การโคลนยีน การแสดงออก และลักษณะสมบัติของไลเพสจาก *Staphylococcus warneri*

นางสาว แก้วใจ สังคะหะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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GENE CLONING, EXPRESSION AND CHARACTERIZATION OF LIPASE FROM *Staphylococcus warneri*

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แก้วใจ สังคะหะ : การโคลนยีน การแสดงออก และลักษณะสมบัติของไลเพสจาก *Staphylococcus warneri* (GENE CLONING, EXPRESSION AND CHARACTERIZATION OF LIPASE FROM *Staphylococcus warneri*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. ปกรณ์ วินะยานุวัติคุณ, 97 หน้า.

ไลเพส (ไตรเอซิลกลีเซอรอล เอซิลไฮโดรเลส EC 3.1.1.3) เป็นเอนไซม์ที่มีอยู่แพร่หลาย เร่งการย่อยสลายไตรกลีเซอไรด์ ได้เป็นกลีเซอรอลและกรดไขมันอิสระ นอกจากนี้ไลเพสยังมีประสิทธิภาพในการเร่งปฏิกิริยาต่างๆ เช่น ปฏิกิริยาเอสเทอริฟิเคชัน ทรานเอสเทอริฟิเคชัน และ อะมิโนไลซิส ในตัวทำละลายอินทรีย์ ดังนั้นเอนไซม์เหล่านี้จึงเป็นที่ศึกษากันอย่างกว้างขวางในปัจจุบันเพื่อนำไปประยุกต์ใช้ในอุตสาหกรรม ไลเพสพบได้ทั่วไปในธรรมชาติ แต่ไลเพสจากจุลินทรีย์เท่านั้นที่มีความสำคัญในเชิงพาณิชย์ อย่างไรก็ตามจุลินทรีย์ที่อยู่ในธรรมชาติไม่สามารถผลิตไลเพสได้เพียงพอกับการประยุกต์ใช้ในระดับอุตสาหกรรม การโคลนยีนไลเพสเพื่อให้ผลิตไลเพสได้ปริมาณมากเพื่อนำไปศึกษาคุณสมบัติ เช่น ความจำเพาะ ความเสถียร และนำไปประยุกต์ใช้ในกระบวนการอุตสาหกรรม ดังนั้นวัตถุประสงค์ของงานวิจัยนี้คือ การโคลนยีนไลเพสจากแบคทีเรียเพื่อนำไปประยุกต์ใช้ในการผลิตไบโอดีเซล การทดสอบด้วยเทคนิคทางชีวเคมีและพันธุศาสตร์ยืนยันว่าแบคทีเรียที่คัดเลือกโดยหน่วยวิจัยเชื้อเพลิงชีวภาพด้วยตัวเร่งทางชีวภาพคือ เชื้อ *Staphylococcus warneri* ทำการโคลนยีน lipWY ของเชื้อ *S.warneri* เข้าสู่พลาสมิด pET-17b ได้เป็นรีคอมบีแนนท์พลาสมิด pET17b-lipWY ยีน lipWY ประกอบด้วย 1,053 คู่เบส แปลรหัสเป็นกรดอะมิโนได้ 350 ตัว แต่ lipWY ไม่สามารถแสดงออกใน *Escherichia coli* BL21(DE3) ทำการโคลนยีน gehWA จากเชื้อ *S. warneri* เข้าสู่พลาสมิด pET-28a ได้เป็นรีคอมบีแนนท์พลาสมิด pET28a-gehWA โดยยีนgehWAประกอบด้วย 1,140 คู่เบส แปลรหัสเป็นกรดอะมิโนได้ 379 ตัว ทำการถ่ายโอนและแสดงออกพลาสมิด pET28a-gehWA ใน *E.coli* โดยการเหนี่ยวนำด้วย 0.1 mM IPTG ที่อุณหภูมิ 18 องศาเซลเซียส เป็นเวลา 5 ชั่วโมง ทำรีคอมบีแนนท์ไลเพสให้บริสุทธิ์ด้วยขั้นตอนเดียว โดยใช้ Ni-NTA affinity chromatography พบว่ามีความบริสุทธิ์ขึ้น 38 เท่า และมีน้ำหนักโมเลกุลประมาณ 40 กิโลดาลตัน ค่าความเป็นกรดและด่างที่เหมาะสมของรีคอมบีแนนท์ไลเพสบริสุทธิ์คือ 8.5 และทนต่อความเป็นกรดและด่างในช่วงระหว่าง 8.0 ถึง 10.0 อุณหภูมิที่เหมาะสมคือ 30 องศาเซลเซียสแต่จะเสถียรที่อุณหภูมิต่ำกว่า 30 องศาเซลเซียส การศึกษาความจำเพาะของสารตั้งต้นพบว่าเอนไซม์มีความจำเพาะต่อสารตั้งต้นที่มีจำนวนคาร์บอนเท่ากับ 4 (พารา-ไนโตรฟีนิล บิวทิเรท) รีคอมบีแนนท์ไลเพสมีความเสถียรต่อสารละลายอินทรีย์แตกต่างกันโดยจะเสถียรในสารละลายอินทรีย์ที่ไม่มีขั้วมากกว่าสารละลายอินทรีย์ที่มีขั้ว Ca^{2+} ช่วยในการทำงานของไลเพส ส่วน Zn^{2+}, Hg^{2+}, Ag^{+} และ Co^{2+} จะยับยั้งการทำงานของเอนไซม์เช่นเดียวกับ SDS, EDTA และ Tween–80 รีคอมบีแนนท์ไลเพสสามารถย่อยสลายได้เพียงน้ำมันมะพร้าวเท่านั้น โดยมีค่าแอกทิวิตีจำเพาะเท่ากับ 0.157 ไมโครโมลต่อนาทีต่อมิลลิกรัมโปรตีน อย่างไรก็ตามเอนไซม์ไลเพส gehWA ไม่สามารถเร่งปฏิกิริยาในการผลิตไบโอดีเซลได้ ซึ่งไลเพสนี้น่าจะเหมาะสมสำหรับใช้เป็นตัวเร่งทางชีวภาพในด้านอุตสาหกรรมอาหาร ยา หรือใช้ในการบำบัดสารกำจัดศัตรูพืช

