days and the lipase activities were measured at various times as show in Figure 4-6. From the results, the lipase activity increased when methanol concentration increased from 0.5 to 2% (v/v) for 5 days after methanol addition, the highest lipase activity was 12.33 ± 0.11 μmol/min/ml when 2% (v/v) of methanol was added every 24 hours for 5 days at the beginning of stationary phase (Figure 4-7). However, when 3% (v/v) of methanol was added, the lipase activity was found to be lower than at 1 and 2% (v/v) of methanol. This could be explained that higher methanol concentration can decrease the induction of lipase gene expression. Although higher

methanol concentration can induce the high expression of lipase gene but too high methanol concentration denatures lipase and also leads to cell lysis (Jahic et al., 2003, Trentmann et al., 2004). After 6 and 7 days, the lipase activities were decreased when methanol was added at all concentrations since methanol as inducer of the lipase gene expression is also a substrate with high oxygen demand for anabolic and catabolic purposes. Therefore, the unlimited methanol supply can lead to sudden oxygen depletion that negatively affects the expression of lipase gene (Khatri and Hoffmann, 2006). In addition, the AOX gene in *Pichia pastoris* was induced by methanol for alcohol oxidase production. This enzyme catalyze the oxidation of methanol in methanol metabolism using molecular O_2 as an electron acceptor to yield formaldehyde and H_2O_2 (Scheme 3) that is highly toxic to the living cells (Yurimoto et al., 2002).

$$CH_3OH + O_2 \xrightarrow{\text{alcohol oxidase}} HCHO + H_2O_2$$

Scheme 3 Oxidation reaction of methanol catalyzed by alcohol oxidase

From the results, the optimal conditions for the expression of lipase gene in recombinant *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* were the addition of 2% (v/v) of methanol every 24 hours for 5 days after cultured in BMMY. These optimal conditions were used to prepare the recombinant *Aureobasirium pullulans* lipase (rAPL) in later experiments.

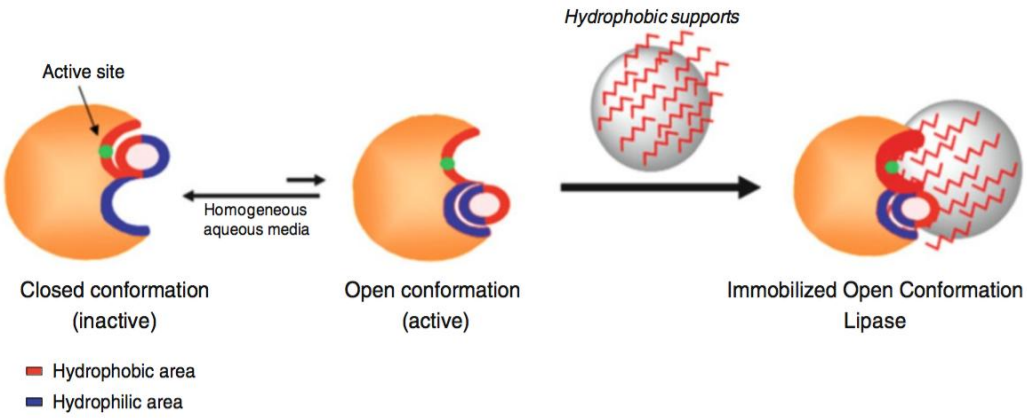
5.3 Preparation of rAPL

After the optimal conditions for lipase gene expression were obtained, the rAPL solution was prepared. The *Pichia pastoris* containing lipase gene of *Aureobasidium pullulans* was cultured in BMMY for 5 days and 2% (v/v) of methanol were added for every 24 hours. Then, the supernatant was frozen at -80°C and concentrated by lyophilization for 24 hours. The solution was centrifuged and the rAPL solution was collected. In this experiment, the lyophilization was performed to increase protein concentration. Therefore, the opportunity of interaction between rAPL and supports was increased. In lyophilization

process, the lipase stability was increased in dry state. Moreover, the water was removed without excessive heating. To avoid the lipase denaturation (Jadhav and Moon, 2015).

