การโคลนยีน การแสดงออก และลักษณะสมบัติของไลเพสจาก *Fusarium solani* สำหรับการผลิตไบโอดีเซล

นาย วีระศักดิ์ ถเกิงการกิจ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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GENE CLONING, EXPRESSION AND CHARACTERIZATION OF LIPASE FROM *Fusarium solani* FOR THE PRODUCTION OF BIODIESEL

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Field of Study	Biotechnology
Thesis Advisor	Associate Professor Tikamporn Yongvanich
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วีระศักดิ์ ถเกิงการกิจ : การโคลนยีน การแสดงออก และลักษณะสมบัติของไลเพสจาก *Fusarium solani* สำหรับการผลิตไบโอดีเซล (GENE CLONING, EXPRESSION AND CHARACTERIZATION OF LIPASE FROM *Fusarium solani* FOR THE PRODUCTION OF BIODIESEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทิฆัมพร ยงวณิชย์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร. ปกรณ์ วินะยานุวัติคุณ, 107 หน้า.

ไบโอดีเซลเป็นผลิตภัณฑ์ที่เกิดจากไตรกลีเซอไรด์ในปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นที่มีกรดหรือเบสเป็นตัวเร่ง อย่างไรก็ตาม ตัวเร่งปฏิกิริยาทางชีวภาพเช่นไลเพสได้รับความสนใจมากขึ้น ด้วยเหตุนี้จึงมีการคัดเลือกไลเพสจากจุลินทรีย์ในธรรมชาติเพื่อนำมาใช้ในการผลิตไบโอดีเซลด้วยปฏิกิริยาดังกล่าว โดยนำพันธุวิศวกรรมมาใช้พัฒนาและเพิ่มปริมาณการผลิตไลเพส งานวิจัยนี้ทำการโคลนยีนไลเพสที่ผลิตได้จาก เชื้อรา *Fusarium solani* เข้าสู่ pPICZαA พบว่ายีนที่ได้มีขนาด 1002 คู่เบส และแปลรหัสเป็นกรดอะมิโนได้ 333 หน่วย จากนั้นถ่ายโอนยีนไลเพสดังกล่าวเพื่อแสดงออกใน *Pichia pastoris* สายพันธุ์ KM71 พบว่า ภาวะที่เหมาะสมในการเหนี่ยวนำการแสดงออกได้แก่การบ่มในเมทานอลปริมาณ 3% ที่อุณหภูมิ 30 องศาเซลเซียส เวลา 5 วัน จากนั้นทำรีคอมบิแนนท์ไลเพสที่ได้ให้บริสุทธิ์เพื่อศึกษาลักษณะสมบัติ พบว่าเอนไซม์มีความบริสุทธิ์เพิ่มขึ้น 2.5 เท่า และมีน้ำหนักโมเลกุลประมาณ 40 กิโลดาลตัน จากการศึกษาความจำเพาะของสารตั้งต้น พบว่า เอนไซม์มีความจำเพาะต่อสารตั้งต้นที่มีจำนวนคาร์บอนตั้งแต่ 4 ถึง 14 อะตอม ภาวะที่เหมาะสมของการทำงานคือความเป็นกรดด่างเท่ากับ 9 ช่วงอุณหภูมิ 35-40 องศาเซลเซียส อีกทั้งมีความเสถียรที่ความเป็นกรดด่าง 5.0 ถึง 10.0 ที่อุณหภูมิต่ำกว่า 35 องศาเซลเซียส เมื่อศึกษาสารที่มีผลต่อการทำงานของเอนไซม์ พบว่า ไอออนของโลหะ ตัวทำละลายที่ไม่มีขั้วและสารลดแรงตึงผิวส่วนใหญ่ไม่มีผลต่อค่าการทำงานของรีคอมบิแนนท์ไลเพส แต่ถูกยับยั้งอย่างรุนแรงด้วย SDS และสารรีดิวซิ่ง β- mercaptoethanol เมื่อนำรีคอมบิแนนท์ไลเพสมาเร่งปฏิกิริยาไฮดรอลิซิสของน้ำมันพืช 7 ชนิด ได้แก่ มะพร้าว เงาะ ปาล์ม มะละกอ มะกอก สบู่ดำ และดอกคำฝอย พบว่าน้ำมันมะพร้าวเป็นสารตั้งต้นที่ให้ค่าการทำงานจำเพาะสูงสุดเท่ากับ 1.14 ไมโครโมลต่อนาทีต่อมิลลิกรัมโปรตีน จากนั้นศึกษาการเร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นของเอนไซม์ โดยใช้น้ำมันที่ให้ค่าการทำงานจำเพาะสูงจากปฏิกิริยาไฮดรอลิซิส 4 ชนิด ได้แก่มะพร้าว เงาะ มะละกอ และปาล์ม เป็นสารตั้งต้น พบว่าน้ำมันที่สกัดจากเงาะสามารถผลิตไบโอดีเซลได้ประมาณ 45 เปอร์เซ็นต์ซึ่งสูงกว่ามะละกอ ปาล์ม และมะพร้าว ตามลำดับ

จากผลการศึกษาทั้งหมดสรุปได้ว่าสามารถโคลนยีนไลเพสจากเชื้อรา *Fusarium solani* ซึ่งมีความสามารถแสดงออกในการเร่งปฏิกิริยาไฮดรอลิซิสและทรานส์เอสเทอริฟิเคชั่นสำหรับการผลิตไบโอดีเซลได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5072598223 : MAJOR BIOTECHNOLOGY
KEYWORDS: LIPASES / *Fusarium solani* / GENE CLONING /BIODIESEL

WEERASAK THAKERNKARNKIT: GENE CLONING, EXPRESSION AND CHARACTERIZATION OF LIPASE FROM *Fusarium solani* FOR THE PRODUCTION OF BIODIESEL. ADVISOR: ASSOC. PROF.TIKAMPORN YONGVANICH. CO-ADVISOR: PAKORN WINAYANUWATTIKUN, Ph.D., 107 pp.

Biodiesel is the product derived from triglycerides by transesterification catalyzed by acid or base. However, biological catalyst such as lipase has become more attractive. The screening for natural microbial lipases with the transesterification activities together with gene technology can therefore be applied to develop and increase the production of large quantities of the enzyme. In this research, the lipase gene was cloned from lipase producing fungus namely, *Fusarium solani* into the pPICZαA. The result revealed that the gene was composed of 1,002 bp and could be deduced into 333 residues of amino acids. The lipase gene was then transformed and expressed in *Pichia pastoris* strain KM71. It was found that the incubation in the presence of 3 % of methanol at 30 °C for 5 days was optimal for the induction of the expression. The expressed recombinant lipase was subsequently purified and 2.5 purification folds with the molecular weight of approximately 40 kDa were obtained. The study of substrate specificity showed that the enzyme was specific towards the substrates with carbon chain lengths between 4-14. The optimal conditions were found to be pH 9 at 35-40 °C while the enzyme was stable at pH between 5.0-10.0 and the temperature below 35 °C. The effect of chemicals revealed that the activity of recombinant lipase was not influenced by most studied metal ions and detergents but strongly inhibited by SDS and reducing agent, β-mercaptoethanol. When the recombinant lipase was tested for the hydrolysis of 7 types of plant oils including coconut, rambutan, palm, papaya, olive, physic nuts and safflowers, the highest specific activity of 1.14 μmol/min/mg protein was obtained from the coconut oil as substrate. Finally, the transesterification of 4 types of oils with high hydrolytic activities ie. coconut, rambutan, palm and papaya was investigated. The results indicated that approximately 45% of biodiesel were obtained from rambutan oil which was higher than papaya, palm and coconut respectively.

From this study, it could therefore be concluded that lipase gene from *Fusarium solani* was successfully cloned and expressed. The obtained recombinants could catalyze both the hydrolysis and transesterification for the production of biodiesel.

Field of Study : Biotechnology Student's Signature .....
Academic Year : 2010 Advisor's Signature .....
Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

bp	basepair	µg	microgram
°C	degree celcius	µl	microliter
et al.	et alia (latin)	µM	micromolar
FAME	Fatty Acid Methyl Ester	mg	milligram
FFA	Free Fatty Acid	ml	milliliter
Fig.	figure	mM	millimolar
g	gram	min	minute
hr.	hour	M	Molar
ITS	Internal transcribes spacer	mol	mole
kb	kilobase	nm	nanometer
kDa	kilodalton	$1b/in^2$	pound (force) per square inch
kg	kilogram	s	second
L	liter	v/v	volume by volume
m	meter	w/w, wt	weight by weight

CHAPTER I

INTRODUCTION

1.1 Statement of purpose

The growth in petroleum consumption throughout the world has caused an urgent economic, security, and environmental problems. One of the best solutions to alleviate such problem is to develop the alternative fuels such as biodiesel (Guan *et al*., 2009). Biodiesel is a natural replacement of diesel fuel generated from various types of organic feedstocks including fresh or waste vegetable oils, animal fats, and oilseed plants (Jeong *et al*., 2009; Patil and Deng, 2009 and Rosa *et al*., 2008). Biodiesel releases significantly lower emissions than petroleum-based diesel when it is burned, both in its pure form or blended with petroleum diesel. It does not contribute to an increase in the level of carbon dioxide in the atmosphere but also minimizes the intensity of greenhouse effect (Antolin *et al*., 2002 and Vicente *et al*., 2004). In addition, biodiesel is better than diesel fuel in terms of sulfur content, flash point, aromatic content and biodegradability. Biodiesel can be produced by transesterification (alcoholysis) of vegetables oils or animal fats. This reaction can be catalyzed by chemical or biological catalysts such as acid, base and lipase, respectively. However, biodiesel produced by chemical catalyst has several drawbacks such as difficulties in recovery of glycerol, removal of basic catalyst from product and the treatment of alkaline wastewater. On the other hand, the enzymatic reaction by lipase has attracted much more attention for biodiesel production as it produces high purity product and enables easy separation from the byproduct, glycerol. Lipases (Triacylglycerol acylhydrolases EC 3.1.1.3) are enzyme catalysing the hydrolysis of triglycerides at the oil-water interface. Generally, lipases are very

applicable by many industries e.g., detergent, oil and fats, baking, organic synthesis, hard surface cleaning, leather and paper industries. Lipases are normally found in plants, animals and microorganisms (Cihangir and Sarikaya, 2004 and Deng *et al*., 2005). Microbial lipases display great potential for commercial applications because they show wide range of pH and temperature stability, as well as broad substrate specificity. In addition, microbial lipases are extracellular thus it is easy for the separation. Furthermore, microorganisms are easy to culture because they can be grown in small amount of media. Nevertheless, natural microbial lipases are inadequate for industrial applications. Hence, the modification of the gene encoding the enzyme by gene technology should be promising to increase the yield of enzyme production. This technology offers several benefits to the enzyme industries such as; the use of safe and well documented host organisms resulting in efficiency enhancement, higher product purity and finally, the enzymes with improved stability and activity (Falch, 1991).

From the previous studies, 70 fungal strains isolated from the samples of oil contaminated soil and waste water were screened for lipase production. After the determination of lipase activity, the isolate NAN 103 exhibited the highest specific activity of lipase, 88.73 ± 0.99 U/mg (Malilas, 2006). When the production of biodiesel using the palm oil and methanol as substrates catalyzed by purified lipases from isolate NAN 103 was determined at 40 ^{o}C for 48 hours, the percent conversion was found to be 23.98 ± 3.21 (Winayanuwattikun *et al* , 2011). Therefore, this research attempted to clone and express the lipase gene from this isolated NAN 103 to be used as the catalyst for the production of biodiesel.

1.2 Objectives

To clone the gene, express, purify and characterize lipases from *Fusarium solani* as the catalyst for the biodiesel production

1.3 Scopes of the study

1.3.1 To identify the lipase producing fungi

1.3.2 To clone lipase gene

1.3.3 To express lipase gene

1.3.4 To purify recombinant lipase

1.3.5 To characterize recombinant lipase

1.4 Expected results

This research should provide the lipase which can be produced from *Fusarium solani* in order to catalyze transesterification for the production of biodiesel.

1.5 Thesis organization

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

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Biodiesel

Biodiesel is a diesel fuel substitute produced from renewable sources such as vegetable oils, animal fats and recycled cooking oils. Chemically, it is defined as the monoalkyl ester of long chain fatty acids (chain length C_{14}–C_{22}) (Demirbas, 2009). This fuel has many advantages over petroleum diesel namely; the raw materials used are natural and renewable, biodegradable, non- toxic and results in low exhaust emission of carbon monoxide, particulates and hydrocarbon so it is an environmental friendly fuel (West *et al.,* 2008; Albuquerque *et al.,* 2009; Eevera *et al.,* 2009; Demirbas, 2009; Ramos *et al.,* 2009). Biodiesel can be produced by several processes and the property is similar to petrodiesel as shown in Table 2-1.

Table 2-1. Comparative properties of diesel and biodiesel

Fuel properties	Diesel	Biodiesel
Fuel Standard	ASTM D975	ASTM D6751
Lower Heating Value, Btu/gal	~129,050	~118,176
Kinematic Viscosity, at 40^{o}C	1.3 - 4.1	4.0 – 6.0
Specific Gravity Kg/l, at 60^{o}F	0.85	0.88
Density, lb/gal, at 15^{o}C	7.079	7.328
Water and Sediment, vol%	0.05 max	0.05 max
Carbon, wt%	87	77
Hydrogen, wt%	13	12
Oxygen, by dif. wt%	0	11
Sulfur, wt%	0.05 max	0.0 to 0.002

Boiling Point, ^{o}C	180 to 340	315 to 350
Flash Point, ^{o}C	60 to 80	100 to 170
Cloud Point, ^{o}C	-15 to 5	-3 to 12
Pour Point, ^{o}C	-35 to -15	-15 to 10
Cetane Number	40 - 55	48 – 65
Lubricity SLBOCLE, grams	2000-5000	>7,000
Lubricity HFRR, microns	300 – 600	<300

From Dwivedi *et al*., 2006

2.1.1 Biodiesel production

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

(Joelianingsih *et al.,* 2008; Sinha *et al.,* 2008; Demirbas, 2009). In particular, they are applied to encounter the problems of high fuel viscosity (about 11–17 times higher than diesel fuel) (Meher *et al.,* 2006).

2.2 Lipase

Lipases or triacylglycerol acylhydrolases (EC 3.1.1.3) are water soluble enzymes which catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids at an oil-water interface (Cardenas *et al.,* 2001[a], Betigeri and Neau, 2002; Hung *et al.,* 2003; Saxena *et al.,* 2003). From the EC number of lipase, 3.1.1.3, whose components indicate the following groups of enzymes:

E.C.3. Hydrolases

E.C.3.1. Acting on ester bonds

E.C.3.1.1. Carboxylic ester hydrolases

E.C.3.1.1.3 Triacylglycerol lipases

In some literature, lipases are defined as carboxyl-esterases which hydrolyze preferentially long-chains of fatty acids higher than 8 at alkaline pH (Côté and Shareck, 2008; Sun and Xu, 2009). Lipases are activated only the interface of oil and water and do not hydrolyze dissolved substrates in the bulk fluid (Vakhlu and Kour, 2006). Generally, the catalytic triad of lipase is mostly composed of serine, histidine and aspartate or glutamate also found in serine proteases (Reis *et al.,* 2009). Moreover, most lipases carry a lid-like structure rich in non-polar residues located around the active site and covered in the inactive form of the lipase. On the other hand, when lipases attach to the nonpolar substrate, the lid will move leading to the open form which makes the binding site accessible to the substrate (Côté and Shareck, 2008).

2.2.1 Sources of lipases

The success of lipases in industrial applications is due to their specific properties and price depending on its source. Lipases are widely found in most organisms from the animals, plants and microbes (Cardenas *et al.,* 2001[a,b], Reis *et al.,* 2009). The animal lipase obtained from pancreas of mammalian such as pancreatic lipase of porcine origin is one of the earliest recognized and is still the best known lipase (Vakhlu and Kour, 2006). Moreover, the most important source of mammalian lipase is the pancreas of human, cattle, sheep and guinea pig. In plants, lipases have been purified from the seeds of plants such as maize (*Zea mays*), castor (*Ricinus communis*), rapeseed (*Brassica napus),* ironweed (*Vernonia galamensis*), wheat (*Triticum aestivum*), cotton (*Gossypium spp*.) and soy *(Glycine max)* (Eastmond, 2006). The plant lipases are not commercially used while the animal and microbial lipases are more applied (Vakhlu and Kour, 2006). Nonetheless, microbial lipases show greater potential for commercial applications due to their stability, selectivity and broad substrate specificity (Betigeri and Neau, 2002). Both intracellular and extracellular lipases can be produced by a widespread number of microorganisms namely, bacteria, fungi and yeast. In particular, lipases produced by bacteria such as *Pseudomonas* sp. (Karadzic et al., 2006) *Burkholderia cepacia*; fungi belonging to the genera *Penicillium, Rhizopus* and *Rhizomucor* and especially yeast such as *Candida sp.*, one of the most well-known industrial lipase producers.

2.2.2 Properties of lipases

Lipases are amongst the most important biocatalysts that carry out novel reactions in aqueous and non-aqueous media (Vakhlu and Kour, 2006). Animal lipases show high activity at an optimum pH range from 8.0–9.0, according to the type of substrates, salt and emulsifiers (Malcata *et al.,* 1992). Most of the microbial lipases show the stability around pH 6.0–7.5. Extracellular lipase from *Aspergillus niger*, *Chromobacterium viscosum* and *Rhizopus* sp. are active at acidic pH whereas an alkaline lipases active at pH 11.0 have been isolated from *Pseudomonas nitroreducens* (Saxena *et al.,* 2001). Morever, microbial lipases are more stable in high temperature than lipases from animals and plants (Hasan *et al.,* 2006). The pancreatic lipases lose activity upon storage at temperatures above 40 ^{o}C while lipases of *Aspergillus niger*, *Ranunculus japonicus*, and *Chromobacterium viscosum* are stable at 50 ^{o}C. Lipases from thermotolerant *Humicola lanuginosa* and *Pseudomonas nitroreducens* are stable at 60 ^{o}C and 70 ^{o}C, respectively. Nonetheless, the maximum activities of *Calvatia gigantea* and other lipases from mesophiles were at 30–35 ^{o}C. Moreover, purified lipase from *Aspergillus terreus* retained 100% of its activity at 60 ^{o}C after 24 hr. In general, lipases show considerable variations in their reaction specificities: this property is generally referred as enzyme specificity. Thus, some lipases have affinity for short-chain fatty acids (acetic, butyric, capric, caproic, caprylic, etc.), some have preference for unsaturated fatty acids (oleic, linoleic, linolenic, etc.), while many others are nonspecific and randomly split the fatty acids from the triglycerides. From the glycerol side of the triglycerides, lipases often show positional specificity and attack the fatty acids at 1 or 3 carbon position of

glycerol or at both positions but not the fatty acid at the 2nd position of the glycerol molecule. The specific characteristics of lipase presented by enzymes are high level of catalytic efficiency and high degree of specificities including substrate specificity, region-specificity, stereo-specificity and enantioselectivity. Activity of lipases can be assayed by many methods namely: spectrophotometry or titrimetry, radiolabelling assay, fluorimetry, surface tension method and estimation of free fatty acids by high performance liquid chromatography (HPLC). Tributyrin plate assay and titrimetry are the most commonly used methods for screening of lipase producers and estimation of lipase activity, respectively (Saxena *et al.,* 2001).

2.2.3 Enzymatic reaction of lipase

The biological function of lipases is to catalyze the hydrolysis of triglycerides, especially long chain triacylglycerols, as act on ester bonds (Al-Zuhair *et al.,* 2008) to yield diglycerides, monoglycerides, glycerol and fatty acids. Lipases are powerful tools for catalysis of not only hydrolysis, but also various reverse reactions, such as esterification, interesterification, alcoholysis, acidolysis, and aminolysis (Fig. 2-1) (Reis *et al.,* 2009; Saxena *et al.,* 2003 Foresti and Ferreira, 2007). The reaction is reversible and the direction of the reaction depends upon the water content available in the reaction. In low water media, lipases catalyse esterification, interesterification and transesterification (Vakhlu and Kour, 2006). The two main categories of reactions with lipase catalysis can be classified as follows;

i. Hydrolysis:

$$R_1COOR_2 + H_2O \longrightarrow R_1COOH + R_2OH$$

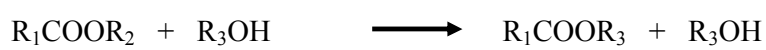
ii. Synthesis:

Reactions under this category can be further classified as:

(a) Esterification

(b) Interesterification

(c) Alcoholysis



(d) Acidolysis

Fig. 2-1 Enzymatic reactions of lipases (Reis *et al.,* 2009; Saxena *et al.,* 2003)

The last three reactions are often grouped together into the single term of transesterification. Furthermore, lipase efficiently catalyzes the aminolysis of different acrylic esters and aliphatic amines shown in Fig. 2-2.

Aminolysis

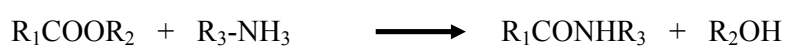


Fig. 2-2 Aminolysis reaction catalyzed by lipases (Villeneuve *et al.*, 2000; Saxena *et al.,* 2003)

2.2.4 Application of lipases

Since lipase have many advantages (Vakhlu and Kour, 2006) and they are capable of preserving their catalytic activity in organic solvents, the activities of lipases as catalysts have been investigated to determine their potential for the conversion of fats and oils into higher value products for food industrial uses. Hence, lipases are generally applied in a variety of other

biotechnological industries such as dairy enrichment (cheese ripening, flavour development, enzyme-modified cheese (EMC) technology), fruit juice, baked food, vegetable fermentation, detergent, pharmaceutical (naproxen, ibuprofen), agrochemical (insecticide, pesticide) and oleochemical industries (fat and oil hydrolysis, biosurfactant synthesis) industries (Saxena *et al.,* 2001), cosmetics, flavors, single cell protein production and biosensor preparation. They are also used in leather industry for processing hides and skins (bating) and for environmental treatment of activated sludge and other aerobic waste products where they remove the thin layer of the fats and by so doing provide for oxygen transport (Hung *et al.,* 2003; Vakhlu and Kour, 2006; Chang *et al.,* 2007; Abdel-fattah and Gabaiia, 2008; Côté and Shareck, 2008). Ultimately, lipases can be further applied in many newer areas where they can serve as potential biocatalysts.

2.3 DNA technology

DNA technology has revolutionized modern science. Deoxyribonucleic acid (DNA), or an organism's genetic material is inherited from one generation to the next. It holds many clues that have unlocked some of the mysteries behind human behavior, disease, evolution, and aging. As technological advances lead to a better understanding of DNA, new DNA-based technologies will emerge. Recent advances in DNA technology including cloning, PCR, recombinant DNA technology, DNA fingerprinting, gene therapy, DNA microarray technology, and DNA profiling have already begun to shape medicine, forensic sciences, environmental sciences, and national security. In 1956, the structure and composition of DNA was elucidated and confirmed previous studies more than a decade earlier demonstrating DNA is the

genetic material that is passed down from one generation to the next. A novel tool called PCR (polymerase chain reaction) was developed not long after DNA was discovered representing one of the most significant discoveries or inventions in DNA technology.

PCR (polymerase chain reaction) is a technique in which cycles of denaturation, annealing with primer, and extension with DNA polymerase, are used to amplify the number of copies of a target DNA sequence by more than 100 times in a few hours. American molecular biologist Kary Mullis developed the techniques of PCR in the 1970s. For his ingenious invention, he was awarded the 1993 Nobel Prize in physiology or medicine. PCR amplification of DNA is like any DNA replication by DNA polymerase *in vivo.* (in living cells) The difference is that PCR produces DNA in a test tube. For a PCR reaction to proceed, four components are necessary: template, primer, deoxyribonucleotides (adenine, thymine, cytosine, guanine) and DNA polymerase. In addition, part of the sequence of the targeted DNA has to be known in order to design the according primers. In the first step, the targeted double stranded DNA is heated to over 90 °C for denaturation. During this process, two strands of the targeted DNA are separated from each other. Each strand is capable of being a template. The second step is carried out around 50 °C. At this lowered temperature, the two primers anneal to their complementary sequence on each template. The DNA polymerase then extends the primer using the provided nucleotides. As a result, at the end of each cycle, the numbers of DNA molecules double (Fig. 2-3).

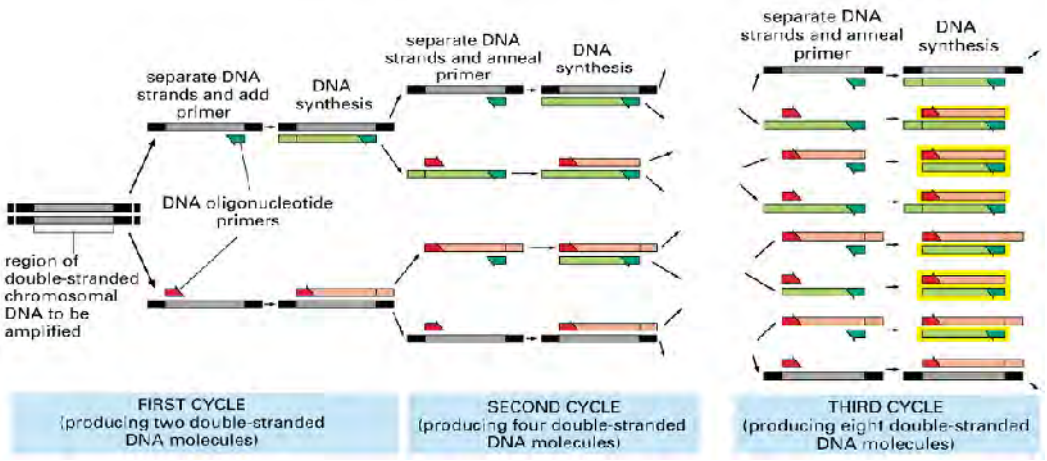


Fig. 2-3 Polymerase Chain Reaction (PCR)

Not long after PCR technology was developed, genetic engineering of DNA through recombinant DNA technology quickly became possible. Genetic engineering is the alteration of genetic material with a view to producing new substances or creating new functions. The most common form of genetic engineering involves the insertion of new genetic material at an unspecified location in the host genome. This technique requires three elements: the gene to be transferred, a host cell in which the gene is to be inserted, and a vector to effect the transfer. This is accomplished by isolating and copying the genetic material of interest, generating a construct containing all the genetic elements for correct expression, and then inserting this construct into the host organism. The utilization of gene technology and of new production technologies have made industrial enzymes with improved properties or better cost performance available. The benefits to the customers are considerable: cost savings in the application process, improved product quality, and in most cases also a significantly reduced impact on the environment. Gene technology offers several benefits to the enzyme industry. This technology enables the use of safe, well documented host organisms easy to cultivate, the microbial production of enzymes from animal and plant origins, the realization of enhanced efficiency and high product

purity, and also the production of enzymes with improved stability and activity, for example, lipase from *Burkholderia cepacia* strain G63 (Hasan *et al*., 2006). The purified lipase can be kept stable at a temperature range of 40-70 ^{o}C. After 10 hr. of incubation at 70 ^{o}C, the optimal temperature of this enzyme, it still retained 86.1 % of its activity. In addition, the enzyme was also highly tolerant to a series of organic solution. Incubated in 50 % methanol solution up to 48 hr, the enzyme still kept 98.3 % of its activity. The transesterification activity of soybean oil to fatty acid methyl esters (FAMEs) reached 87.8 % after 72 hr. (Yang *et al*., 2007).

2.4 Protein purification

Protein can be purified by various methods depending on its properties. The following methods were the example of protein purification using four characteristics.

A. Protein solubility

(1) Isoelectric precipitation

(2) Ionic strength change

(3) Dielectric constant decrease

B. Protein size

(1) Gel filtration

(2) Ultrafiltration

(3) Dialysis

C. Protein charge

(1) Ion- exchange chromatography

(2) Isoelectric focusing

D. Specific biological properties

Affinity chromatography

In this study, some of the methods shown in the Table 2-2 were applied to purify the obtained recombinant lipase.

Table 2-2 Lipase purification (Saxena *et al.,* 2003)

Bacillus spp.	Purification steps	Recovery (%)/ purification fold	Molecular weight (kDa)	Reference
Bacillus spp.	ammonium sulfate fractionation, treatment with acrinol, DEAE-Sephadex A-50, Toyopearl HW-55F and butyl-Toyopearl 650 M	9%/7762	22	Sugihara et al., 1991
Bacillus thermocatenulates (lipase gene cloned into *Escherichia coli*)	cell disintegration, heat precipitation, ion exchange chromatography and hydrophobic interaction chromatography	312	16	Schmidt-Dannert et al., 1996
Bacillus spp. THL027	ultrafiltration and Sephadex G-100	2.6	69	Dharmsthiti and Luchai, 1999
Bacillus pumulus	ammonium sulfate fractionation and gel filtration on Sephadex G-100	75		Jose and Kurup, 1999
Bacillus alcalophilus	ammonium sulfate precipitation, Sephadex G-100	111		Ghanem et al., 2000
Bacillus stearothermophilus (lipase gene cloned into *Escherichia coli*)	CM-Sepharose and DEAE-Sepharose	62.2%/11.6		Kim et al., 2000
Bacillus spp.	acetone fractionation, two acetone precipitations and, octyl-Sepharose CL-4B, Q-Sepharose and Sepharose-12	20%/3028	25	Imamura and Kitaura, 2000
Pseudomonas fragi	acidification, ammonium sulfate fractionation. DEAE-Toyopearl 650 M and DEAE-Sepharose CL-6B	48%/68	33	Nishio et al., 1987
Pseudomonas fluorescens	ammonium sulfate precipitation and chromatography on DEAE-cellulose and octyl-Sepharose CL-4B	21%/3390	45	Sztajer et al., 1992
Pseudomonas spp. ATCC 21808	Q-Sepharose, octyl-Sepharose and the enzyme eluted with isopropanol	56%/159	35	Kordel et al., 1991
Pseudomonas cepacia	liquid–liquid (10% PEG 6000 and 10% Dextran 500) extraction and chromatography using Q-Sepharose	30%/55	58	Dominguez et al., 1991
Pseudomonas cepacia	polyoxyethylene detergent C14EO6-based aqueous two-phase partitioning	76%/24	60	Terstappen et al., 1992
Pseudomonas putida 3SK	DEAE-Sephadex A-50 and Sephadex G-100	21%/5.3	45	Lee and Rhee, 1993
Pseudomonas fluorescens	ultrafiltration, ammonium sulfate precipitation, DEAE-Toyopearl 650 M and phenyl-Toyopearl 650	42%/6.1	33	Kojima et al., 1994
Pseudomonas spp. Yo103	ammonium sulfate precipitation, DEAE-cellulose and Sephadex G-200	3.7%/62	38	Kim et al., 1997
Pseudomonas aeruginosa	ammonium sulfate precipitation, hydroxyapatite column chromatography	518	30	Sharma et al., 1998
Rhizopus japonicus NR 400	hydroxyapatite, octyl-Sepharose and Sephacryl S-200	31%/93	30	Suzuki et al., 1986
Rhizopus oryzae	acetone precipitation (80%), Sephadex G-100	64%/160		Razak et al., 1997
Rhizopus delemar	oleic acid affinity chromatography, CM-Sephadex	30%/10.3	30.3	Haas et al., 1992
Rhizopus arrhizus	ammonium sulfate fractionation and Sephadex G-100 gel filtration	42%/720	67	Chattopadhyay et al., 1999
Rhizopus chinensis	CM-Cellulofine C-500, ether Toyopearl 650 M, Super Q Toyopearl and CM-Cellulofine C-500	27.6%	28.4	Yasuda et al., 2000
Rhizopus oryzae	ammonium sulfate fractionation, sulfopropyl-Sepharose, Sephadex G-75 and again on sulfopropyl-Sepharose	22%/1260	32	Hiol et al., 2000

CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

Agarose gel electrophoresis	(BioRad, USA)
Autoclave	(Ta Chang Medical instrument, Taiwan)
Balance	(Sartorius, Germany)
Digital Balance	(Mettler Toledo, USA)
Digital Dry Bath	(Labnet International, Inc., USA)
Gel Documentation	(UVP, UK)
High performance liquid chromatography	(Shimudzu, Japan)
Incubator	(Gallenkamp, UK)
Laminar flow	(Thermo electron corporation, USA)
Magnetic stirrer	(KIKA Labortechnik, Malaysia)
Magnetic bar	(Lio Lab Limited Partnership)
Microcentrifuge	(Satorious, Germany)
Microplate reader spectrophotometer	(ASYS Hitech GMBH, Austria)
Microrefrigerated centrifuge	(Hettich, USA)
Microwave	(Sharp, Thailand)
pH meter	(Mettler Toledo, USA)
Peristaltic pump	(LKB-Pump P.1 Pharmacia, Sweden)
Refrigerated incubator shaker	(New Brunswick Scientific Co., Ltd, China)
Slab gel electrophoresis equipment	(Biorad, USA)
Thermal Cycler	(Biorad, USA)
UV-VIS spectrophotometer	(Thermo scientific, UK)

Vortex	(Scientific industries, USA)
Water bath	(T.S. Instrument, Thailand)

3.2 Chemicals

In all experiments, the analytical grade and/or molecular biological grade chemicals and reagents were purchased from various manufacturers; namely, Sigma (USA), Merk (Germany), Fluka (Switzerland), Carlo Erba (Italy), Ajax Finechem (Australia), Difco (USA), Usb (USA), Biorad (USA) and Invitrogen (Canada).

3.3 Bacterial and fungal strains

Fusarium solani was used as the donor of chromosomal DNA.

E.coli DH5α (F-Ø80*lac*ZΔM15Δ (*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-) was used as host for plasmid propagation.

Pichia pastoris strain KM71 was used as host for expression of the gene constructed in pPICZαA.

3.4 Enzymes

*Bam*HI	New England Biolabs (USA)
*Eco*RI	New England Biolabs (USA)
*Hin*dIII	New England Biolabs (USA)
*Not*I	New England Biolabs (USA)
*Sac*I	New England Biolabs (USA)
Taq DNA polymerase	New England Biolabs (USA)
T4 DNA ligase	New England Biolabs (USA)

3.5 Commercial plasmids

pGEM®-T Easy vector (Fig. 3-1)	Promega (USA)
pPICZαA (Fig. 3-2)	Invitrogen (USA)

3.6 Commercial kits

QIAprep Spin Miniprep Kit	QIAGEN (Germany)
QIAquick Gel extraction Kit	QIAGEN (Germany)
Genomic DNA Extraction Kit	QIAGEN (Germany)

3.7 Synthetic oligonucleotides

All synthetic oligonucleotides, used as primers, were purchased from Bio Basic Inc. (Canada). The primers information was shown in Table 3-1.

Table 3-1 List of primers

Primers	**Sequences**	**T_m°C**
FSF1	5'- GGAATTCATGATGCTCATCCTATCTATTCTTTC –3' (*Eco*RI)	59.4
FSRS1	5'-TATCAAATGCGGCCGCCTAAGTCATCTGCTTAACAAA TTC-3'(*Not*I)	64.5
ITS1-F	5'- CTTGGTCATTTAGAGGAAGTAA -3'	49.2
ITS4	5'- TCCTCCGCTTATTGATATGC -3'	49.7

3.8 Purification column

DEAE column	GE Healthcare Bio-Science AB (Sweden)

3.9 Oils

Coconut oil	(Pumedin natural products, Thailand)
Olive oil	(Rafael Salgado, Spain)
Palm oil	(Morakot industry, Thailand)
Papaya oil	(Extracted in laboratory)
Physic nut oil	(Extracted in laboratory)
Rambutan oil	(Extracted in laboratory)
Safflower oil	(OHIO, Mexico)

3.10 Miscellaneous

TriDye ^{TM} 1 kb DNA Ladder	New England Biolabs (USA)
Prestained Protein Ladder, Broad Range	Fermentas (Canada)
6X DNA loading dye	Fermentas (Canada)
pUC Mix Marker	Fermentas (Canada)

3.11 Data analysis program

Sequence analysis program	(BioEdit, Chromas Lite and BLAST program)
Statistical analysis program	(Graph Pad InStat3)
Graph analysis program	(Graph Pad Prism4)

3.12 Research methodology

The research methodology is as follows**:**

3.12.1 Identification of the lipase producing fungi

3.12.2 Cloning of the lipase gene

3.12.3 Expression of the lipase genes

3.12.4 Purification of the lipase

3.12.5 Characterization of the lipase

3.12.1 Identification of the lipase producing fungi

3.12.1.1 Molecular identification

3.12.1.1.1 Genomic DNA extraction

The genomic DNA of NAN 103 was extracted by cetyl trimethyl ammonium bromide known as CTAB method (Zhou *et al*., 1999). The hyphae suspended in broth were collected and the cells were later lysed by freezing in liquid nitrogen and ground by mortar and pestle. The powdered samples were thawed and homogenized in 1000 µl of washing buffer (Appendix A) followed by centrifugation at 13,000 rpm for 5 min. Then, the pellets were twice washed with the same amount of washing buffer and centrifugation condition. These pellets were washed by 700 µl 2X CTAB (Appendix A) and subsequently incubated at 65 ^{o}C for 1 hr. Next, the complete extraction was achieved by an equal volume of chloroform-isoamyl alcohol mixture (24:1 v/v). The obtained DNAs were precipitated by adding an equal volume of chilled isopropanol and left on ice for 30 min. After centrifugation at 8,000 rpm for 5 min, the precipitated DNAs were washed by 500 µl of 70 % ethanol and centrifuged again at the same condition. The precipitation was repeated by resolubilizing the DNAs obtained from the previous step in 100 µl of sterilized distilled water before treating with 1 µl of 10 mg/ml RNase for

30 min at room temperature. Subsequently, 60 µl of 20 % PEG (Appendix A) was added and later centrifuged at 13,000 for 10 min before washing by 70 % ethanol. The genome of NAN 103 were finally resuspended in 100 µl of TE buffer (Appendix A).

3.12.1.1.2 ITS gene amplification

The ITS gene was amplified from the obtained genome by using oligonucleotide primer ITS1-F 5'CTTGGTCATTTAGAGGAAGTAA-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3'. PCR amplification was performed in 50 µl reaction mixture containing 10 ng of DNA template, 0.5 µM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer and 2.5 U Taq DNA polymerase. The 50 µl reaction mixture was incubated at 94 ℃ for 5 min, followed by 38 cycles of incubation at 94 ℃ for 1 min, 51 ℃ for 1 min and 72 ℃ for 1 min and finally at 72 ℃ for 5 min, respectively. Then, the obtained PCR products were detected by 1% agarose gel electrophoresis (Appendix A).