5.4 Immobilization of rAPL

The structure of lipase has a central L-sheet with active site consisting of serine on a nucleophilic elbow placed in a groove that is covered by a peptide lid. Lipases exist in certain equilibrium between two different structural forms namely, closed conformation (inactive) and open conformation (active). For the closed conformation, a polypeptide chain (lid or flat) isolates the active site from the medium and another open conformation where this lid moves and the active site is fully exposed to the reaction medium. In the homogeneous aqueous media, this equilibrium is mainly shifted to the closed conformation. However, this equilibrium is shifted to the open conformation in the presence of hydrophobic surfaces where the lipase becomes adsorbed by the large hydrophobic pocket around their active site and the internal face of the lid following a mechanism in Scheme 4. Moreover, lipases can be adsorbed to other hydrophobic surfaces droplets of oils, hydrophobic proteins, and on the surface of hydrophobic supports. The immobilization of lipases by their interfacial activation on hydrophobic supports permit to fix the open conformation on a solid phase representing suitable and simple method (Palomo et al., 2013).



Scheme 4 Immobilization of lipase on hydrophobic surfaces

Notably, any support can be applied for enzyme adsorption. However, not every enzyme can be immobilized on all support. For the successful adsorption of the enzyme to occur, the optimal conditions must be met. The characteristics of immobilized enzyme preparations are governed by the properties of both the enzyme and the support (Guldhe et al., 2015). In general, various types of the supports have been used for the lipase immobilization by adsorption such as silica (Wu et al., 2012, Yu et al., 2013), chitosan (Nasratun et al., 2010, Xie and Wang, 2012), celite (Brem et al., 2012) and agarose (Mendes et al., 2012). A wide range of available compounds can be successfully used as the enzyme carriers. The criteria of the choice suitable for a given immobilized enzyme including the cost, availability, stability in specific conditions and physicochemical properties of the support such as the surface area, particle size, pore size, porosity and type of functional groups present on the surface (Jesionowski et al., 2014). Typically, porous support is advantageous since lipase can be adsorbed at both the outer surfaces and within the pores. The support 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 support (Zhao et al., 2015). In general, porous support can be classified according to their pore sizes in which correlations between pore characteristics and protein loading: microporous support (less than 2 nm), mesoporous support (2-50 nm), macroporous support (50-200 nm) and gigaporous support (more than 200 nm) (Li et al., 2010). In 2013, the effect of pore size on the performance of immobilized enzymes was studies. From the results, concluded that increase of support pore diameter will increase the degree of immobilization (Bayne et al., 2013). For the surface area of support, there is probably significant contribution of the microporous regions and the external surface area of the support is also accessible to the enzyme molecules. The support can affect the partitioning of products, substrates, and water in the reaction mixture and thereby can influence the catalytic properties of the enzyme (Palomo et al., 2013). From previous research of Biofuels by Biocatalysts Research Unit, the immobilized commercial *Candida rugosa* lipase (CRL) was studied to catalyze biodiesel production using palm oil as feedstock. The CRL was immobilized on seven types of commercial hydrophobic

supports with pore diameters of around 10-60 nm. The results indicated that dramatic differences existed in the activity of lipases on different supports (Winayanuwattikun et al., 2011).

In this research, Amberlite XAD7HP was selected as the supporting materials for immobilization of rAPL. They are highly porous macroreticular aliphatic crosslinked 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 lipase. From the literature, immobilized lipase from commercial *Candida rugosa* by adsorption on hydrophobic support, Amberlite XAD7HP demonstrated that adsorption on Amberlite XAD7HP significantly stabilizes lipase. The stability of immobilized lipase was tested, 5 mg of immobilized CRL was incubated at 40°C for 24 hours. The results showed that thermal stability of lipase was enhanced by the immobilization. Moreover, using this kind of support presents an additional advantage that is the possibility of reuse of the support due to the reversible adsorption of the enzyme on the support. In this study, the used conditions for initial immobilization followed the previous studies of Wasinee Thaipanich about the optimal conditions for immobilization of CRL (Thaipanich, 2014).

5.5 Optimization of immobilization

Previous studies have shown that many factors such as properties of enzyme, type of support, enzyme to support ratio and immobilization conditions have significant influences on the immobilization efficiency. In this research, Amberlite XAD7HP was used as the supporting materials for immobilization of rAPL. The optimal conditions for the immobilization were studied. The effect of following factors were investigated namely, pH, ionic strength, enzyme loading, time and temperature.