3.12.1.1.3 Ligation of ITS gene into vector

The purified PCR products were ligated into pGEM-T Easy vector. The ligation condition was performed in 10 µl reaction mixture containing 1X T4 ligation buffer, PCR product, pGEM-T Easy vector and 5U of T4 DNA ligase. The molar ratio of PCR product to vector was 3:1 (Appendix D).

The 10 µl reaction mixture was incubated overnight in water bath at 16 °C.

3.12.1.1.4 Preparation of competent cells by calcium chloride ($CaCl_2$) method

A single colony of *E.coli* strain DH5α was inoculated into 3 ml of LB broth before incubation at 37 °C, 250 rpm overnight. 500 µl of culture cell was transferred into 50 ml LB broth in 250 ml flask and later incubated at 37 °C, 250 rpm until the absorbance at 600 nm was 0.3 to 0.4 and then placed on ice for 10 min followed by cell harvesting with centrifugation at 4,000 rpm, 4 °C for 10 min. The pellets were resuspended with 10 ml of chilled 0.1 M $CaCl_2$ and put on ice for 10 min. Then, the mixture was centrifuged at 4,000 rpm, 4 °C for 10 min. The previous step was repeated; namely, resuspending in the same amount of chilled 0.1 M $CaCl_2$, storing on ice and centrifugation condition. However, the pellets were resuspended with 2 ml of chilled 0.1 M $CaCl_2$ and added with 900 µl of steriled glycerol. The solution was gently mixed and stored on ice for 15 min before the aliquots were transferred into microcentrifuge tubes (100µl/tube) and stored in -80 °C until use.

3.12.1.1.5 Transformation into *E.coli* DH5α by heat shock method

10 µl of recombinant plasmids were mixed with 100 µl of competent cells. The mixture was stored on ice for 30 min and immediately heated at 42 °C for 90 sec. The reaction was quickly chilled on ice for 5 min. Next, 900 µl of LB broth were added and incubated at 37 °C, 250 rpm for 1 hr. The transformed cells were observed by spreading on the ITPG screening plates containing 100 µg/ml ampicillin and further incubated overnight at 37 °C.

3.12.1.1.6 Screening of the recombinant clones by rapid size screening method

E.coli strain DH5α containing recombinant plasmids were visible as white colonies on agar plate. These clones were picked for master plate preparation. Next, the colonies were then selected from the obtained master plates and later screened by rapid size screening method. The cells were lyzed with 30 µl of lysis buffer (Appendix A). The obtained mixture was incubated at 37 °C for 5 min, followed by chilling on ice for 5 min and centrifuged at 13,000 rpm for 5 min at room temperature. Finally, 20 µl of upper phase was further analyzed by agarose gel electrophoresis.

3.12.1.1.7 Extraction of plasmid

E.coli strain DH5α containing expected recombinant plasmids were inoculated in 3 ml LB broth containing

100 µg/ml ampicillin and incubated at 37 °C, 250 rpm for 14 to 16 hr. Then, the cells were harvested by centrifugation at 5,000 rpm, at 4 °C for 10 min. The recombinant plasmids were extracted by the commercial extraction kit, Qiagen spin miniprep kit.

3.12.1.1.8 Restriction analysis

The expected recombinant plasmids were checked by digesting with 3 units of *Eco*RI. The 10 µl of reaction medium consisted of certain restriction enzymes in the NEBuffer EcoRI as indicated in Table 3-2 and incubated at 37 °C for 3 hr. The digested recombinant plasmids were analyzed by agarose gel electrophoresis and detected by Gel Documentation machine.

Table 3-2 Restriction enzymes

Restriction enzymes	Recognition sequence (5'-3')	Reagents Supplied
*Bam*HI	G´GATCC	NEBuffer 3 + BSA
*Eco*RI	G´AATTC	NEBuffer EcoRI
*Hin*dIII	A´AGCTT	NEBuffer 2
*Not*I	GC´GGCCGC	NEBuffer 3 + BSA

3.12.1.1.9 DNA sequencing and analysis

The expected recombinant clones containing fragment were verified by nucleotide sequencing using automated DNA sequencer (1st BASE, Malaysia). Then, the obtained sequences were selected by Chromas Lite and BioEdit program and were later aligned to

GenBank database using the BLAST program to determine the most relevant sequence of lipase gene.

3.12.2 Cloning of the lipase gene

3.12.2.1 Preparation of the lipase gene

The hyphae from NAN 103 in medium broth were collected and the cells were later lyzed by freezing in liquid nitrogen and ground by mortar and pestle. Finally, RNA was extracted using the extraction kits. The cDNA of lipase gene was synthesized by reverse transcriptase using mRNA as template. This cDNA was then used as template for amplifying lipase gene by nucleotide primer FSF1 (5′GGAATTCATGATGCTCATCCTATCTATTCTTTC-3′) and FSRS1 (5′TATCAAATGCGGCCGCCTAAGTCATCTGCTTAACAAATTC3′). The underlined sequences represented the *Eco*RI and *Not*I restriction sites. The reaction was carried out in a thermocycler by incubating at 94 ^{o}C for 3 min, followed by repeating 32 cycles of 94 ^{o}C for 1 min, 55 ^{o}C for 30 sec and 72 ^{o}C for 90 sec and final incubation was performed at 72 ^{o}C for 7 min. The PCR products were finally purified by 1 % agarose gel using commercial gel extraction kit, QIAquick Gel Extraction Kit.

3.12.2.2 Preparation of the plasmids

A single colony of pPICZαA plasmid was inoculated into 5 ml of low salt LB medium containing 25 μg/ml zeocin and subsequently shaken at 37 °C, 250 rpm for 14 to 16 hr. The suspended cells were

collected by centrifugation at 4 °C, 5,000 rpm for 10 min. The obtained pellets were resuspended in 400 µl of STET buffer (Appendix A) before transferring to 1.5 ml microcentrifuge tube. Next, 10 µl of 50 mg/ml lysozyme was added and then incubated at 37 °C for 10 min. After incubation, the mixture was boiled for 45 sec followed by centrifugation at 13,000 rpm for 15 min and the pellets were removed, respectively. Subsequently, 5 % CTAB was added and centrifuged at 13,000 rpm for 15 min. The pellets were resuspended in 300 µl of 1.2 M NaCl and mixed well. 3 µl of 10 mg/ml RNase A was then added and incubated at 37 °C for 1.5 hr. Before centrifugation at 12,000 rpm for 5 min, the mixture was added with 300 µl of chloroform-isoamyl alcohol mixture (24:1 v/v). The obtained solution was separated into two phases. The upper phase was then transferred to the new tubes before precipitating with two volumes of cold absolute ethanol and freezing at -20 °C for 30 min. After freezing, this mixture was later centrifuged at 13,000 rpm for 15 min. The pellet was twice washed with 100 µl of 70 % ethanol followed by drying at 55 °C for 10 min and resuspended in 20-30 µl of distilled water.

3.12.2.3 Double digestion of lipase gene and pPICZαA plasmids by restriction enzyme

Both pPICZαA plasmids and fungal lipase gene were double digested with 5 units of *Eco*RI and *Not*I restriction enzymes in 50 µl of appropriate reaction buffer (Table 3-2) and incubated at 37 °C for 3 hr. Both digested pPICZαA plasmids and lipase gene were analyzed by

agarose gel electrophoresis and visualized by Gel Documentation machine.

3.12.2.4 Ligation of lipase gene into pPICZαA vectors

The digested lipase gene was ligated into digested pPICZαA vector. The ligation condition was performed in 10 µl reaction mixture containing 1X T4 ligation buffer, lipase gene, pPICZαA vector and 5U of T4 DNA ligase. The molar ratio of PCR product to vector was 3:1. The mixture was later incubated overnight at 16 °C.

3.12.2.5 Transformation of recombinant plasmids into *E.coli* DH5α competent cells

10 µl of recombinant plasmids were transformed into *E.coli* DH5α by heat shock method. The cells were then spread on low salt LB agar plate containing 25 µg/ml zeocin before incubation overnight at 37 °C.

3.12.2.6 Screening of the recombinant clones

Recombinant clones were preliminarily checked by rapid size screening method as described in 3.12.1.1.6 and further verified by restriction enzyme analysis using *Eco*R I and *Not* I, according to the method in 3.12.2.3.

3.12.2.7 DNA sequencing and analysis

The expected recombinant clones with lipase gene fragments were verified by nucleotide sequencing using automated DNA sequencer (1st BASE, Malaysia). Nucleotide sequences were checked and analyzed by the method described in 3.12.1.1.9.

3.12.3 Expression of lipase gene in *Pichia pastoris*

3.12.3.1 Preparation of recombinant plasmid

The *E.coli* containing an expected recombinant plasmid were grown in LB low salt medium containing 25 µg/ml zeocin and incubated at 37 °C, 250 rpm for 14 to16 hr. The cells were collected by centrifugation at 5,000 rpm, 4 °C for 10 min before extraction by CTAB method as described in 3.12.2.2 and later digested by *Sac*I to linearise the obtained recombinant plasmid. These plasmids were subsequently purified by phenol/chloroform extraction method followed by ethanol precipitation (Appendix B).

3.12.3.2 Preparation of the competent cells

A single colony of *Pichia pastoris* strain KM71 was grown into 5 ml YPD and then incubated at 30 °C, 250 rpm until the absorbance at 600 nm reached 1.3 to 1.6. The cells were collected by centrifugation at 2,000 g, 4 °C for 5 min. The obtained pellets were further resuspended in 40 ml YPD and 8 ml of 1 M HEPES, pH 8.0. The mixture was added with 1 ml of 1 M DTT and 1 ml of 5 M LiAc pH 7.0, and then gently mixed and incubated at 30 °C for

15 min. 150 ml of cold water was then added and centrifuged at 4 °C, 250 rpm for 10 min. The obtained pellets were washed by 100 ml of cold water and then centrifuged at 4 °C, 250 rpm for 10 min. The cell pellets were resuspended twice, firstly in 8 ml of cold 1 M sorbitol and later in 0.4 ml cold 1 M sorbitol. Each resuspension was followed by centrifugation at 4 °C, 250 rpm for 10 min.

3.12.3.3 Transformation of recombinant plasmids into *Pichia pastoris* by electroporation

Pichia pastoris strain KM71 was mixed with 10 µg of recombinant plasmid linearized by *Sac*I and 4 µl DNA carriers and was chilled on ice for 1 min. The mixture was then transferred to cold electroporation cuvette. After pulsation, 1 ml of 1 M ice-cold sorbitol was immediately added into the cuvette. Then, the mixture was transferred to 15 ml tube and incubated at 30 °C followed by the addition of 1 ml YPD and shaken at 30 °C, 250 rpm for 3 hr. Next, the mixture was later spread on YPDS plate containing 200 µg/ml zeocin before incubation overnight at 30 °C.

3.12.3.4 Screening of recombinant clones by rhodamine B plate visualization

Pichia pastoris containing recombinant clones grown on BMGY plate (Appendix A) at 30 °C for a day were then picked and transferred onto BMMY plate supplemented with the mixture of 1% olive oil and 0.0002 % rhodamine B. The BMMY plates were later

incubated at 30 °C. The induction of expression was conducted every 24 hr. by the addition of 150 µl fresh methanol on the plate lid. The lipase activity was detected as orange fluorescence under UV light.

3.12.3.5. Optimization of the expression

Recombinant clones with the activity were inoculated into 5 ml YPD and then incubated at 30 °C, 250 rpm until the absorbance at 600 nm was 7 to 8. Next, cell culture was transferred to 6 ml of BMGY medium before shaking at 250 rpm, 30 °C until the absorbance at 600 nm was 7 to 8 again. The cells were harvested by centrifugation at 4 °C, 5,000 rpm for 10 min before resuspending in 1 ml BMMY. The cell medium was shaken at 30 °C, 250 rpm for 5 days.

3.12.3.5.1 Effect of the methanol concentration

The cell medium was shaken at 30 °C, 250 rpm for 5 days. The induction was conducted by the separate addition of 1% to 5% fresh methanol every 24 hr. before the assay of hydrolytic activity as described in 3.12.4.1.

3.12.3.5.2 Effect of the time

To investigate the optimal time of the induction, the cells were incubated for 1 to 7 days at 30 °C, 250 rpm and added every 24 hr. with optimal concentration of methanol obtained from 3.12.3.5.1. Finally, the lipase activity was assayed according to the protocol in 3.12.4.1.

3.12.4 Purification of the lipase

The recombinant strain was grown according to the method described in 3.12.3.5 using the optimal conditions obtained from 3.12.3.5.1 and 3.12.3.5.2. After centrifugation at 5000 rpm, 4 °C for 1.30 hr., supernatant was concentrated by viva flow and then purified using DEAE Hitrap column (5ml) previously equilibrated with 5 column volumes of 50 mM Tris buffer pH 7.5 with 60 ml/min flow rate. The concentrated solution was loaded to a column. Then, the column was washed with 5 column volumes of 50 mM Tris buffer to remove the unbound protein. The 5 ml fractions were collected and the adsorbed protein was eluted with linear gradient of 0-1 M ammonium sulfate. The fractions with high lipase activity were pooled and concentrated. The homogeneity and the approximate molecular weight of the purified lipase were further analyzed by SDS-PAGE.

3.12.4.1 Lipase assay

Lipase activity was determined by spectrophotometry using two solutions; namely A and B (Appendix A). The assay reagent was prepared by dropwisely adding 1 ml of solution A to 9 ml of solution B to get an emulsion that remained stable for 2 hr. The assay mixture contained 180 µl of the emulsion and 20 µl of appropriately diluted enzyme solution. The liberated *p*-nitrophenol was measured at 410 nm. One unit of enzyme was defined as the amount of released 1 µmol *p*-nitrophenol from the substrate per min.

3.12.4.2 Protein determination

The protein concentration was determined by Bradford's protein assay method. 5 µl of sample and 300 µl of Bradford reagent were mixed in 96 well plates before incubating at room temperature for 5 min and later measured for the absorbance at 595 nm. The protein concentrations were determined from the standard curve of bovine serum albumin (BSA) shown in the Appendix C.

3.12.5 Characterization of lipase

3.12.5.1 Substrate specificity of the purified lipase

Various substrates with different hydrocarbon chain lenghts such as *p*-nitrophenyl esters (*p*NP-acetate (C2), *p*NP-butyrate (C4), *p*NP-caprylate (C8), *p*NP-caprate (C10), *p*NP-laurate (C12), *p*NP-myristate(C14), *p*NP-palmitate (C16) and *p*NP-stearate (C18) were tested for the specificity of the obtained enzyme.

3.12.5.2 Effect of pH on the activity of lipase

The effect of pH on lipase activity using the optimal substrate obtained from 3.12.5.1. was studied in various pH buffers from 5.0 to 11.0; namely 50mM acetate/Na-acetate buffer for pH 5.0, 50 mM K_2HPO_4/KH_2PO_4 buffer for pH 6.0 to 7.0, 50 mM Tris–HCl buffer for pH 8.0 to 10.0 and 50 mM $NaCO_3$/HCl for pH 11.0.

3.12.5.3 Effect of temperature on the activity of lipase

The effect of temperature was also determined by varying the incubating temperature from 30 to 60 °C using the obtained optimal pH buffer from the above 3.12.5.2. The lipase activity was then determined.

3.12.5.4 Stability of recombinant lipase

There are 2 factors which may have the effect on the stability of recombinant lipase ie. pH and temperature. Therefore, the effects of these factors were investigated by the following methods.

3.12.5.4.1 Effect of pH

The effect of pH on lipase stability was determined by incubating 0.5mg/ml purified lipase in various pH buffers varying from 4.0 to 11.0 at 4 °C for 20 hr. and residual activity was assayed at room temperature.

3.12.5.4.2 Effect of temperature

To determine the influence of temperature on the enzyme stability, 1.0 mg/ml the purified lipase dissolved in the optimal pH buffer obtained from 3.12.5.2 above was incubated at various temperatures ranging from 20–60 °C for 5 hr. Subsequently, the residual activity was assayed at room temperature.

3.12.5.5 Effect of metal ions on the activity of lipase

The effect of metal ions on the lipase activity was determined by incubating 1.0 mg/ml purified lipase dissolved in optimal pH buffer and incubating temperature obtained from 3.12.5.4.2 with various metal ions such as 1 and 10 mM of K^{+}, Na^{+}, Li^{+}, Ag^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+} and metal-chelating agent, EDTA for 1 hr. The activity in the presence of non-metal ions was defined as control. The remaining activity was further measured as method described above.

3.12.5.6 Effect of chemicals on the activity of lipase

The chemicals studied were DMSO; detergents such as Triton X-100, Tween-80, and SDS together with reducing agents like DTT and β- mercaptoethanol. The effect of these chemicals on enzyme activity were comparatively studied by using the concentrations at 1% and 5% (v/v) of all chemicals. 1.0 mg/ml of purified lipase was dissolved in optimal pH buffer and incubating temperature obtained from 3.12.5.4.2. The activity of the mixture without chemicals was defined as control. The remaining activity was later checked as described above.

3.12.5.7 Effect of organic solvents on the activity of lipase

To study the effect of organic solvents on the lipase activity, 13 types of the solvents namely; methanol, ethanol, iso-propanol, glycerol, butanol, acetone, hexane, cyclohexane, n-heptane,

chloroform, isooctane, benzene and diethyl ether were selected. 1.0 mg/ml of purified lipase was mixed with an equal volume of organic solvent followed by incubating temperature obtained from 3.12.5.4.2 and stirred at 300 rpm for 1 and 6 hr. The purified lipase with the same amount of optimal pH buffer from 3.12.5.2 was used as control. The residual activity was measured and the percentage of the organic solvent tolerance of enzymes was calculated relative to control.

3.12.5.8 Effect of alcohol on the activity of lipase

The alcohol tolerance was then tested by varying the concentrations of methanol and ethanol from 0.5, 1, 2 and 3 % under the same conditions for 12 and 24 hr. Finally, the residual activity was measured and calculated as percentage relative to control.

3.12.5.9 Hydrolysis of oil

Hydrolysis was performed by using seven types of oil as substrates. Three types were obtained from local groceries such as palm, olive, and safflower oil while the other four were obtained from extraction by hexane namely; coconut, physic nut, papaya and rambutan. The fatty acid contents obtained from reaction were quantitated by titration method. The reaction was prepared by mixing 0.5 ml of buffer at the optimal pH (obtained from 3.12.5.2) with 0.4 ml of the mixture of an equal volume of 2.0 % polyvinyl alcohol and various oils before sonicating and incubating at room temperature for 5 min. Subsequently, 0.1 ml of the purified lipase was later added into

the reaction mixture. This reaction was stirred at 300 rpm and incubated at temperature obtained from 3.12.5.4.2 for 5 hr. Next, 1 ml of 95 % ethanol was immediately added to stop the reaction. The liberated free fatty acids were quantified by titrating with 5 mM NaOH using phenolphthalein as indicator. One unit of lipase activity was defined as the amount of released 1 μmol of free fatty acids per min under the above conditions.

3.12.5.10 Transesterification for the production of biodiesel

Transesterification reactions were carried out in 20 ml screw-capped vials containing 8 mg/ml purified lipase and 0.5 g suitable oil from 3.12.5.9 in the molar ratio of oil to methanol at 1:3 using three steps addition of methanol at 0, 4 and 8 hr. This reaction was carried out by stirring the mixtures with magnetic stirrer at 300 rpm for 24 hr. at 35 °C. The samples were taken from the reaction mixture and the obtained fatty acid methyl esters were later determined by high performance liquid chromatography (HPLC).

3.12.5.10.1 High performance liquid chromatography analysis

Samples were taken from the reaction mixture at specified time and centrifuged at 12,000 rpm 10 min 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 x 4.6 mm x 5 μm) from Alltech (Deerfield, IL) and ELSD-LT Evaporative Light Scattering Detector (Shimadzu Corp., Kyoto). Two mobile phases were employed: phase A consisted of hexane, 2-propanol, ethyl acetate and formic acid (80:10:10:0.1 v/v) and phase B consisted of hexane and formic acid (100:0.05 v/v). The flow rate was 1.5 ml/min and the injection volume was 20 μl. The protocol employed for the mobile phase involved a linear elution gradient of 1 % (v/v) phase A increasing to 98 % (v/v) in 20 min. The final mixture (A:B, 98:2 v/v) was employed for 3 min. Next, the system was restored to initial condition by passing the A:B, 1:99 (v/v) mixture through the column for 15 min. Biodiesel yield was calculated as the percentage of the actual amount of methyl ester detected in the reaction process divided by the theoretical quantity of methyl ester. Calculation of the biodiesel yield was described in Appendix D (Winayanuwattikun *et al*., 2008).

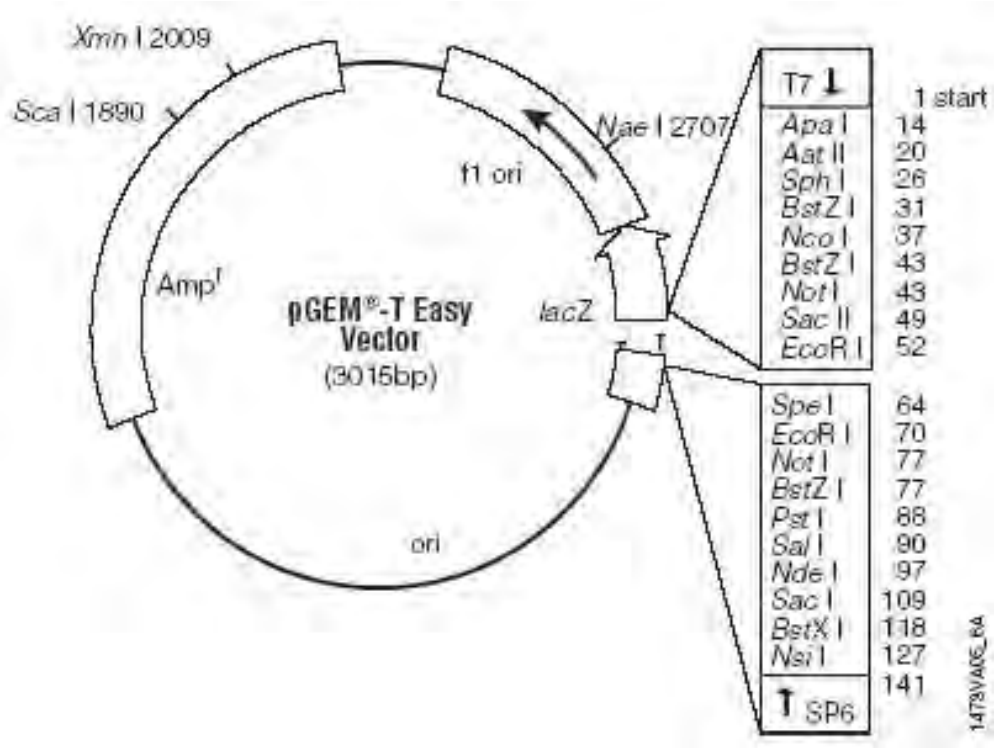


Fig. 3-1 Physical map of pGEMT® - T Easy vector

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

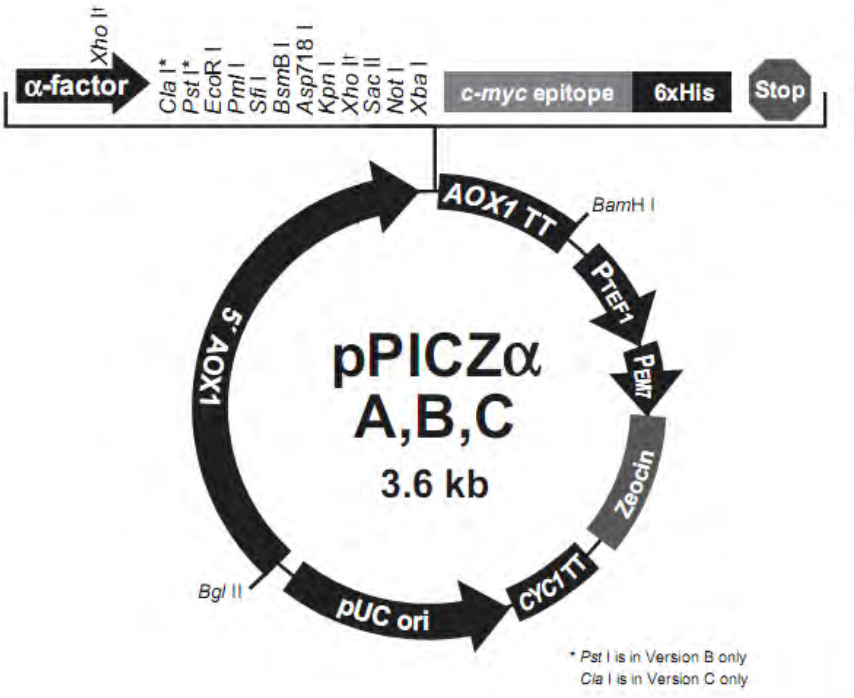


Fig. 3-2 Physical map of pPICZαA vector

5' *AOX1* promoter region	1-941
5' *AOX1* priming site	855-875
α- factor signal sequence	941-1207
Multiple cloning site	1208-1276
c-myc epitope	1275-1304
Polyhistidine (6xHis) tag	1320-1337
3' *AOX*1 priming site	1423-1443
*AOX*1 transcription termination region	1341-1682
*TEF*1 promoter	1683-2093
EM 7 promoter	2095-2162
Sh ble ORF	2163-2537
CYC 1 transcription termination region	2538-2855
pUC origin	2866-3539

CHAPTER IV

RESULTS

4.1 Identification of the lipase producing fungi

4.1.1 Molecular identification

The ITS gene was amplified by PCR technique and the result from agarose gel electrophoresis showed that the size of ITS gene was 564 kilobase (kb) (Fig. 4-1). The gene was then purified and ligated into pGEM-T Easy vector. The expected recombinant clones containing ITS fragment were verified by nucleotide sequencing and aligned to GenBank database by using the BLAST program. The results showed that the submitted sequence was 99% identical to the ITS gene of *Fusarium solani* (accession number, AM412642.1) (Fig. 4-2)

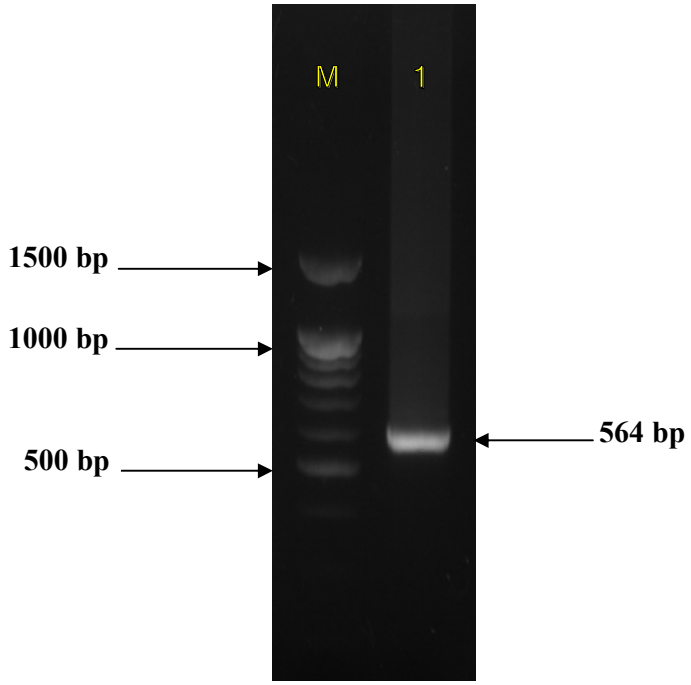


Fig. 4-1 PCR amplification of ITS gene. Lane M, DNA markers; lane 1, ITS gene was indicated by black arrow at 564 bp.

```
>gb|FJ719812.1|  Fusarium solani strain MTCC 9622 18S ribosomal RNA gene, partial
sequence; internal transcribed spacer 1, 5.8S ribosomal
RNA gene, and internal transcribed spacer 2, complete sequence;
and 28S ribosomal RNA gene, partial sequence
Length=594

 Score = 1020 bits (552),  Expect = 0.0
 Identities = 560/564 (99%), Gaps = 0/564 (0%)
 Strand=Plus/Minus

Query  1    TTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACATTCAGAAGTTGGGTGTTTTA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  573  TTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACATTCAGAAGTTGGGTGTTTTA  514

Query  61   CGGCGTGGCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCTACTACGCAATGGAAGCTGCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  513  CGGCGTGGCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCTACTACGCAATGGAAGCTGCG  454

Query  121  GCGGGACCGCCACTGTATTTGGGGGACGGCGTTGTGCCCACAGGGGGCTTCCGCCGATCC  180
            |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
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Query  181  CCAACGCCAGACCCGGGGGCCTGAGGGTTGTAATGACGCTCGAACAGGCATGCCCGCCAG  240
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  393  CCAACGCCAGGCCCGGGGGCCTGAGGGTTGTAATGACGCTCGAACAGGCATGCCCGCCAG  334

Query  241  AATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  333  AATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCA  274

Query  301  CATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  273  CATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTT  214

Query  361  GAAAGTTTTGATTTATTTGCTTGTTTACTCAGAAAAAACATTATAGAAACAGAGTTAGGG  420
            ||||||||| ||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  213  GAAAGTTTTAATTTATTTGCTTGTTTACTCAGAAGAAACATTATAGAAACAGAGTTAGGG  154

Query  421  GGTCCTCTGGCGGGGGCGGCCCGTGTTACGGGGCCGTCTGTTCCCGCCGAGGCAACGTTT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  153  GGTCCTCTGGCGGGGGCGGCCCGTGTTACGGGGCCGTCTGTTCCCGCCGAGGCAACGTTT  94

Query  481  TAGGTATGTTCACAGGGTTGATGAGTTGTATAACTCGGTAATGATCCCTCCGCTGGTTCA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93   TAGGTATGTTCACAGGGTTGATGAGTTGTATAACTCGGTAATGATCCCTCCGCTGGTTCA  34

Query  541  CCAACGGAGACCTTGTTACGACTT  564
            |||||||||||||||||||||||
Sbjct  33   CCAACGGAGACCTTGTTACGACTT  10
```

Fig. 4-2 Alignment of ITS gene to database of GenBank using BLAST program

4.2 Cloning of the lipase gene

The lipase gene was amplified by PCR in order to clone the gene of *Fusarium solani* into pPICZαA. From Fig. 4-3, their sizes were approximately 1 kb and 3.6 kb, respectively. Lipase gene and pPICZαA were then ligated and transformed into *E.coli* DH5α. The recombinant plasmids were checked by the restriction enzyme analysis using *Eco*RI and *Not*I. The result was shown in Fig. 4-3. Moreover, the DNA

sequencing analysis by BLAST program resulted in 92% identity to extracellular lipases in *Nectria haematococca* or *Fusarium solani* (Eddine *et al*., 2001). The deduced amino acid sequence revealed a protein of 333 amino acid residues with a calculated molecular mass approximately 36 kDa as shown in Fig. 4-4 and 4-5, respectively.