5.5.1 Effect of pH

The optimal pH for lipase immobilization varies with the enzyme species. The pH of buffer at which the adsorption is conducted is important since the electrostatic forces are crucial for immobilization. Typically, the maximum adsorption is observed at pH values

close to the isoelectric point (pI) of the enzyme (Zhao et al., 2015). Changes in pH over the isoelectric point of the enzyme will have a large impact on the enzyme binding constant. The protonation and deprotonation of the altered functional groups are dependent upon pH of the solution (Lei et al., 2009). From the results, the effect of pH on lipase activity of immobilized rAPL and percentage of protein loading were shown in Figure 4-8 and Figure 4-9. It could be seen that the shape of the graph is bell-shaped curve. The lipase activity and the protein loading increased with the increment of pH values and the maximal values were obtained at pH 5. From the Protein Isoelectric Point database (PIP-DB), there have not been the previous studies reporting about isoelectric point of lipase from *Aureobasidium pullulans*. However, the theoretical pI can be obtained from compute pI tool (ExPASy Bioinformatics Resource Portal) by calculation of amino acid sequences. In 2016, Wongwatanapaiboon et al studied characterization of *Aureobasidium melanogenum* lipase in *Pichia pastoris*, the results showed the amino acid residues and can be calculated to obtain the theoretical pI value that is 5.24 (Wongwatanapaiboon et al., 2016a). These results indicated that at pH 5, overall net charge of rAPL is close to 0 causing the maximal adsorption since the lipase can be easily adsorbed to the nonionic or hydrophobic support by hydrophobic interaction. When the pH value was below or above the isoelectric point, it was not expected to be an effective combination of the lipase and support. Moreover APL is widely stable in various pH values between 4 to 9 (Liu et al., 2008). However, the lipase activity and the protein loading started to decrease when the pH subsequently rose to 9. At higher or alkali pH, the lipase denaturation tends to be increasing like the other enzyme.

5.5.2 Effect of ionic strength

The effect of ionic strength on activity of immobilized rAPL and protein loading were shown in Figure 4-10 and Figure 4-11. Various concentrations of acetate buffer at pH 5 were tested from 10 to 500 mM. From the results, it was found that the lipase activity significantly rose in the presence of 10 mM acetate buffer, pH 5 and kept rising until the highest activity was reached in the presence of 50 mM. This means that 50 mM of buffer effectively represents the suitable concentration for rAPL to attach on Amberlite XAD7HP.

At low ionic strength, 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 to 250 mM, lipase activities declined dramatically by 3.5 folds and then slightly decreased when ionic strength was 500 mM. This was consistent to the previous studies that adsorption of lipases was decreased when the ionic strength increased (Fernandez-Lafuente et al., 1998). There have not been reporting about the immobilization of APL. However, the study of Biofuels by Biocatalysts Research Unit in 2014, Wasinee Thaipanich studied the effect of ionic strength on activity of immobilized CRL by adsorption on the same support, Amberlite XAD7HP. The result indicated that also at low ionic strength (20 mM phosphate buffer at pH 6) gave highest lipase activity (Thaipanich, 2014). The similar optimal ionic strength obtained for CRL and rAPL immobilized on Amberlite XAD7HP possibly resulted from the same support which the immobilized efficiency of enzyme also depend on the support properties such as hydrophobic surfaces area, adsorption capacity, porosity and pore size. In addition, at low ionic strength condition, the equilibrium between closed and open structures of lipase may be shifted towards the open structure higher than the closed structure. In this condition, high rate of lipase adsorption and strong adsorption were found (Fernandez-Lafuente et al., 1998).

5.5.3 Effect of enzyme loading

The amount of enzyme loaded on the surface of support has a large effect on the performance of biocatalytic surface. In this study, various amounts of rAPL were immobilized on the support by varying the protein loading of rAPL solution from 3 to 27 mg. The effect of enzyme loading on activity of immobilized rAPL and percentage of protein loading were shown in Figure 4-12 and Figure 4-13. The results showed that the activity of immobilized rAPL increased with an increase in protein loading of rAPL solution. This indicated that there were enough spaces in the support for rAPL molecules to be accommodated. Lipase activity reached the maximum when the protein loading was 27 mg. However, the increment of lipase activity tends to decrease when the protein loading increases from 15 to 27 mg. Therefore, in order to reduce the cost for further applications,

15 mg was the optimum protein loading of rAPL solution for immobilization. In addition, the increment of lipase activity tends to decrease at high protein loading probably due to excessive adsorption of rAPL. It could be explained that binding sites on the surface areas of the support are limited and close to support saturation (Ramos et al., 2015). Moreover, the enzyme molecules also need enough space for catalyzing the reaction of the substrate and the multilayer adsorption might have occurred resulting in possible inhibition of access to enzyme active sites (Yücel, 2012). Similar results have been reported by Egwim et al. They found that the activity of the immobilized enzyme increased as more lipase was loaded onto the support until it reaches a particular threshold (Egwim et al., 2012). Therefore, 15 mg protein of rAPL solution was selected as the optimal enzyme loading for subsequent experiment.