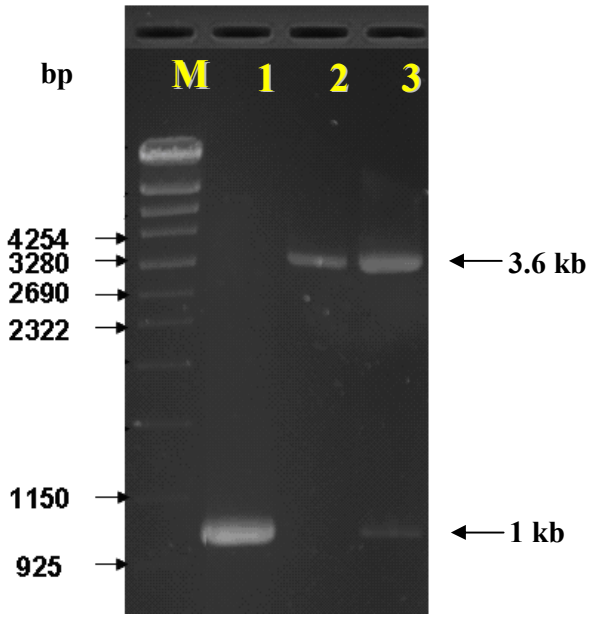

Fig. 4-3 Agarose gel electrophoresis represented pPICZαA and the restriction enzyme analysis. Lane M, DNA marker. Lane 1, a lipase gene. Lane 2, a pPICZαA plasmid. Lane 3, a restriction enzyme analysis by *Eco*RI and *Not*I.

```
>emb|CAC19602.1|  extracellular lipase [Nectria haematococca]
 gb|EEU34629.1|  predicted protein [Nectria haematococca mpVI 77-13-4]
Length=333

 Score =  639 bits (1647),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 308/333 (92%), Positives = 325/333 (97%), Gaps = 0/333 (0%)

Query  1    MMLILSILSIIAFTAAGPVPSVDENTRVLEHRAVTVTTQDLSNFRFYLQHADAAYCNFNT  60
            MMLILSILSIIAF AA PVPS+DEN RVLEHRAVTVTTQDLSNFRFYLQHADAAYCNFNT
Sbjct  1    MMLILSILSIIAFAAASPVPSIDENIRVLEHRAVTVTTQDLSNFRFYLQHADAAYCNFNT  60

Query  61   AVGKPVYCSAGNCPDIEKDAAIVVKSVIGTKTGIGAYVATDNARKEIVVSVRGSINVRNW  120
            AVGKPV+C AGNCPD+EKD+AIVV SV+GTKTGIGAYVATDNARKEIVVSVRGSINVRNW
Sbjct  61   AVGKPVHCGAGNCPDVEKDSAIVVGSVVGTKTGIGAYVATDNARKEIVVSVRGSINVRNW  120

Query  121  ITNFDFGQKACDLVAGCGVHTGFLDAWEEVAANIKAAVTAAKAANPTFKFVATGHSLGGA  180
            ITNF+FGQK CDLVAGCGVHTGFL+AWEEVAANIKAAV+AAK ANPTFKFV TGHSLGGA
Sbjct  121  ITNFNFGQKTCDLVAGCGVHTGFLEAWEEVAANIKAAVSAAKTANPTFKFVVTGHSLGGA  180

Query  181  VATIAAAYLRKDGFPFDLYTYGSPRVGNDFFANFVTQQTGAEYRVTHGDDPVPRLPPIIF  240
            VAT+AAAYLRKDGFPFDLYTYGSPRVGNDFFANFVTQQTGAEYRVTHGDDPVPRLPPI+F
Sbjct  181  VATVAAAYLRKDGFPFDLYTYGSPRVGNDFFANFVTQQTGAEYRVTHGDDPVPRLPPIVF  240

Query  241  GYRHTSPEYWLDGGPLDKDYTVTEIKVCEGMANVMCNGGTVGLDILAHITYFQSMATCAP  300
            GYRHTSPEYWLDGGPLDKDYTV+EIKVC+G+ANVMCNGGT+GLDILAHITYFQSMATCAP
Sbjct  241  GYRHTSPEYWLDGGPLDKDYTVSEIKVCDGIANVMCNGGTIGLDILAHITYFQSMATCAP  300

Query  301  IAIPWKRDMSDEELEKKLTRYSELDQEFVKQMT  333
            IAIPWKRDMSDEEL+KKLT+YSE+DQEFVKQMT
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```

Fig. 4-4 Alignment of amino acid sequence and sequence data from GenBank using BLAST program

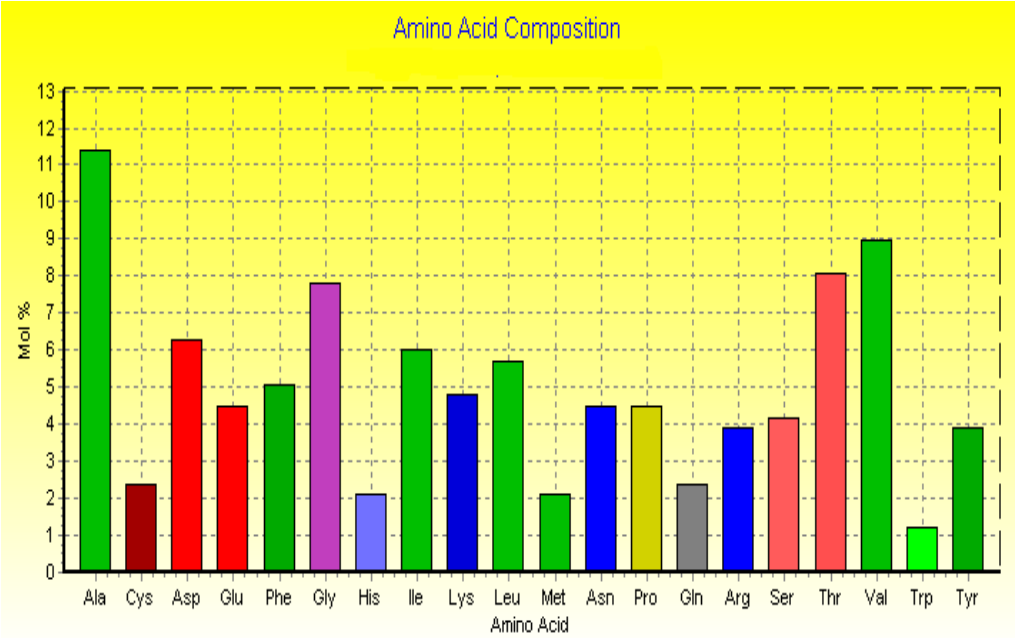

Fig. 4-5 Amino acid composition of lipase from *Fusarium solani* representing the calculated molecular mass of approximately 36 kDa by bioedit program

4.3 Expression of lipase gene in *Pichia pastoris*

The recombinant plasmids were extracted by CTAB method and then digested by *Sac*I to linearise recombinant plasmids. They were later purified and precipitated by phenol/chroloform extraction and ethanol precipitation method, respectively. Then, they were transformed into *Pichia pastoris* strain KM71 which were grown on BMGY plate at 30 °C for a day. The clones were transferred onto BMMY plate which were later incubated at 30 °C. The induction of expression was conducted every 24 hr by adding 150 µl of fresh methanol. The clones with high lipase activities were selected from BMMY plate and were later used to determine the optimal concentration of methanol and optimal time, respectively.

4.3.1 Optimization of the expression

4.3.1.1 Effect of the methanol concentration

The cell medium was shaken at 30 ℃, 250 rpm for 5 days. The optimal concentration of methanol was investigated by adding 1 % to 5 % of fresh methanol to maintain induction every 24 hr. From the results shown in Fig 4-6, the hydrolysis activity gradually increased and the highest activity of 0.114 ± 0.015 μmol/min was reached in the presence of 3% methanol. However, the hydrolysis activity was decreased by approximately 50 % when 4 % of methanol was added and decreased further with 5 % methanol. Hence, the optimal concentration for methanol was 3 %.

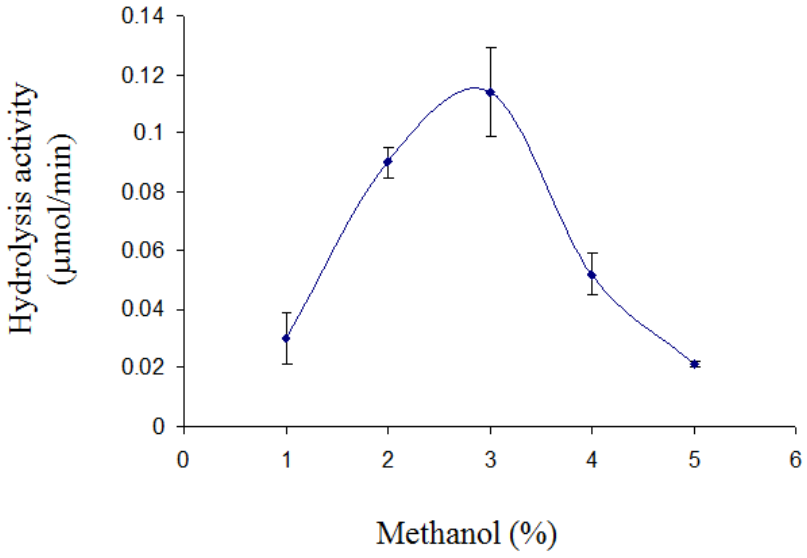

Fig 4-6 The effect of methanol concentration on the hydrolysis activity

4.3.1.2 Effect of the time

Once the optimal concentration of methanol was obtained at 3 %, the optimal time was investigated by adding 3 % of fresh methanol to maintain the induction every 24 hr. The cells were incubated for 1 to 7 days at 30 °C, 250 rpm. From the result, the obtained maximum hydrolysis activity was 0.137 ± 0.012 µmol/min when the cells were incubated for 5 days (Fig 4-7).

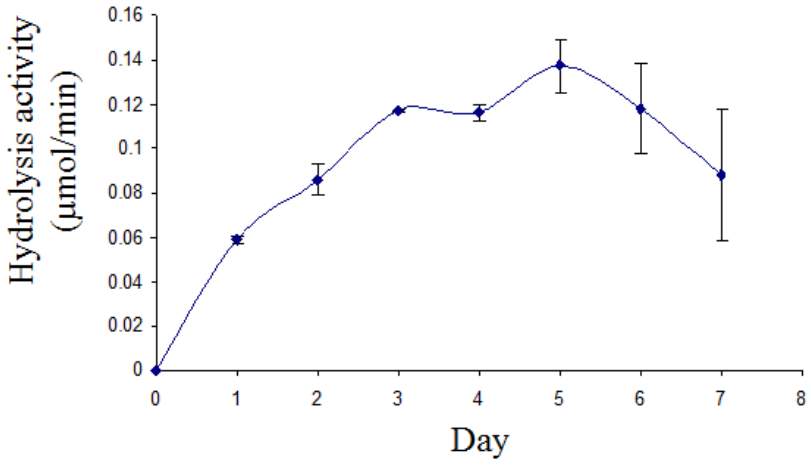

Fig 4-7 The effect of time on the hydrolysis activity

4.4 Purification of the lipase

The recombinant strain was grown at 30 °C, 250 rpm for 5 days and 3 % of fresh methanol was added to maintain the induction every 24 hr. After centrifugation at 5000 rpm at 4 °C for 1.30 hr., the supernatant was concentrated by viva flow and later purified using DEAE Hitrap column. The unbound or the flowthrough proteins were initially obtained by 50 mM Tris buffer pH 7.5 and the adsorbed fractions were

later eluted by the linear gradient of 0-1 M ammonium sulfate as shown in the elution profile in Fig 4-8. From this figure, it was clearly shown that much higher protein content was apparently obtained in the unbound fractions than the eluted. Hence, to confirm the presence of recombinant lipase, all pools of protein fractions were assayed for the lipase activity. From the results, more than ten nmol/min/mg protein of lipase activity was interestingly obtained merely in the flowthrough pool with the presence of recombinant lipase. Table 4-1 summarized the results of the purification and it can be seen that the purity of lipase increased by 2.5 folds after purification which was confirmed by SDS-PAGE shown in Fig 4-9. From the electrophoretic pattern, the single band of recombinant lipase was obtained with the approximate molecular mass of 40 kDa.

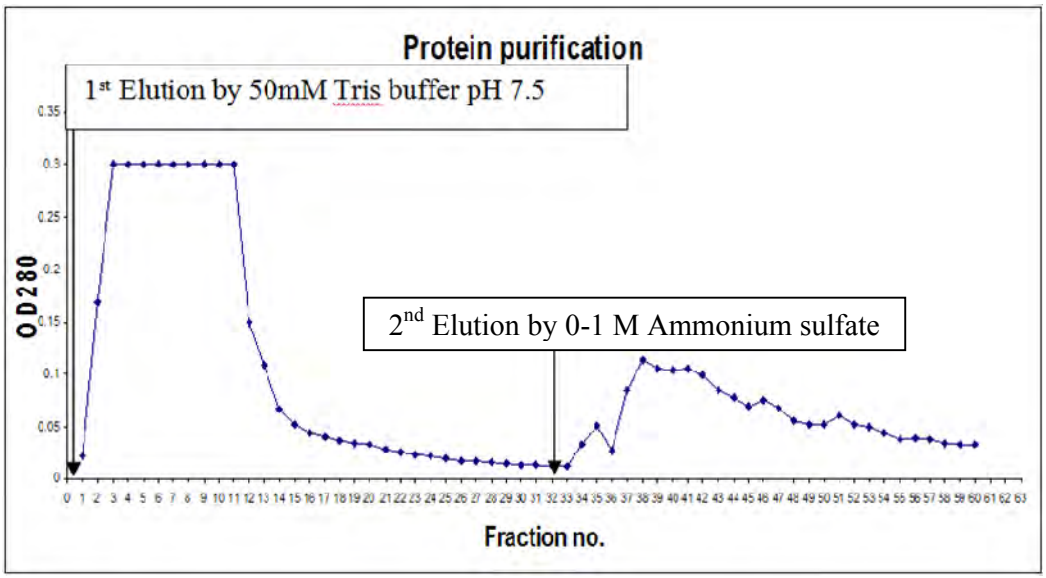

Fig. 4-8 Chromatographic purification profile of recombinant lipase from *Fusarium solani*. Approx. 50 mg of sample was loaded on DEAE Hitrap column (5x1 ml) previously equilibrated with 5 column volumes of 50 mM Tris buffer pH 7.5 with the flow rate of 60 ml/min. 5 ml fractions were collected and the column was finally eluted with linear gradient of 0-1 M ammonium sulfate.

Table 4-1 Purification of lipase from *Fusarium solani*

Fraction	Total protein (mg)	Total activity (μmol/min)	Specific activity (nmol/min/mg protein)	Purification (fold)	Activity Yield (%)
Crude extract	188	2.80	14.9	1	100
Flow through fractions from DEAE	149.5	5.45	36.5	2.5	194.6

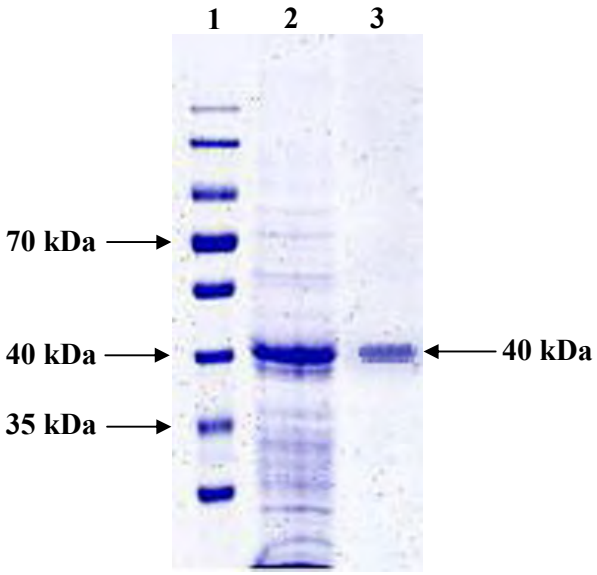

Fig. 4-9 SDS-PAGE analysis of purified lipase from *Fusarium solani*.

Lane 1: molecular weight markers. Lane 2: a crude extract.

Lane 3: purified protein from DEAE Hitrap column chromatography

4.5 Characterization of lipase

4.5.1 Substrate specificity of the purified lipase

Substrate specificity of lipase was studied by using *p*-nitrophenyl esters with various chain lengths as substrates. From Table 4-2, the maximum specific activity was 0.035 ± 0.001 μmol/min/mg protein when *p*NP-caprate (C10) was used as substrate while the minimum was 0.011 μmol/min/mg protein from *p*NP-acetate (C2). From Fig. 4-10, the relative activity was 99.6 ± 0.6 and 98.4 ± 1.9 % when *p*NP-caprate (C10), *p*NP-laurate (C12) were used as substrates, respectively. *p*NP-acetate (C2) gave the lowest relative activity, approximately 32.3 ± 0.8 %. Thus, *p*NP-caprate (C10) and *p*NP-laurate (C12) were specific substrate for purified lipase. Since *p*NP-caprate (C10) is more expensive than *p*NP-laurate (C12), *p*NP-laurate (C12) was therefore selected as the substrate for the subsequent experiments.

Table 4-2 Effect of substrate chain lengths on the specific activity of the purified recombinant lipase

Substrates	Specific activity (μmol/min/mg protein)
C2 (acetate)	**0.011 ± 0**
C4 (butyrate)	**0.031 ± 0**
C8 (caprylate)	**0.032 ± 0.001**
C10 (caprate)	**0.035 ± 0.001**
C12 (laurate)	**0.034 ± 0.001**
C14 (myristate)	**0.033 ± 0**
C16 (palmitate)	**0.025 ± 0.001**
C18 (stearate)	**0.013 ± 0.001**

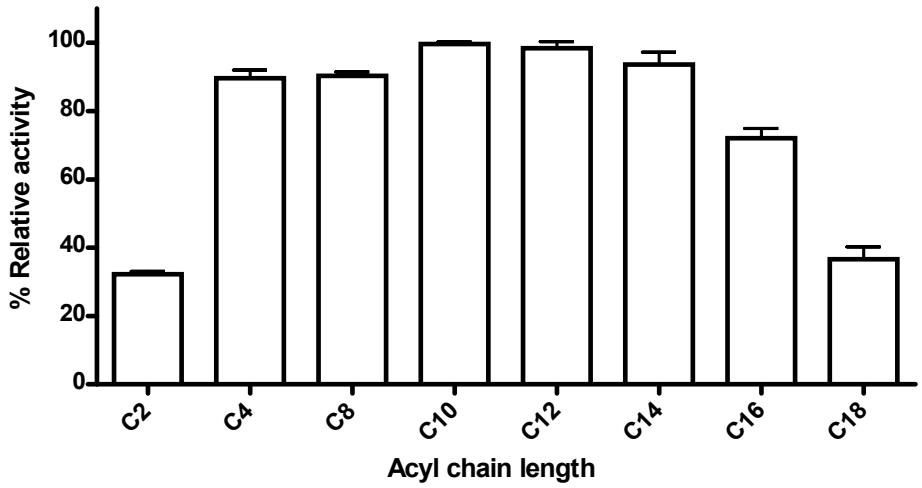

Fig. 4-10 Effect of substrate chain lengths on the specific activity of the purified recombinant lipase

4.5.2 Effect of pH on the activity of lipase

The effect of pH on the purified lipase activity was determined by using *p*-nitrophenyl laurate as the substrate. The optimum pH of enzyme activity was determined in triplicates at room temperature using various buffers with pH ranging from 5.0 to 11.0. The results showed that purified lipase was active at slightly alkaline pH range from 8.5–9.5 and the obtained optimal pH was 9 (Fig. 4-11). Therefore, 50 mM Tris–HCl buffer, pH 9.0 was used to determine the lipase activity in the following experiment.

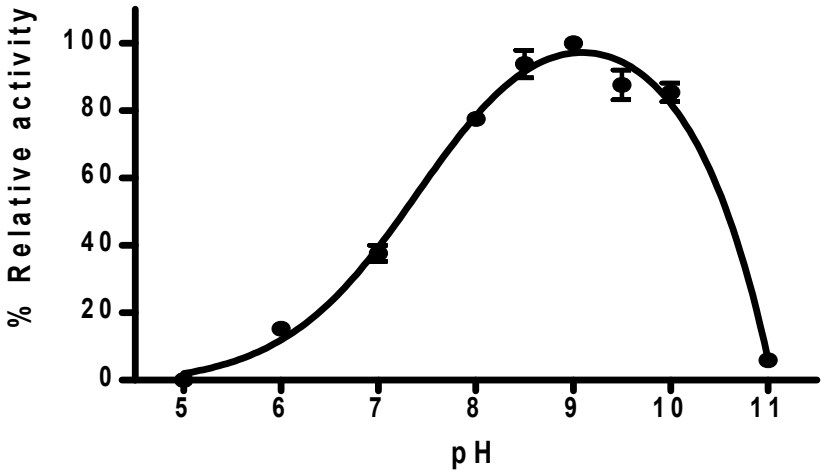

Fig. 4-11 Effect of pH on the activity of the purified recombinant lipase. 0.5 mg/ml of purified lipase was added to various pH buffers from 5.0 to 11.0.

4.5.3 Effect of temperature on the activity of lipase

The effect of temperature on the purified lipase activity was determined by using *p*-nitrophenyl laurate as the substrate. The optimal temperature of lipase activity was determined by measuring the rate of reaction at temperatures ranging from 30 to 60 °C under the standard assay conditions and the results were illustrated in Fig. 4-12. The enzyme exhibited high activities at temperatures range of 35–40 °C.

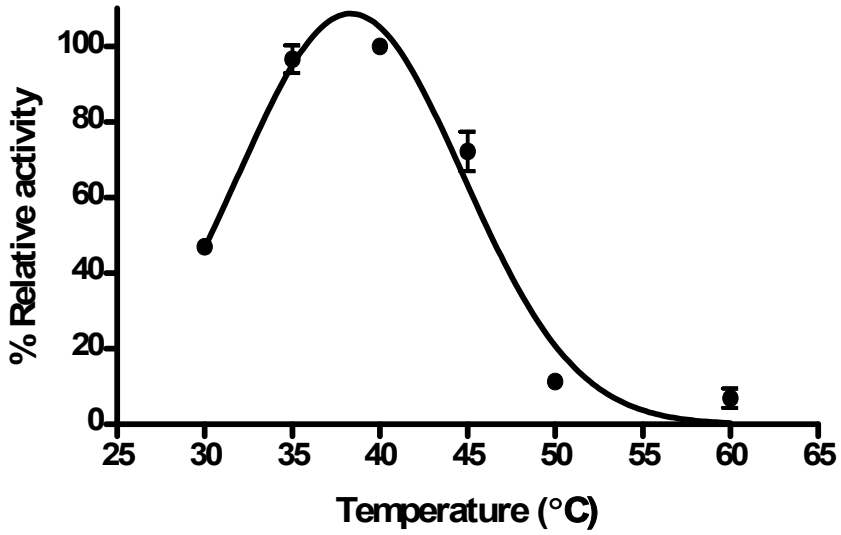

Fig. 4-12 Effect of temperature on the relative activity of the purified recombinant lipase. 0.5 mg/ml of purified lipase was incubated at 30 to 60 ℃.

4.5.4 Stability of recombinant lipase

4.5.4.1 Effect of pH

The effect of pH on lipase stability was determined by incubating the purified lipase (0.5 mg/ml) in the different pH buffers (pH 4.0-11.0) at 4 °C for 20 hr. The residual activity was assayed by spectrophotometric method at room temperature. From Fig. 4-13, it can be seen that 80 % of lipase activity were still retained at pH 5.0 to 10.0 revealing that the obtained recombinant enzyme was relatively stable for the wide range of pH. Nevertheless, pH at which the maximal activity of purified lipase was obtained equaled 8.0

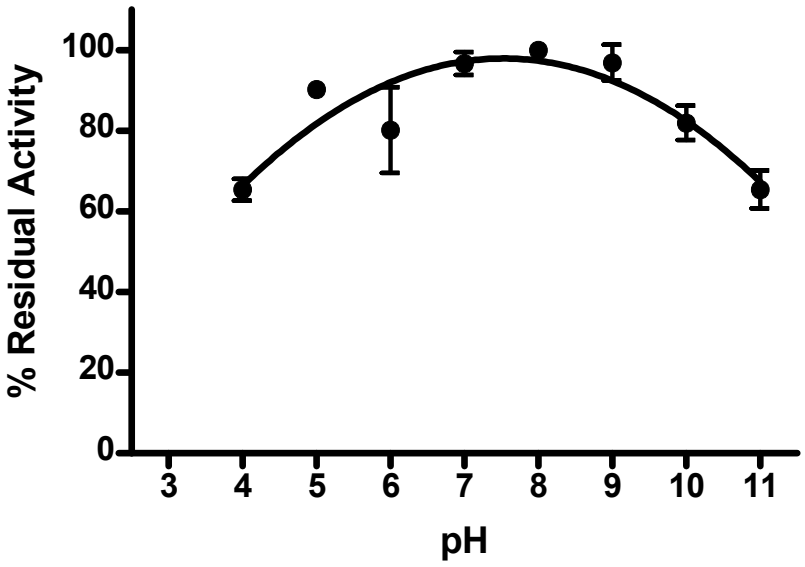

Fig. 4-13 Effect of pH on lipase stability. 0.5 mg/ml of purified lipase was incubated in pH buffer (pH 4-11) at 4 °C for 20 hr.

4.5.4.2 Effect of temperature

To determine the influence of temperature on the enzyme stability, the purified lipase (1.0 mg/ml) was preincubated in 50 mM Tris–HCl buffer, pH 9 at temperature ranging from 20 to 60 °C for 5 hr. Subsequently, the residual activity was analyzed at room temperature. From the result, the enzyme activity retained above 80 % at 20 to 35 °C. However, the residual activity was the highest at 35 °C, approximately 99.9 ± 6.1 %. The activity of the enzyme declined dramatically when the temperature was elevated more than 35 °C and finally lost all of the activity at 40 °C (Fig. 4-14). These results demonstrated that the recombinant lipase is reasonably stable below 35°C.

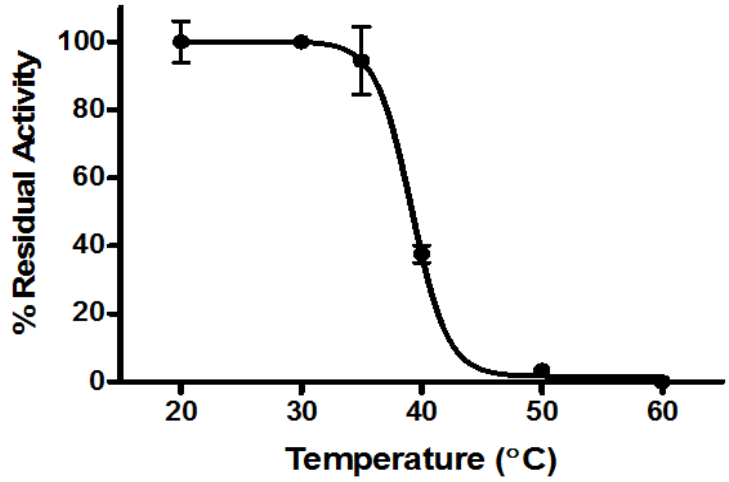

Fig. 4-14 Effect of temperature on lipase stability. The purified lipase (1.0 mg/ml) was preincubated in 50mM Tris–HCl buffer, pH 9 at temperature ranging from 20 to 60 °C for 5 hr.

4.5.5 Effect of metal ions on the activity of lipase

The effect of various metal ions and metal chelating agent, EDTA on the activity of the purified recombinant lipase was studied. The enzyme solution was incubated with various kinds of metal ions and EDTA at 35 °C for 1 hr. The residual activity was measured at room temperature and expressed as the % residual activity relative to the control. The remained activity of purified recombinant lipase after 1 hr. incubation was shown in Fig. 4-15. Both concentrations, 1 mM and 10 mM of metal ions such as K^{+}, Na^{+}, Li^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} showed no significant effect on the lipase activity. This indicated that the recombinant lipase was metal ion independent with the positive confirmation from the result of EDTA which also had no effect on the activity of lipase. Expectedly, 90 % of the activity was sharply inhibited by 10 mM Hg^{2+}.

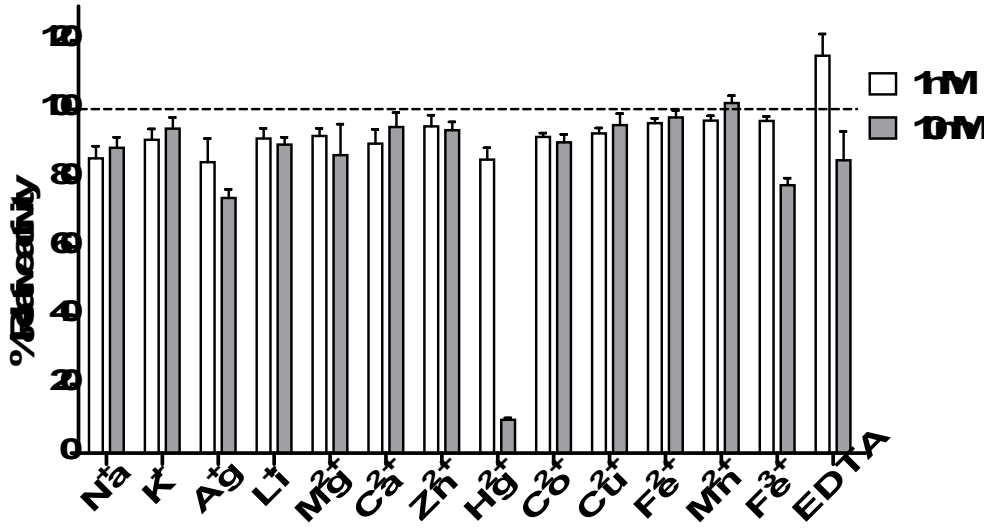

Fig. 4-15 Effect of metal ions on lipase activity. 1 mg/ml of purified lipase was mixed with 50mM Tris–HCl buffer, pH 9 containing 1 or 10 mM of various metal ions such as of K^{+}, Na^{+}, Li^{+}, Ag^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+} and EDTA. The reactions were carried out at 35 ^{o}C for 1 hr.

4.5.6 Effect of chemicals on the activity of lipase

The effect of various kinds of chemicals on the activity of the purified recombinant lipase was studied by mixing enzyme solution with 1 % and 5 % (v/v) of detergents as well as reducing agent incubated at 35 °C for 1 hr. The residual activity was measured at room temperature. The effect of these chemicals on the percentage of enzyme activity was calculated by comparing the residual activity to the control. The comparison of residual activity from both 1 % and 5 % (v/v) chemicals was shown in Fig. 4-16. From the results, most chemicals gave quite similar effect except for SDS and

β-mercaptoethanol. It could be seen that, in the presence of most of the 1 % (v/v) chemicals, 10 to 27 % increase of the residual activity were obtained. The high residual activity, approximately 27 %, 20 % increase were obtained under the influence of reducing agent, DTT and detergent Triton X-100. However, higher concentration of 5 % appeared to decrease the activities. Interestingly, 5 % of β-mercaptoethanol showed 80 % inhibition while both concentrations of SDS showed absolute inhibition.

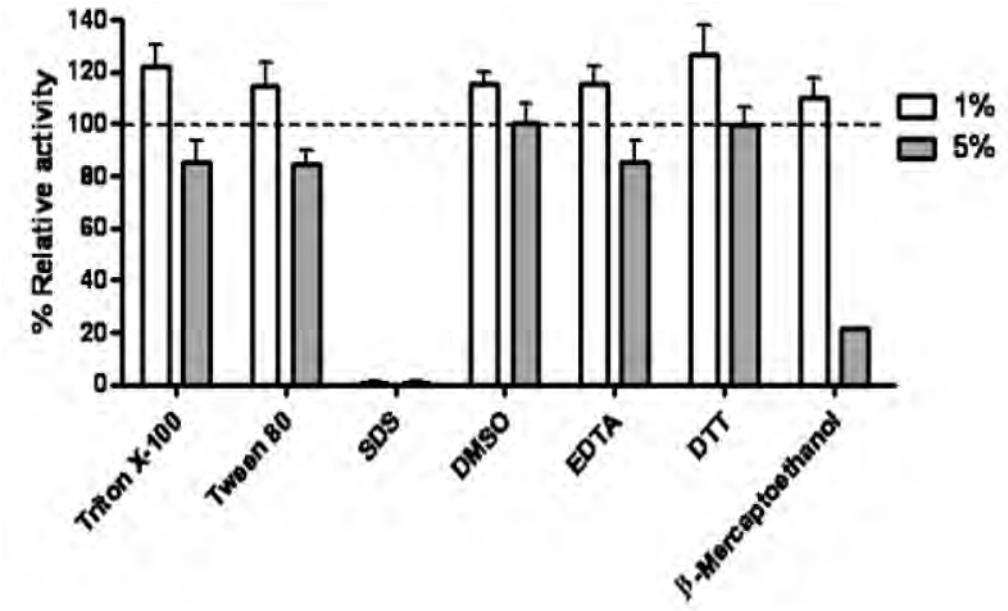

Fig. 4-16 Effect of chemicals on lipase activity. 1.0 mg/ml of purified lipase was dissolved in 50mM Tris–HCl buffer, pH 9 and incubated for 1 hr. at 35 °C with 1 % and 5 % (v/v) of various chemicals.

4.5.7 Effect of organic solvents on the activity of lipase