5.5.4 Effect of immobilization time and temperature

5.5.4.1 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 surface of support. 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 60°C was shown in Figure 4-14. When lipase was incubated at 10 to 40°C, the residual activities of lipase solution decreased approximately 40-50% in the first 90 minutes indicating that rAPL was adsorbed on the surfaces and distributed throughout the pores of the support. Then, the residual activities gradually decreased approximately 10% for the next 60 and 30 minutes at 10 to 25°C and 30 to 40°C, respectively. These results showed that the adsorption rate slowed down probably due to the aggregation of rAPL molecules at the surface and pore mouth of the support in the earlier stage of adsorption. After that residual activity stayed unchanged until 360 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 50 and 60°C, the residual activities of lipase gave rather similar patterns. The residual activities of lipase dramatically decreased in the first 120 minutes and stayed unchanged to 360 minutes. It could be explained that the adsorption of rAPL occurred rapidly and maximal loading was reached at 150 minutes.

From these results, it could be seen that higher temperature usually provides shorter time for the reaction to completion than low temperature. However, the enzyme denaturation causing the loss of activity is inevitable. Therefore, the activities of the immobilized lipase at various temperatures were further studied. As a consequence, the optimal immobilization time has to compromise between the shortest time that provides the bonded protein on the support and enzyme activity. Hence, the decreasing lipase activity in solution indicated that the reacted amount of enzyme to carrier with equilibrium were different at specific time of each temperature.

5.5.4.2 Effect of temperature

The optimal temperature for rAPL immobilization was determined by measuring the activity of immobilized lipase and the protein loading at various temperatures from 10 to 60°C. The results were shown in Figure 4-15. From the results, the lipase activity of immobilized rAPL gradually increased with the increment of temperature at 10 and 20°C indicating that the catalytic site of enzyme is not highly flexible leading to a decrease in lipase activity and the enzyme molecule slowly are transferred to the support. After that, the lipase activity dramatically increased when the temperature increased and reached the maximum at 50°C may due to at high temperature this enzyme can keep their original structure and the lipase molecule in the bulk phase can be rapidly transported to the surface of support. Moreover, the functional groups of amino acids at the catalytic site show higher flexibility than low temperature. However, when the immobilization was conducted at 60°C, lipase activity dropped while protein loading rose because some lipase molecules started to denature from heat inactivation before immobilization. Thus, 50°C was suitable for enzyme immobilization in this study.

From the optimal immobilization time obtained in section 4.4.4.1, the activity of immobilized lipase was found to be steady at 50°C for 120 minutes of incubation period. Therefore, the optimal immobilization time and temperature for subsequent experiment were 2 hours at 50°C.

5.6 Transesterification catalyzed by free and immobilized rAPL

After the optimal conditions for immobilization of rAPL were obtained, the transesterification catalyzed by free and immobilized rAPL was investigated using palm oil as the substrate for biodiesel production. The reactions were conducted as described in section 3.5.7. The optimal conditions for transesterification followed the previous studies of Wasinee Thaipanich (Thaipanich, 2014). The yields were quantitated by HPLC and approximately 56 and 61% were obtained from using free and immobilized rAPL catalyst, respectively. From the results, the immobilized rAPL showed better catalytic ability probably due to the immobilization which could approve thermal stability of enzyme. In biodiesel production reaction performed at high temperature (40°C), free rAPL may start to denature while immobilized rAPL has more rigid in term of conformation (Sun et al., 2010). In addition, there are many factors that affect transesterification reaction such as addition mode of methanol, mole ratio of oil to methanol, enzyme loading, water content, reaction time and reaction temperature. The higher yield of biodiesel may obtain through optimization of the conditions for transesterification catalyzed by immobilized rAPL.