The effect of various organic solvents on the activity of the purified recombinant lipase was studied by incubating enzyme solution in 50 % (v/v) of various polar and non-polar organic solutions at room temperature for 1 and 6 hr. The residual activity was measured at room temperature. The organic solvent tolerance of enzymes was calculated from the % residual activity relative to the control. The retained activities of lipases after incubation were shown in Fig. 4-17. From the results, it can be seen that different polarity of organic solvents showed completely opposite effect on the enzyme. Apparently, the recombinant enzyme displayed more tolerance towards non-polar solvents; namely diethyl ether, benzene, cyclohexane, hexane, n-heptane and isooctane than the polar such as methanol, ethanol, acetone, iso-propanol and butanol. The highest residual activity was obtained after incubation for 1 and 6 hr. (100.8 ± 4 % and 94 ± 15 %) in the presence of cyclohexane and n-heptane, respectively.

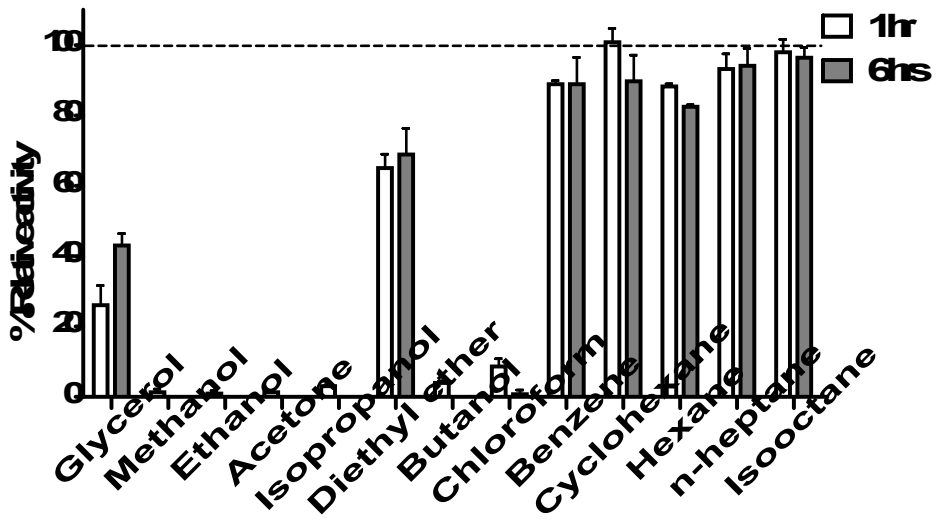

Fig. 4-17 Effect of organic solvents on lipase activity. Purified lipase was incubated with 50mM Tris–HCl, pH 9 in the presence of 13 types of the solvents; methanol, ethanol, iso-propanol, glycerol, butanol, acetone, hexane, cyclohexane, n-heptane, chloroform, isooctane, benzene and diethyl ether. The reaction mixture was incubated at 35 °C and stirred at 300 rpm for 1 and 6 hr.

4.5.8 Effect of alcohol on the activity of lipase

From the results in 4.5.7, the purified lipase was found to be completely inactivated by 50% methanol and ethanol. The concentrations of both organic solvents and the incubation period at which lipase could exhibit tolerance were therefore determined.The results showed that the enzyme could tolerate the concentrations of 0.5-2 % both methanol and ethanol for 12 hr and still retained 80% of the activity.

Considering the effect of the incubation period, the purified lipase was incubated with 0.5, 1, 2 and 3 % of methanol at 35 °C, 300 rpm for 12 and

24 hr in comparison. From Fig. 4-18 and 4-19, it was found that longer incubation period at 24 hr, the lipase could still retain 80 % of the activities in the presence of 0.5 % of both alcohols but decreased further by higher concentrations from 1-3 %. Similarly, the presence of ethanol gave the same trend as methanol in both the concentrations and the incubation time illustrated in Fig 4-19.

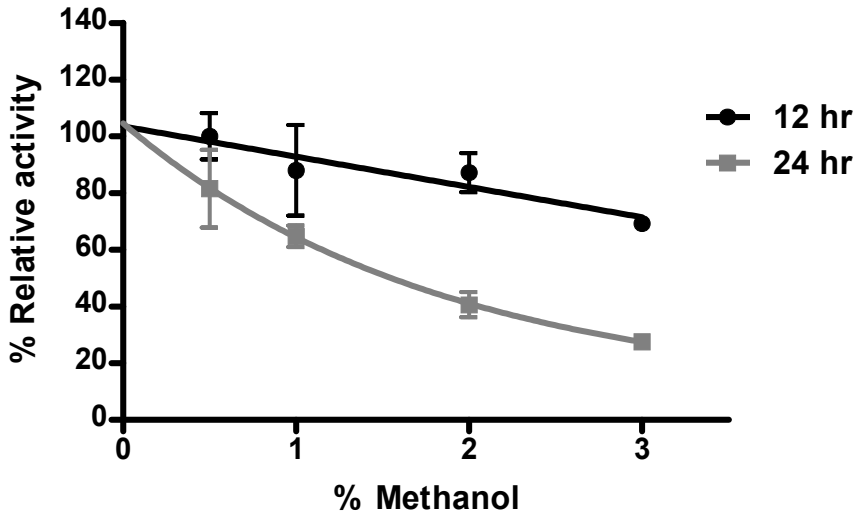

Fig. 4-18 Effect of methanol on lipase activity. 0.5 mg/ml of purified lipase was dissolved in 50mM Tris–HCl buffer, pH 9 containing 0.5, 1, 2 and 3 % methanol. The reaction was carried out by incubating at 35 °C and stirring at 300 rpm for 12 and 24 hr.

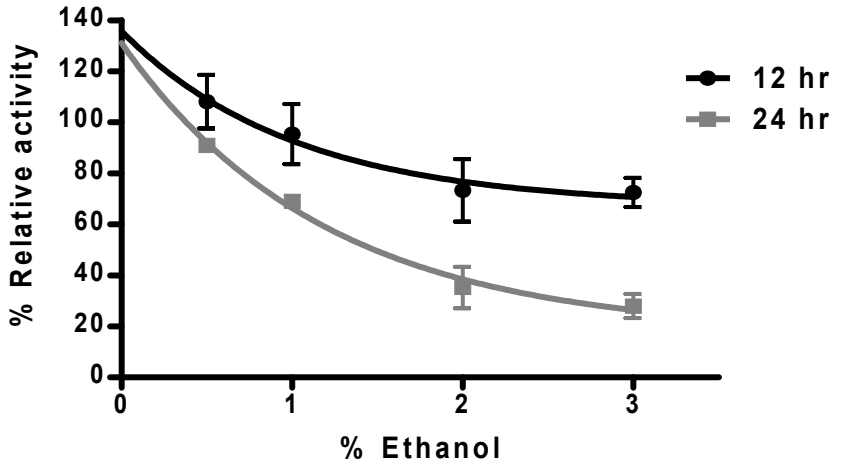

Fig. 4-19 Effect of ethanol on lipase activity. 0.5 mg/ml of purified lipase was dissolved in 50mM Tris–HCl buffer, pH 9 containing 0.5, 1, 2 and 3 % ethanol, and incubated at 35 °C for 12 and 24 hr.

4.5.9 Hydrolysis of oil

Hydrolysis was performed by using seven types of oil namely; coconut, olive, palm, safflower, physic nut papaya and rambutan. The reaction was carried out by incubating at 35 °C, 300 rpm for 5 hr. The results showed that all the oil samples could be hydrolyzed by the purified recombinant lipase and the specific activities obtained were tabulated in Table 4-3. However, the highest specific activity was 1.136 ± 0.084 μmol/min/mg protein when coconut oil was used as substrate. From the table, the coconut, palm, papaya and rambutan oil were therefore subsequently selected as the substrates for transesterification according to their specific activities respectively. The calculation of the lipase activity was described in Appendix D.

Table 4-3 Hydrolysis of oil

Oils	Specific activity µmol/min/mg protein
Coconut	**1.136 ± 0.084**
Olive	**0.639 ± 0.013**
Palm	**0.72 ± 0.010**
Safflower	**0.535 ± 0.009**
Physic nut	**0.558 ± 0.027**
Papaya	**0.705 ± 0.013**
Rambutan	**0.756 ± 0.124**

4.5.10 Transesterification of oil

The oil samples from coconut, palm, papaya and rambutan selected from hydrolysis assay (4.5.9) were used as substrates in transesterification for the production of biodiesel. The mixture was composed of oil, purified lipase and methanol. The reaction was stirred at 300 rpm, 35 °C for 24 hr. and the samples were taken for analysis by HPLC. From the results, about 3.31 ± 4.68 %, 7.03 ± 1.73 %, 9.88 ± 3.14 % and 44.7 ± 2.2 % of fatty acid methyl esters could be obtained from coconut, palm, papaya and rambutan oil, respectively.

From this study, it can be seen that rambutan oil might have been applied as the substrate for transesterification catalyzed by the lipase from *Fusarium solani*.

CHAPTER V

DISCUSSION

In general, the most preferrable source of microbial lipases is from the fungus since the obtained excreted extracellular enzymes can facilitate the extraction from fermentation media. The production of lipase by several strains of *Fusarium sp*. has been studied in terms of enzyme production, protein, properties and purification. Among all of studied *Fusarium sp*., *Fusarium solani* is the most common species which can be recovered in humans, animals, plants and soil. In this study, the fungal samples, NAN 103, were obtained from the culture collection of Biofuels By Biocatalysts Research unit, Faculty of Science, Chulalongkorn University (Malilas, 2006). The samples were initially confirmed for the identification and were further studied for cloning, expression, purification and characterization of the obtained recombinant lipases.

5.1 Identification of the lipase producing fungi

The fungi, NAN103 were morphologically identified. When observed under the microscope, the colonies of white puffy fibers with cross walls that divide the cytoplasm into segments called septate hyphae were detected (Malilas, 2006).

After the samples were morphologically identified, they were genetically confirmed using the internal transcribed spacer (ITS); the nucleotide sequences localized at 5.8 ribosomal RNA. In this work, such conserved region was used to specify the definite species of fungi. The ITS gene was amplified by a pair of primers namely; ITS1F and ITS4 by PCR technique. The 564 bp PCR products were obtained and sequenced. The submitted sequence showed 99% identity with the closest known

relative of *Fusarium solani* . Hence, the identification of the samples was confirmed as follows.

Kingdom: Fungi

Phylum: Ascomycota

Class: Pyrenomycetes

Order: Hypocreales

Genus: *Fusarium*

Species: *Fusarium solani*

5.2 Cloning of the lipase gene

In this study, the fragment containing the lipase operon was amplified by PCR technique with the primers FSF1 and FSRS1. From Fig. 4-3, the result showed that the size was 1,002 bp containing an ORF of 999 bp. The recombinant plasmid was successfully constructed by ligating the purified lipase gene into pPICZαA vector and later subjected for sequencing. The obtained sequences were selected by Chromas Lite and BioEdit program and aligned to GenBank database using the BLAST program. The results showed 92 % identity to extracellular lipases of *Fusarium solani* (Fig. 4-4). At neutral pH, the deduced amino acid sequence revealed the protein of 333 amino acid residues with the same numbers of 36 negatively charged (Asp and Glu) and 36 positively charged (Arg, Lys and His) residues. The total number of charged residues (Asp, Glu, Arg, Lys and His) apparently account for 72 (21.6 %) from the total number of amino acids in the protein molecule. On the other hand, it was found that the hydrophobic amino acids (Ala, Ile, Leu, Met, Phe, Pro, Trp and Val) make up 45 % of the protein. According to the amino acid composition (Fig. 4-5), the calculated molecular mass was approximately 36 kDa. From all of the

mentioned above, the obtained lipase gene was closely related to that of *Fusarium solani* which consists of 999 bp in an ORF and the encoded protein consists of 333 amino acids with a molecular weight of 35 kDa (Eddine *et al.*, 2001). Hence, the primary morphological identification of the fungus was positively confirmed.

5.3 Expression of lipase gene in *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast which has been shown to be a more effective production host than *Saccharomyces cerevisiae* (Shen *et al.*, 2009). This microorganism is well suited for the expression of heterologous proteins since it is easy for genetic manipulation. The cells are also easier to culture with high density than the mammalian cells. Purification procedure required for the secreted heterologous proteins is also simple (Daly and Hearn, 2005). Moreover, it can carry post-translational modification of foreign proteins such as glycosylation similar to mammalian and insect cells (Abdelmoula-souissi *et al*., 2007). The yeast, *Pichia pastoris* is capable of metabolizing methanol as its sole carbon source. The metabolism of methanol is the oxidation of methanol to formaldehyde and hydrogen peroxide using oxygen by the enzyme alcohol oxidase. The optimal methanol concentration and incubation time are the important factors for the expression. Too high the concentration of methanol may lead to low expression of protein or even death. On the other hand, too low the concentration of methanol added in the reaction may not be sufficient. Similarly, the shorter time may not be suitable for expression of yeast cell whereas the longer time may attribute to cell damage. In this research, the recombinant plasmid was transformed into *Pichia pastoris* strain KM71 as expression vector. The expressed protein was secreted into the culture medium and the expression level of protein was induced in the presence of different methanol

concentrations and time of induction. The results showed that the optimal condition for the expression of lipase from *Fusarium solani* in *Pichia pastoris* strain KM71 was 3 % of methanol for the period of 5 days. This is not consistent with the results from *Galactomyces geotrichum* Y05 in which 0.5 % of methanol for 6 days were necessary (Yan *et al*., 2007). Thongekkaew and Boonchird studied the induction in *Candida thermophilia*, the results showed that 0.5 % methanol for the period of 7 days were required for the optimization (Thongekkaew and Boonchird, 2007).

5.4 Purification of the lipase

From the results in Fig. 4-8, the activities of lipase from DEAE cation exchanger column from the pools of negatively charged recombinant proteins was obtained from the unbound flowthrough pool and the purity increased by 2.5 folds. When the sample was tested for homogeneity from SDS-PAGE, the single band was obtained with the approximate MW of 40 kDa (Fig 4-9). The calculated molecular mass was approximately 36 kDa which was closed to 39 KDa of an extracellular lipase from *Yarrowia lipolytica* (Yu *et al*., 2007) and 45 kDa of mature lipase (SAL3) from *Staphylococcus aureus* (Horchani *et al*., 2009). The obtained higher molecular weight may have been the result from the post-translational glycosylation in which the site of N-glycosylation (Asn-Pro-Thr) was found at position-165 according to amino acid sequences (Rotticci-Mulder *et al*., 2001).

5.5 Characterization of lipase

5.5.1 Substrate specificity of the purified lipase

Generally, lipases can hydrolyze *p*-nitrophenyl esters with various chain lengths (C2-C18). Moreover, the specificity for the acyl chain length of

substrate fatty acids varies according to the shape and size of the active site groove or pocket (Lee and Swaisgood, 1998). The substrate specificity of the recombinant lipase in this work was performed with substrates, *p*-nitrophenyl esters, with the various numbers of carbon chain lengths ranging from C2-C18. From the results, the approximately 90-nearly 100 % residual activities were obtained from C4-C14. The highest activity of the enzyme was obtained towards both C10 and C12 similar to the bacterial lipase from psychrotrophic *Pseudomonas* sp. (Kumar *et al*., 2005). In addition, medium chain lengths (*p*NP-C4 - *p*NP-C8), *p*-nitrophenyl laurate (*p*NP-C12) and *p*-nitrophenyl myristate (*p*NP-C14) appeared to be the good substrates for purified lipase whereas the poor activities were obtained towards *p*NP-C2 and *p*NP-C18. These results demonstrated that the recombinant lipase has the strong catalytic ability to the substrates with medium chain lengths but displayed low activities towards the substrates with short and long chains. In contrast, the native lipase from *Fusarium solani* reported by Winayanuwattikun *et al* exhibited a narrow range of specificity towards only *p*-nitrophenyl laurate (*p*NP-C12) (Winayanuwattikun *et al*., 2011). This contradictory results may have been the consequence from the obtained higher molecular weight of purified lipase resulted from the N-glycosylation of the lipase molecule. This might have altered the amino acid sequence at the active site resulting in the differences of the substrate specificity.

5.5.2 Effect of pH on the activity of lipase

From the result in 4.5.2, the optimal pH of purified lipase was pH 9.0. This result was similar to *Fusarium solani* FS1 lipase in which

low activity was obtained from pH lower than 8.0. The other lipase producing fungi such as *Aspergillus sp*. and *Mucor sp.* also showed the optimal pH at alkaline pH approximately at 10 (Savitha *et al.,* 2007).

However, extracellular lipase of *Fusarium oxysporum* f. sp. *line* and *Fusarium oxysporum* f. sp. *vasinfectum* showed optimal pH at 7.0 and 5.8, respectively (Maia *et al*., 1999).

5.5.3 Effect of temperature on the activity of lipase

The optimal temperature of purified recombinant lipase was between 35 to 40 ℃, slightly higher than at 37 ℃ of native lipase from *Fusarium solani* (Eddine *et al.*, 2001). Nevertheless, the result was consistent to extracellular lipase, YlLip7, from *Yarrowia lipolytica* (Yu *et al*., 2007).

5.5.4 Stability of recombinant lipase

5.5.4.1 Effect of pH