5.7 Stability of immobilized lipase

5.7.1 Thermal stability for transesterification

The resistance of immobilized lipase to temperature is an important potential advantage for practical applications of this enzyme. Since the temperature for transesterification was 40°C, this experiment was performed by incubating 5 mg of immobilized rAPL at 40°C for 24 hours. Afterwards, the incubated immobilized rAPL were periodically sampled at intervals. The percentages of residual activities relative to the untreated control obtained were shown in Figure 4-17. The results revealed that the percentages of residual activity at 40°C gradually decreased from 100 to 93% within 1

hour after that continually decreased to 62% within 4 hours and stayed unchanged until 24 hours. According to Liu et al, they reported that the residual activity of free rAPL at 40°C rapidly decreased to approximately 70% within 1 hour (Liu et al., 2008). 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 rAPL was investigated, the results were expressed as the percentages of relative of the residual activity and half life time as shown in Figure 4-18. It was shown that the half life of the immobilized rAPL at 40°C was 5.78 hours whereas the soluble free rAPL was approximately 1.95 hours (Liu et al., 2008). Evidently, the immobilization on Amberlite XAD7HP has considerably increased the thermal stability of lipase approximately 3 folds higher than the free lipase. This perhaps due to lipase located inside the macroporous pores which offer a good protection against alterations. In addition, the previous studies of Živkovic et al about the immobilization inside the narrow pores of support is expected to improve thermal stability of lipase (Izrael Živkovic et al., 2015).

5.7.2 Repeated use of the immobilized rAPL 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 rAPL in transesterification as it can deactivate enzymes, particularly in 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, transesterification were conducted to obtain the production of biodiesel and repeated every 12 hours. Approximately 61% of FAME content was obtained. From Wasinee Thaipanich concluded that unwashed immobilized CRL represents the advantage that it can be reused for transesterification (Thaipanich, 2014). Therefore, after completion of each cycle of the reaction for 12 hours, the immobilized rAPL 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 4-19. 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, it was found that the yields of FAMEs gradually decreased to 86% in the 2nd cycle (first reuse). These results suggested that immobilized rAPL on Amberlite XAD7HP could be reactivated and reused even after losing some of its activity. However, the yield of FAMEs continually dropped to 23% in the 3rd cycle. It could be explained that leaching of lipase from supporting surface was the main problem in the reaction process. Then, the yields of FAMEs decreased to approximately 9% of its initial activity in the 4th 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 (Winayanuwattikun et al., 2014).

CONCLUSION

In this research, the recombinant *Pichia pastoris* with the expression of lipase gene from *Aureobasidium pullulans* was selected as the potential lipolytic microorganisms from two natural sources and two recombinant lipases. The overexpression of the rAPL gene was optimized by the addition of 2% methanol every 24 hours for 5 days. The immobilization of this rAPL on Amberlite XAD7HP was successful by physical adsorption. The optimal conditions for the immobilization were obtained at pH 5, 50 mM ionic strength, 15 mg protein of rAPL at 50°C for 2 hours. When the transesterifications catalyzed by free and immobilized rAPL were carried out for the production of biodiesel, the %yield of 56 and 61 were achieved, respectively under the conditions of four steps addition mode of methanol, 1 to 3 mole ratio of oil:methanol, 20% (w/w) of oil for immobilized rAPL loading or equal activity of free rAPL at 40°C for 12 hours. The obtained immobilized rAPL was found to be stable at 40°C and could be reused 2 times with the half life of approximately 6 hours. Nevertheless, the higher yield of biodiesel may be obtain by optimization of the conditions for transesterification catalyzed by immobilized rAPL.

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APPENDIX

APPENDIX A

Properties of Amberlite XAD7HP

Table A-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:	≤ 2.0
Fines content:	< 0.300 mm: 7.0% max.
Coarse beads:	> 1.180 mm: 8.0% max.
Porosity[2]:	≥0.5 ml/ml
Surface area[2]:	≥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 Nutrient Broth (NB)

Beef extract	3 g
Peptone	5 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 Nutrient Agar (NA)

Beef extract	3 g
Peptone	5 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 Yeast Peptone Dextrose (YPD)

Yeast extract	10 g
Peptone	20 g
Dextrose	20 g

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

1.4 Yeast Peptone Dextrose Agar (YPD Agar)

Yeast extract	10 g
Peptone	20 g
Dextrose	20 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.

1.5 Lipase production agar with Rhodamine B

Bacto-tryptone	10 g
Yeast extract	3 g
Beef extract	5 g
NaCl	5 g
KH_2PO_4	7 g
Palm oil	1% (v/v)
Rhodamine B	0.001% (w/v)
Agar	20 g

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

1.6 Lipase production media (for bacteria)

Bacto-tryptone	10 g
Yeast extract	3 g
Beef extract	5 g
NaCl	5 g
KH_2PO_4	7 g
Palm oil	1% (v/v)

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

1.7 Lipase production media (for yeast)

Yeast extract	3 g
$MgSO_4$	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.

1.8 Stock solution for BMGY and BMMY

1.8.1 1.4x YP

Yeast extract	14.3 g
Peptone	28.6 g

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

1.8.2 1 M Na-K Phosphate Buffer pH 6

KH_2PO_4	59 g
K_2HPO_4	11.5 g

All components were dissolved in 450 ml distilled water and adjusted to pH 6 ± 0.1 with KOH or H_3PO_4. The solution was adjusted to 500 ml with distilled water and then steriled at 121°C, 15 lb/in^2 for 15 min.

1.8.3 10x YNB (13.4% Yeast Nitrogen Base without amino acid)

YNB	33.5 g

YNB was dissolved in 250 ml distilled water and then steriled by filter (0.2 μm).

1.8.4 500x Biotin (0.02% biotin)

Biotin	10 mg

Biotin was dissolved in 50 ml distilled water and then steriled by filter (0.2 μm).

1.8.5 10x Glycerol (10% glycerol)

Glycerol	25 ml

Glycerol was mixed with 225 ml distilled water and then steriled at 121°C, 15 lb/in^2 for 15 min.

1.8.6 10x Methanol (5% Methanol)

MeOH	5 ml

Methanol was mixed with 95 ml distilled water then steriled by filter (0.2 μm)

1.9 Buffered Glycerol-complex Medium (BMGY)

1.4 YP	70 ml
1 M Phosphate Buffer pH 6	10 ml
10x YNB	10 ml
500x Biotin	0.2 ml
10x Glycerol	10 ml

All components were mixed in Laminar Flow Clean Cabinet.

1.10 Buffered Methanol-complex Medium (BMMY)

1.4 YP	70 ml
1 M Phosphate Buffer pH 6	10 ml
10x YNB	10 ml

500x Biotin	0.2 ml
10x Methanol	10 ml

All components were mixed in Laminar Flow Clean Cabinet.

2. Preparation of solutions for hydrolysis assay

2.1 Phosphate buffer solution

1 M Phosphate Buffer pH 7

KH_2PO_4	26.2 g
K_2HPO_4	53.5 g

All components were dissolved in 450 ml distilled water and adjusted to pH 7 with KOH or H_3PO_4. Then, solution was adjusted to 500 ml with distilled water.

2.2 *p*-nitrophenyl laurate solution

p-nitrophenyl laurate	40.2 mg
Absolute ethanol	5 ml

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

APPENDIX C

Protein determination

1. Preparation of solutions for protein assay

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

2. Standard curve of BSA

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

1. Prepare stock bovine serum albumin with concentration at 10 mg/ml.
2. 10 mg/ml BSA was diluted with distilled water as 0.1-0.6 mg/ml (Table C-1).

Table C-1 Composition for standard BSA

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

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

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 microplate reader.

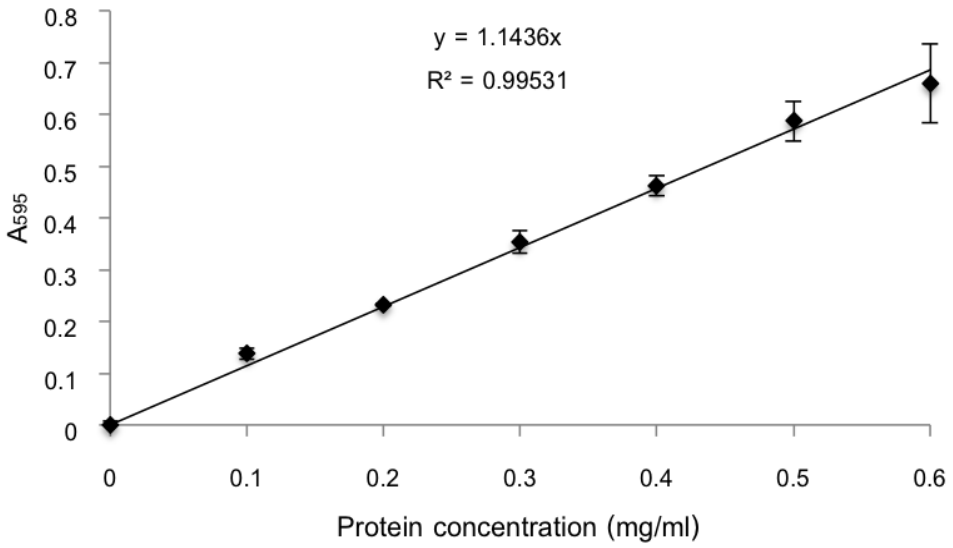


Figure C-1 Calibration curve for protein determination by Bradford's method

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

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

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

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

C_i = Initial protein concentration (mg/ml)