From Fig. 4-13, regarding the trend of the results obtained, the purified recombinant lipase was apparently stable in a broad range of pH values between pH 5.0 to 10.0 by retaining over 80 % of the residual activity. This indicated that the recombinant enzyme was stable at both slightly acid and more alkaline pH which was quite similar to the extracellular lipase from *Fusarium oxysporum* (Maia *et al*., 1999).

5.5.4.2 Effect of temperature

For the thermal stability study in 4.5.4.2, the results illustrated that the residual activity of the purified lipase was highest at 35 °C and still retained the activity above 80 % at the temperature between 20-35 °C. The obtained recombinant lipase was obviously more stable in wider range of temperature than the lipase of *Fusarium solani* FS1 in which the maximum of stability was observed in the temperature range from 25-30 °C (Maia *et al*., 1999).

5.5.5 Effect of metal ion on the activity of lipase

The effect of various metal ions on the activity of the purified recombinant lipase was studied by incubating enzyme solution with various kinds of metal ions at 35 °C for 1 hr. From the results (Fig. 4-15), almost all studied metal ions had no effect on lipase activity indicating that *Fusarium solani* lipases activities were metal ion independent. This is consistent to the previous reports that Ca^{2+} and Mg^{2+} cations did not produce adverse effect. On the other hand, calcium is generally known to stabilize lipolytic enzymes (Côté and Shareck, 2008). Nevertheless, strong inhibition by Hg^{2+} on the activity of the recombinant lipase was expected due to the alteration of enzyme conformation resulting from heavy metal denaturation (Yan *et al*., 2007). Moreover, when 1mM of EDTA was tested, 20% increase of the activity was clearly observed more than control. However, increase of the concentration to 10 mM resulted in the significant drop of lipase activity. This indicated that only low concentration of EDTA was likely to enhance the activity of recombinant lipase. Similarly, the EDTA was reported to have no

effect on the lipase from *Bacillus subtilis* suggesting that the enzyme was not a metalloenzyme (Kamini *et al*., 2000). However, our results were opposite to psychrotrophic bacterium, *Pseudomonas* sp. strain KB700A reported by Rashid *et al*., 2001 in which addition of EDTA completely abolished lipase activity.

5.5.6 Effect of chemicals on the activity of lipase

The effects of various types together with the concentrations of the chemicals on the activity of the purified recombinant lipase were studied by incubating enzyme solution with 1 % and 5 % (v/v) of chemicals at 35 °C for 1 hr. two categories of chemicals were studied namely; the detergents such as Triton X-100, Tween-80, and SDS and the reducing agents such as DTT and β- mercaptoethanol and together with DMSO. From the effect of concentration (Fig. 4-16), the results showed that 1 % v/v of most chemicals tended to enchance the activity of recombinant lipase whereas slight inhibition was observed at higher concentration of 5 % (v/v). However, both 1 and 5 % of SDS exhibited 100 % inhibitory effect and 5 % β-mercaptoethanol showed 80 % inhibition. SDS is the anionic detergent with the ability to disrupt the structure of proteins which can unfold the structural protein or polypeptide to the single strand with negative charges. β-mercaptoethanol affects native protein structure via its ability to cleave disulfide bonds. Nevertheless, the purified recombinant lipase was not inhibited by DTT, it has high conformational propensity to form a six-membered ring with an internal disulfide bond contributing to the difficulty to react with disulfide bonds in protein molecule. Moreover, many studies have shown that disulfide bonds are

not needed for the enzyme activity but rather for stability and correct folding formation of the enzyme structure (Côté and Shareck, 2008). In consistence, almost all studied detergents such as Triton X-100 and Tween-80, as well as reducing agent like DTT showed slight enhancement (approximately 10-20 %) on the activity of lipase. Similar to the previous report, DMSO was found to have a marked stimulatory effect on lipase activity by increasing the proportion of substrate present in a monomeric form. Since the lipase was active in both soluble and emulsified substrates, this effect of DMSO could partly be accounted for by an effect of apparent increase in substrate concentration, i.e. by an increase in the concentration of substrate accessible to the lipase. Moreover, it possibly enhances the activity of lipase via the disaggregation of the lipase (Kamini *et al.,* 2000).

5.5.7 Effect of organic solvents on the activity of lipase

The effect of various organic solvents on the activity of the purified recombinant lipase was studied by incubating enzyme solution in 50 % (v/v) of various polar and non-polar organic solutions at 35 °C for 1 and 6 hr. From the results (Fig. 4-17), the recombinant enzyme appeared to display more tolerance towards non-polar solvents; namely hexane, cyclohexane, n-heptane, isooctane, benzene and diethyl ether than the polar such as methanol, ethanol, iso-propanol, butanol and acetone. In general, lipases are diverse in their sensitivity to solvent but there is general agreement that polar water miscible solvents are more destabilizing than water immiscible solvents. In polar organic solvents group, the highest residual activity was shown in the presence of glycerol. It has been proposed that a thin layer of water molecules tightly

bound to the enzyme acting as a protective sheath along the enzyme's hydrophilic surfaces and allowing retention of the native conformation. Conversely, methanol, ethanol, iso-propanol, butanol, chloroform and acetone were shown to be the strong inhibitors by causing the rapid protein denaturation or disturbance of the reaction mixture interface (Yan *et al*., 2007).

5.5.8 Effect of alcohol on the activity of lipase

The concentration required for both methanol and ethanol, plays important role in transesterification for the production of biodiesel since the excessive alcohol content will certainly inactivate lipase (Ma and Hanna, 1999; Fukuda *et al.,* 2001). Hence, the percentage of alcohol tolerance for the recombinant lipase was studied in comparison. The purified lipase was mixed with 0.5, 1, 2 and 3 % of alcohol and incubated at 35 °C, 300 rpm for 12 and 24 hr. Various short-chain alcohols (C1-C2) were the acyl acceptors in transesterification (Yang *et al*., 2007). From the results in 4.5.8 (Fig. 4-18 amd Fig. 4-19) the high residual activity was obtained from incubating purified lipase with 0.5 - 2 % of both alcohols retaining 80% of activity.

5.5.9 Hydrolysis of oil

Hydrolysis was performed by using seven types of oil such as palm, olive, safflower, coconut, physic nut, papaya and rambutan. With regard to the suitability of such oils as the feedstock of biodiesel production, palm and coconut have been widely used for this purpose. Additionally, physic nut, papaya and rambutan were the agricultural waste from the industrial sector.

The property of biodiesel produced from these oils have passed the specification among the three biodiesel standards; Biodiesel standards of Thailand (2007), USA (ASTM D6751-07a) and European Standards Organization (EN 14214:2003) (Winayanuwattikun *et al*., 2008). From the results shown in Table 4-3, the purified recombinant lipases revealed the hydrolytic activities towards all of the studied oils. The specific activity of more than 0.5 μmol/min/mg protein was obtained. From this study, it could therefore be concluded that lipase from *Fusarium solani* can catalyze many substrates with a wide variety of hydrocarbon chain lengths. Nevertheless, the highest specific activity of 1.136 ± 0.084 μmol/min/mg protein was obtained when coconut oil was used as substrate. The main composition of fatty acids are as follows: 12:0 (34.37), 14:0 (13.75), 16:0 (9.29), 18:0 (10.53), 18:1 (12.34), 18:2 (6.46), 18:3 (0.72) and 20:0 (7.72) (Winayanuwattikun *et al*., 2008). From this, the C-12 substrate yielded the best hydrolysis of oil which was positively correlated to the result from substrate specificity of the enzyme recombinant in 5.5.1. Hence, C-12 was the most suitable substrate for hydrolysis consistent with the high content in palm and papaya oils. This is different from the quite low content of C-12 but high specific activity from the rambutan oil.

5.5.10 Transesterification of oil

The four types of plant oils with higher specific activities for hydrolysis were investigated as the substrates in transesterification for the production of biodiesel namely; coconut, palm, papaya and rambutan oils respectively. The reaction mixture was composed of oil, purified lipase and

methanol, continuously stirred at 300 rpm, 35 °C for 24 hr. The samples were taken for analysis by HPLC. From the results, the highest biodiesel production yield of 44.7 ± 2.2 % was obtained from rambutan oil whereas the lowest fatty acid methyl ester of 3.31 ± 4.68 % was obtained from coconut oil. Notably, the transesterification of coconut oil gave the contradictory result from hydrolysis. This may be the results from the high percentage of water content and the differences in composition of fatty acids in the coconut oil causing the reaction to favour the hydrolytic reaction instead of transesterification.

CHAPTER VI

CONCLUSION

From this study, the lipase producing fungus was genetically confirmed as *Fusarium solani.* The genome was extracted and the lipase gene was amplified by specific primers. The gene was successfully cloned into pPICZαA using *E.coli* DH5α as the competent cells. After analysis by BLAST program, the open reading frame was composed of 999 bp encoding 333 amino acids. The lipase gene was then expressed by linearizing the recombinant plasmids with *Sac* I digestion and later transformed into *Pichia pastoris* strain KM71. Additionally, the expression conditions were optimized in which 3 % of methanol for the period of five days was the most suitable regarding the lipase activity. The expressed product was later purified and the obtained lipase was 2.5 folds higher in purity, molecular mass of approximately 40 kDa with 194.6 % of activity yield. The recombinant lipase showed the strong catalytic ability towards the broad range of substrates with carbon numbers of medium chain lengths between C4-C14. Nevertheless, the highest activity was obtained from *p*-nitrophenyl caprate, *p*NP-C10 and *p*-nitrophenyl laurate, *p*NP-C12 with optimal pH and temperature at 9 and 35-40 °C respectively. The lipase was found to be stable at pH range between 5.0 and 10.0 and temperature below 35 °C. The studies on the influence of various chemicals showed that the activity of the enzyme was metal ion independent. Furthermore, almost all studied detergents resulted in the slight enhancement of the activity and the lipase displayed tolerance towards non-polar solvents. However, the enzyme was strongly inhibited by SDS and reducing agent, β-mercaptoethanol. When the enzyme was assayed for hydrolytic activity with 7 types of plant oils namely, palm, coconut, olive, safflowers, physic nuts, papaya and rambutan, the highest specific activity of

1.136 ± 0.084 μmol/min/mg protein was obtained with coconut oil as a substrate. Finally, the transesterification with 4 types of oils with high hydrolytic activities; coconut, rambutan, palm and papaya as substrates was investigated for the production of biodiesel. The highest production of 45 % biodiesel was obtained from rambutan oil which was higher than papaya, palm and coconut respectively.

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APPENDICES

APPENDIX A

REAGENT PREPARATION

1. Preparation for media

1.1 Buffered Glycerol-complex Medium (BMGY) 1 L

Peptone	20	g
Yeast extract	10	g

All components were dissolved in 700 ml distilled water and then steriled at 121 ° C, 15 1b/in^2 for 15 min. Next, the mixture was cooled down at room temperature and added the following and mix well:

1 M potassium phosphate buffer, pH 6.0	100	ml
10X Yeast Nitrogen Base	100	ml
500X Biotin ($4x10^{-5}$ % biotin)	2	ml
10X Glycerol	100	ml

Stored media at 4 ° C

1.2 Buffered Glycerol-complex Medium plate (BMGY plate) 1 L

Peptone	20	g
Yeast extract	10	g
Agar	15	g

All components were dissolved in 700 ml distilled water and then steriled at 121 ° C, 15 1b/in^2 for 15 min. Next, the mixture was cooled down at room temperature and added the following and mix well:

1 M potassium phosphate buffer, pH 6.0	100	ml

10X Yeast Nitrogen Base	100	ml
500X Biotin ($4x10^{-5}$ % biotin)	2	ml
10X Glycerol	100	ml

Stored media at 4 ° C

1.3 Buffered Methanol-complex Medium (BMMY) 1 L

Peptone	20	g
Yeast extract	10	g

All components were dissolved in 700 ml distilled water and then steriled at 121 ° C, 15 1b/in^2 for 15 min. Next, the mixture was chilled at room temperature and added the following and mix well:

1 M potassium phosphate buffer, pH 6.0	100	ml
10X Yeast Nitrogen Base	100	ml
500X Biotin ($4x10^{-5}$ % biotin)	2	ml
10X M (0.5 % methanol)	100	ml

Stored at 4 ° C

1.4 Buffered Methanol-complex Medium plate (BMMY plate) 1 L

Peptone	20	g
Yeast extract	10	g
Agar	15	g

All components were dissolved in 700 ml distilled water and then steriled at 121 ° C, 15 1b/in^2 for 15 min. Next, the mixture was chilled at room temperature and added the following and mix well:

1 M potassium phosphate buffer, pH 6.0	100	ml

10X Yeast Nitrogen Base	100	ml
500X Biotin ($4x10^{-5}$ % biotin)	2	ml
10X M (0.5 % methanol)	100	ml

Stored media at 4 ° C

1.5 Low Salt Luria-Bertini (LB) broth 1 L

Tryptone	10	g
Yeast extract	5	g
NaCl	5	g

All components were dissolved in 950 ml distilled water. The pH of solution was adjusted to 7.5 with NaOH and then brough the volume up to 1,000 ml by distilled water. Subsequently, the mixture was autoclaved at 121 ° C, 15 1b/in^2 for 15 min.

1.6 Luria-Bertini (LB) broth 1 L

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g