C_f = Protein concentration of filtrate (mg/ml)

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

V_i = Initial volume of enzyme solution (ml)

V_f = Volume of filtrate (ml)

V_w = Volume of washing solution (ml)

m_s = Weight of the support (g)

APPENDIX D

Calculation of the lipase activity

1. Standard curve of *p*-nitrophenol

p-nitrophenol standards should be prepared in the same buffer of the lipase activity assay reaction. The method is as follows:

1. Prepare stock *p*-nitrophenol with concentration at 10 mM.
2. 10 mM *p*-nitrophenol was diluted with buffer as 2 mM and continually diluted as 0.04-0.24 mM (Table D-1).

Table D-1 Composition for standard *p*-nitrophenol

p-nitrophenol (mM)	Reagent volume (μl)	
	2 mM *p*-nitrophenol	Buffer
0	-	1000
0.04	20	980
0.08	40	960
0.12	60	940
0.16	80	920
0.20	100	900
0.24	120	880

3. Pipette 250 μl of each standard into 96 wells microplate, *p*-nitrophenol solutions are normally measured the absorbance at 410 nm in triplicate.

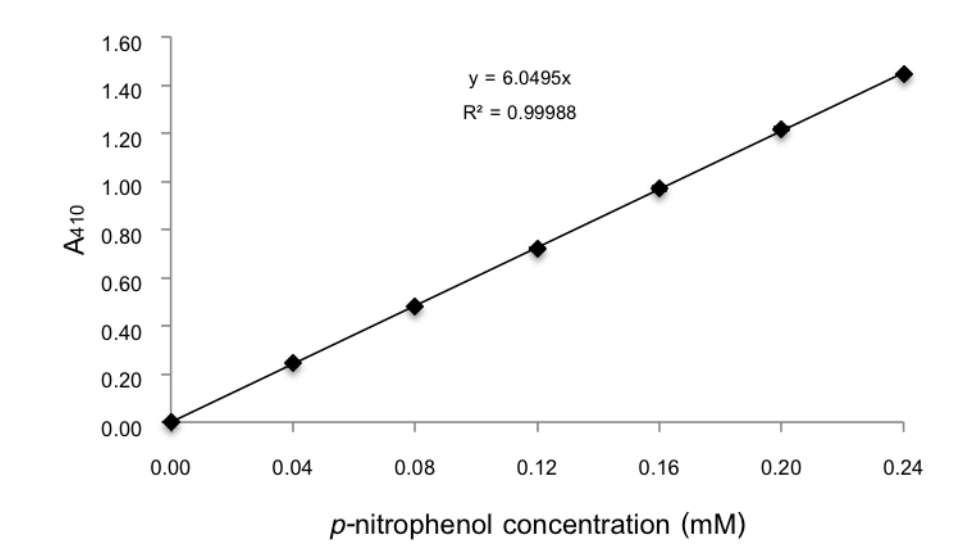


Figure D-1 Calibration curve of *p*-nitrophenol for the lipase activity assay

2. Calculation of lipase activity for free and immobilized enzyme

The absorbance value at 410 nm was calculated by: Y = aX + b

Where X axis = Standard *p*-nitrophenol concentration (mM)

Y axis = Absorbance at 410 nm

One unit (1 U) was defined as that amount of enzyme that liberated 1 μmol of *p*-Nitrophenol per minute under the test conditions.

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

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

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

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

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

3. Calculation of thermal stability

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

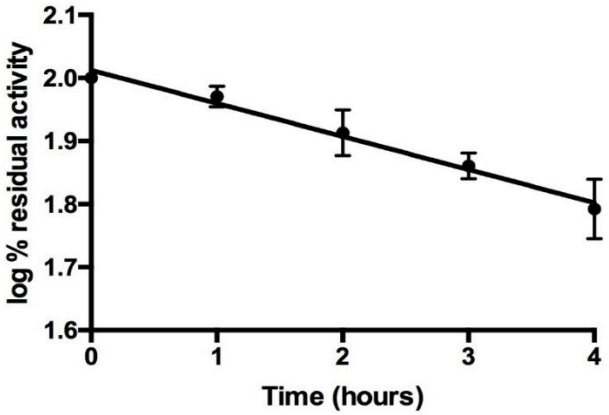


Figure D-2 Half life time ($t_{1/2}$) of immobilized rAPL. 5 mg of immobilized rAPL were incubated at 40°C. The results were average values of triplicate experiments.

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

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

Where A_{in} = the hydrolytic activity at given time

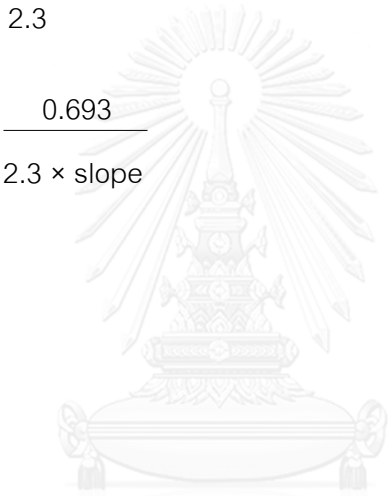
A_{in0} = the initial hydrolytic activity at given time

K_d = thermal deactivation constant

t = the incubation time

Since, slope = $\frac{k_d}{2.3}$

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



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 used.

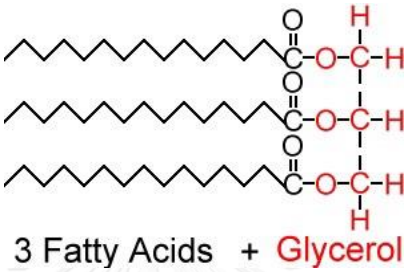


Figure E-1 Molecular structure of triglyceride

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

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

Where, Mw_{TG} = Molecular weight of triglyceride = Molecular weight of palm oil

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

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

Mw_n = Molecular weight of three fatty acid –COOH

= Mw_{FA}- 45 (from main structure of triglyceride) (Figure E-1)

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

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

Molecular weight of palm oil can be calculated as follows;

$$R_{Aver} = \left(\frac{0.59 \times 155}{100}\right) + \left(\frac{0.96 \times 183}{100}\right) + \left(\frac{38.67 \times 211}{100}\right) + \left(\frac{0.11 \times 20}{100}\right) + \left(\frac{3.32 \times 239}{100}\right)$$

$$+ \left(\frac{45.45 \times 237}{100}\right) + \left(\frac{10.87 \times 235}{100}\right) + \left(\frac{0.20 \times 233}{100}\right) + \left(\frac{0.23 \times 267}{100}\right) + \left(\frac{0.02 \times 295}{100}\right)$$

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

$$= 226.823$$

$$Mw_{TG} = (3 \times 226.823) + 173 = 853.469$$

2. Volume of methanol

The stoichiometry of reaction requires 3 mole methanol per mole triglyceride to yield 3 mole fatty acid methyl ester or biodiesel and 1 mole 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-2. The applied volume of methanol was determined by using the molecular weight of palm oil equal to 853.47.

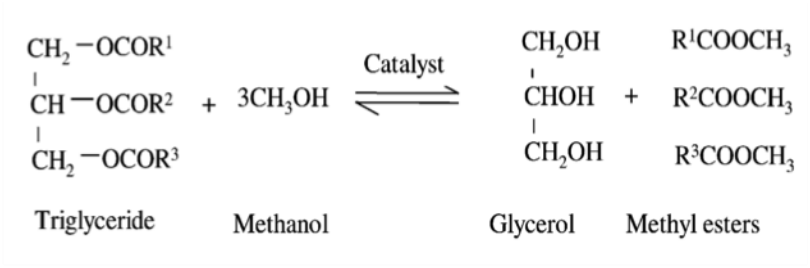


Figure E-2 Transesterification of palm oil and methanol

So, 0.5 g of palm oil = 0.5 / 853.47 ≈ 5.89×10^{-4} mole

Thus, the volume of methanol = $3 \times 5.89 \times 10^{-4}$ = 1.76×10^{-3} mole

Since molecular weight of methanol is equal to 32

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

From D = M/V

D = Density of methanol (0.792 g/ml)

M = Mass of methanol (0.056 g)

V = Volume of methanol (ml) = 0.056 g / 0.792 g/ml = 0.071 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 Rungrawee Impiew was born on November 4, 1990 in Chonburi, Thailand. She graduated with the second class honours in the Bachelor Degree of Science from the Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2013 and furthered her Master Degree of Science in Biochemistry and Molecular Biology at the same department. During her studies, part of her work was presented as poster presentation at Burapha University International Conference 2015 on the topic of “Immobilization of Lipases from Potential Microorganisms on Hydrophobic Amberlite XAD7HP” at The Tide Resort, Bangsaen, Thailand on July 10-12, 2015. She was award with the best paper award for the poster presentation from this conference.