All components were dissolved in 1,000 ml distilled water and then autoclaved at 121 ° C, 15 1b/in^2 for 15 min.

1.7 LB agar 1 L

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g

Agar	15	g

All components were dissolved in 1,000 ml distilled water and then autoclaved at 121 ° C, 15 1b/in^2 for 15 min.

1.8 Yeast Extract Peptone Dextrose Medium (YPD) 1L

Peptone	20	g
Yeast extract	10	g

All components were dissolved in 900 ml distilled water and then autoclaved at 121 ° C, 15 1b/in^2 for 15 min. Next, the mixture was chilled at room temperature and then added 100 ml of 20 % dextrose.

2. Preparation for DNA extraction

2.1 Washing buffer

PVP (polyvinylpyrrolidone)	2	g
Ascorbic acid	1.76	g
1 M Tris buffer pH 8.0	20	ml
2-mercaptoethanol	4	ml

The steriled water was added for adjusting volume to 200 ml and stored at 4 ° C

2.2 2X CTAB lysis buffer

CTAB	4	g
1 M Tris buffer pH 8.0	20	ml
1 M EDTA pH 8.0	8	ml
NaCl	16.36	g

2-mercaptoethanol	1	ml

The steriled water was added for adjusting volume to 200 ml and stored at 4 ° C

2.3 20 % Polyethylene glycol 6000 (PEG)

PEG	20	g
NaCl	14.61	g

The steriled water was added for adjusting volume to 200 ml and stored at 4 ° C

2.4 50 X Tris-Acetate-EDTA buffer (TAE buffer) 1 L

Tris base	242	g
Acetic Acid	57.1	ml
0.5 M EDTA	100	ml

The distilled water was added for making up volume to 1 liter and its pH was adjusted to 8.5 by using KOH. Then, it was autoclaved at 121 ° C, 15 1b/in^2 for 15 min and stored at room temperature.

2.5 50 X Tris-EDTA buffer (TE buffer)

1 M Tris buffer pH 8.0	500	ml
0.5 M EDTA pH 8.0	100	ml

The distilled water was added for adjust volume to 1 liter and autoclaved at 121 ° C, 15 1b/in^2 for 15 min, followed by storing at room temperature.

2.6 1 % Agarose gel

Agarose	1	g
1 X TAE buffer	100	ml

2.7 Lysis buffer

100 mM NaOH

60 mM KCL

5 mM EDTA

10 % (w/v) sucrose

0.25 % (w/v) SDS

0.05 % (w/v) bromphenol blue

The distilled water was added for adjust volume to 30 µl

2.8 STET buffer

8 % sucrose	16	g
50 mM Tris-HCl pH 8.0	10	ml
50 mM EDTA pH 8.0	20	ml
0.1 % Triton X-100	0.2	ml

The distilled water was added for adjust volume to 200 µl and autoclaved at 121 ° C, 15 1b/in^2 for 15 min, followed by storing at room temperature.

3. Preparation of solutions for hydrolysis assays

3.1 1 M Tris buffer, pH 8

Tris base	121	g

Distilled water	800	ml

The pH of solution was adjusted to 8.0 by HCl and brought the volume up to 1,000 ml distilled water. Stored at 4 ° C and was diluted to 50 mM Tris buffer before use.

3.2 Solution A

p-nitrophenyl palmitate	30	µg

p-nitrophenyl palmitate was dissolved with 10 ml of 2-propanol. Then, the solution was mixed well and kept in the brown bottle and stored at 4 ° C.

3.3 Solution B

Triton X-100	0.4	g
Gum Arabic	0.1	g

All components was dissolved with 90 ml of 50 mM Tris buffer pH 8.0 and stored at 4 ° C.

4. Preparation for polyacrylamide gel electrophoresis

4.1 Stock reagent

4.1.1 30 % Acrylamide, 0.8 % bis-acrylamide 100 ml

Acrylamide	29.2	mg
N,N–methylene-bis-acrylamide	0.8	g

4.1.2 1.5 M Tris-HCl, pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

The pH of solution was adjusted to 8.8 by HCl and the volume was brought up to 100 ml distilled water.

4.1.3 1 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

The pH of solution was adjusted to 6.8 by HCl and the volume was brought up to 100 ml distilled water.

4.1.4 10 % Ammonium persulfate

Ammonium persulfate	0.1	mg
Distilled water	1	ml

4.1.5 10 % SDS

SDS	0.1	mg
Distilled water	1	ml

4.2 5 X Sample buffer for SDS-PAGE

1 M Tris-HCl, pH 6.8	0.6	ml
Glycerol	2.5	ml
10 % SDS	2	ml
2-mercaptoethanol	0.5	ml
1 % bromophenol blue	1	ml
Distilled water	3.4	ml

One part of 5 X sample buffer is added to four parts of sample. The mixture is heated at 95 °C for 5 min and centrifuged at 12,000 rpm for 5 min before loading to the gel.

4.3 SDS-PAGE

4.3.1 12 % separating gel

Distilled water	3.3	ml
1.5 M Tris-HCl, pH 8.8	2.5	ml
30 % acrylamide solution	4	ml
10 % SDS	0.1	ml
10 % Ammonium persulfate	0.1	ml
TEMED	0.004	ml

4.3.2 5.0 % stacking gel

Distilled water	1.4	ml
1 M Tris-HCl, pH 6.8	0.25	ml
30 % acrylamide solution	0.33	ml
10 % SDS	0.02	ml
10 % Ammonium persulfate	0.02	ml
TEMED	0.002	ml

4.4 10 X Electrophoresis buffer for SDS-PAGE 1 L

Tris (hydroxymethyl)-aminomethane	30.3	g
Glycine	144	g

SDS	10	g

The distilled water was used for adjusting volume to 1 liter.

4.5 Staining solution

Coomassie brilliant blue R-250	0.5	g
Methanol	250	ml
Glacial acetic acid	50	ml

The distilled water was used for adjusting volume to 500 ml and mixed well.

4.6 Destaining solution

Methanol	100	ml
Glacial acetic acid	100	ml

Add distilled water to 1000 ml and mix.

APPENDIX B

METHODS

1. Phenol/Chloroform Extraction

1) Add an equal volume of phenol: chloroform (1:1) (at least 200 µl) to digestion reaction

2) Mix well, spin at 13000 rpm for 5 min

3) Carefully remove the aqueous layer (upper phase) to a new tube, avoid the interface

4) Repeat step 1-3 until an interface is no longer visible

5) To remove traces of phenol, add an equal volume of chloroform to the aqueous layer

6) Spin at 13000 rpm for 2 min

7) Remove aqueous layer (upper phase) to new tube

8) Clean sample by ethanol precipitation

2. Ethanol Precipitation

1) Add 10% volume of 3M NaOAc pH 4.6

2) Add 3.5 volume of 95% ethanol

3) Spin at 13000 rpm for 20 min

4) Wash with 200 µl of 70% ethanol

5) Air dry

6) Resuspend with 10-20 µl sterile distilled water

APPENDIX C

PREPARATION AND STANDARD CURVE

FOR PROTEIN DETERMINATION

1. Standard curve of BSA

The standard curve of BSA was constructed using Bradford protein assay method for protein determination. The method is as follows;

1. 1 μg/μl BSA was diluted with distilled water as 0.1-0.6 μg (Table C-1).

2. 5 μl BSA from stock solution was added into 96 wells microplate.

Table C-1 Reagent volume for preparation of standard curve

BSA (μg)	**Reagent volume (μl)**	
	1 μg / μl of BSA	**dH_2O**
0	-	1000
0.1	100	900
0.2	200	800
0.3	300	700
0.4	400	600
0.5	500	500
0.6	600	400

3. 300 μl of Bradford's reagent was added and incubated for 5 minutes.

4. The product was measured by an increase in the absorbance at 595 nm.

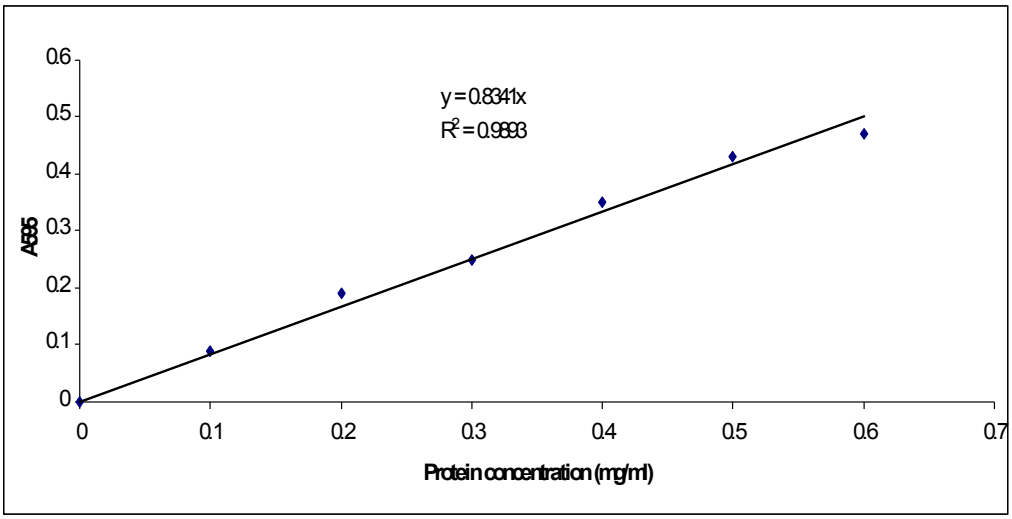

Fig. C-1 Standard curve of BSA

APPENDIX D

CALCULATION METHOD

1. Calculation of the lipase activity

p-nitrophenyl palmitate as substrate

$$\varepsilon_{p\text{-NPP}} \text{ at } 410 \text{ nm} = 15 \text{ mM}^{-1} \text{ cm}^{-1}$$

$$A = \varepsilon lc; \; l = 0.6 \text{ cm}$$

$$\text{Activity} = (\ \Delta \text{mOD } 410 / \text{time}) \text{ x Dilution Factor x } 0.00111)$$

$$\text{Specific activity} = \frac{\text{Activity}}{\text{Concentration of protein}}$$

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

2. Calculation of total protein

The absorbance value at 595 nm was calculated by:

$$Y = aX + b$$

Where

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

Value Y axis = Absorbance at 595 nm

3. Calculation of PCR (insert) volume for ligation

Molar ratio of insert and vector = 3:1

$$\text{Volume of insert (ng)} = \frac{\text{volume of vector (ng) x size of insert x molar ratio}}{\text{size of vector}}$$

4. Calculation of specific activity in hydrolysis by titrate method

$$\text{Mole of NaOH} = \frac{CV}{1000}$$

Mole of NaOH = Mole of fatty acid changed to μmole

Specific activity = μMole of fatty acid/min/mg protein

5. Molecular weight of palm oil

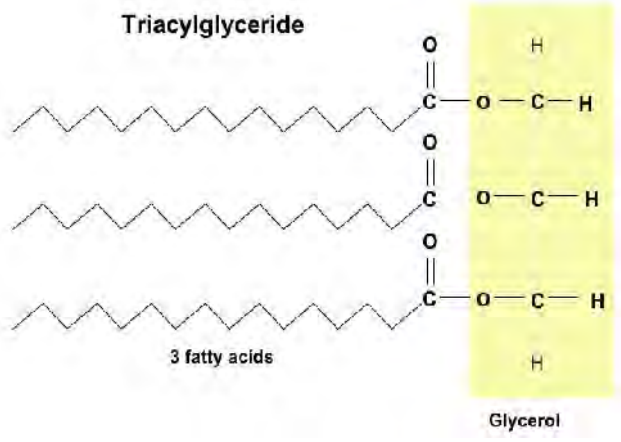

Fig. D-1 Molecular structure of triglyceride

Calculation method was as follows;

$$MW_{TG} = 3R_{Aver} + 173$$

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

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

R_{Aver} = Mass of three fatty acids esterified with glycerol

(minus molecular weight of COOH)

$\%FA_n$ = % fatty acid commposition

MW_n = Molecular weight of three fatty acid – COOH

= MW_{FA} – 45 (from main structure of triglyceride) (Fig. D-1)

Molecular weight of palm oil can be calculated as follows;

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

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

$$= 226.823$$

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

$$= 853.469$$

So, molecular weight of palm oil is 853.47

Table D-1 Fatty acid composition of oils (Winayanuwattikun *et al.,* 2008)

Fatty acids	Abbreviation	% composition			
		Coconut	palm	papaya	rambutan
Lauric acid $C_{12}H_{24}O_2$	12 : 0	34.37	0.59	0.26	0.08
Myristic acid $C_{14}H_{28}O_2$	14 : 0	13.75	0.96	0.46	0.11
Palmitic acid $C_{16}H_{32}O_2$	16 : 0	9.29	38.67	17.12	8.77
Palmitoleic acid $C_{16}H_{30}O_2$	16 : 1	-	0.11	0.45	0.96
Stearic acid $C_{18}H_{36}O_2$	18 : 0	10.53	3.32	2.98	7.25
Oleic acid $C_{18}H_{34}O_2$	18 : 1	12.34	45.45	72.91	55.25
Linoleic acid $C_{18}H_{32}O_2$	18 : 2	6.46	10.87	4.83	3.72
Linolenic acid $C_{18}H_{30}O_2$	18 : 3	0.72	0.20	0.29	0.26
Arachidic acid $C_{20}H_{40}O_2$	20 : 0	7.72	0.23	0.67	22.25
Behenic acid $C_{22}H_{44}O_2$	22 : 0	-	0.02	0.07	1.34

Fatty acids	Abbreviation	% composition		
		physic nut	olive	safflower
Lauric acid $C_{12}H_{24}O_2$	12 : 0	0.14	0.03	0.02
Myristic acid $C_{14}H_{28}O_2$	14 : 0	0.17	0.02	0.11
Palmitic acid $C_{16}H_{32}O_2$	16 : 0	14.82	11.37	6.44
Palmitoleic acid $C_{16}H_{30}O_2$	16 : 1	0.81	0.63	0.06
Stearic acid $C_{18}H_{36}O_2$	18 : 0	4.15	2.58	2.20
Oleic acid $C_{18}H_{34}O_2$	18 : 1	40.98	80.46	14.13
Linoleic acid $C_{18}H_{32}O_2$	18 : 2	38.61	4.17	76.57
Linolenic acid $C_{18}H_{30}O_2$	18 : 3	0.27	0.56	0.15
Arachidic acid $C_{20}H_{40}O_2$	20 : 0	0.06	0.21	0.20
Behenic acid $C_{22}H_{44}O_2$	22 : 0	-	0.01	0.15

6. Volume of methanol

Since a triglyceride has a glycerine molecule as its base with three long chain fatty acids attached, the characteristics of fat are determined by the fatty acids attached to the glycerine. During the transesterification process, the triglyceride reacts with three moles of methanol in the presence of a catalyst to form the mono-alkyl ester, or biodiesel and crude glycerol. So, the ratio of oil and methanol is 1:3 according to the equation in Fig. D-2 (Sinha *et al.,* 2008; West *et al.,* 2008; Ramos *et*

al., 2009). The applied volume of methanol was determined by using the molecular weight of palm oil from section 4 which is equal to 853.47.

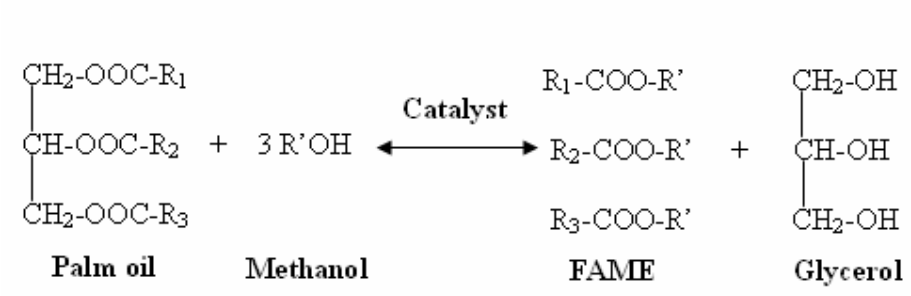

Fig. D-2 Transesterification of palm oil and methanol

So, palm oil 1 g = 0.5/853.47 ~ 5.89×10^{-4} mole

Thus, the volume of methanol = $3\times5.89\times10^{-4} = 1.76\times10^{-3}$ mole

Since molecular weight of methanol is equal to 32,

methanol 1.76×10^{-3} mole = $1.76\times10^{-3}\times32 = 0.056$ g

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

D = Density of methanol (0.792 g/ml)

M = Mass of methanol (0.056 g)

V = Volume of methanol (X ml)

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

So, the volume of methanol is equal to 0.071 ml. (~ 71 µl)

7. % conversion from HPLC analysis

%conversion of fatty acid can be calculated as follows;

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

%conversion of fatty acid methyl ester can be calculated as follows;

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

When: FAME = Fatty Acid Methyl Ester

FFA = Free Fatty Acid

TAG = Triacylglycerol

DAG = Diacylglycerol

MAG = Monoacylglycerol

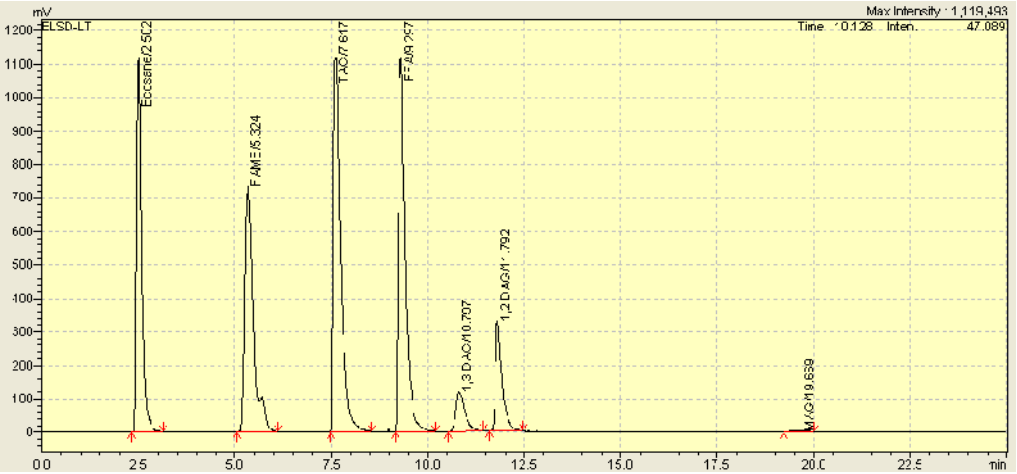

Fig. D-3 Chromatogram of methyl ester from transesterification catalyzed by microbial lipase and analyzed by high performance liquid chromatography

Where

Peak 1 = Eicosane

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

Peak 3 = Triglyceride (TAG)

Peak 4 = Free fatty acid (FFA)

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

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

Peak 7 = Monoglyceride (MAG)

BIOGRAPHY

Mister Weerasak Thakernkarnkit was born on April 19, 1985 in Ratchaburi, Thailand. He graduated with the Bachelor of Science in Biochemistry from Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2006 and furthered his Master's of Science in Biotechnology from Program in Biotechnology at the same institution at which he finished in April of 2011. He was co-author in the publication entitled: "Potential Plant Oil Feedstock for Lipase-catalyzed Biodiesel Production in Thailand" by Winayanuwattikun, P., Kaewpiboon, C., Piriyakananon, K., Tantong, S., Thakernkarnkit, W., Chulalaksananukul, W. and Yongvanich, T. in *Biomass and Bioenergy*, 2008, 32: 1279-1286. In addition, his work had been continuously presented as proceedings together with the posters in both national and international levels. For the proceedings at the international level, at The 7th International Symposium of High Temperature Air Combustion and Gasification (HiTACG 2008), his work was presented on the topic of "Lipase catalyzed biodiesel production from non-edible and waste plant oils" and at The 22nd Annual Meeting of the Thai Society for Biotechnology on the topic of "Gene cloning and expression of lipase from *Fusarium solani* for the production of biodiesel". For national level, at The 2nd BMB Conference: Biochemistry and Molecular Biology for Regional-Sustainable Development on the topic of "Gene cloning, expression of lipase from *Stenotrophomonas sp*. for the production of biodiesel". Furthermore, his posters were presented in both national and international levels. For international level, at The 13th Biological Science Graduated Congress on the topic of "Gene cloning, expression, purification and characterization of lipase from *Stenotrophomonas maltophilia*". For the national level, his poster was presented at The 17th Annual Symposium of Science Forum on the topic of "Gene cloning, expression and characterization of lipase from *Stenotrophomonas sp*. for the production of biodiesel". and at The 4th Annual Symposium of Protein Society of Thailand on the topic of "Gene cloning, expression of lipase from *Stenotrophomonas sp*. for the production of biodiesel".