สาขา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต แก้วใจ สังคะหะ

ปีการศึกษา 2552 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก ปกรณ์ วินะยานุวัติคุณ

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KAEWJAI SANGKHAHA: GENE CLONING, EXPRESSION AND CHARACTERIZATION OF LIPASE FROM *Staphylococcus warneri*.
THESIS ADVISOR: PAKORN WINAYANUWUTTIKUN, Ph.D., 97 pp.

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of triglycerides to glycerol and free fatty acid. Besides this, they are also efficient in various reactions such as esterification, transesterification and aminolysis in organic solvents. Therefore, these enzymes are nowadays extensively studied for their potential industrial applications. Lipases were found widely in nature, but only microbial lipases are commercially significant. However, natural microbial lipases cannot be sufficiently produced for the industrial applications. Utilizing gene technology, cloning of lipase gene is applied in order to produce large volume of lipase to explore suitable characteristic of lipase such as specificity, stability and application for industrial process. Therefore, the aim of this project is cloning lipase gene from lipase producing bacteria. Both biochemical and genetic tests confirmed that the lipase producing bacterium screened by Biofuel by Biocatalyst Research Unit is *Staphylococcus warneri*. The lipWY gene of *S.warneri* was cloned into the plasmid pET-17b producing recombinant plasmid, pET17b-lipWY. The open reading frame of lipWY was composed of 1,053 bp, which encoded 350 amino acids, but the pET17b-lipWY was not expression in *Escherichia coli* BL21(DE3). A mature lipase gene (gehWA) from *S. warneri* was also cloned into the plasmid pET-28a producing recombinant plasmid, pET28a-gehWA. The mature gene was composed of 1,140 bp, encoding 379 amino acids. The plasmid was transformed and expressed in *E.coli* by induction with 0.1M IPTG at 18°C for 5 hr. The expressed recombinant lipase was purified by one step Ni-NTA affinity chromatography yielding 38 folds purity. The 40 kDa purified recombinant lipase was active at pH 8.5 and stable at pH range between pH 8.0-10.0. The lipase exhibited an optimum temperature of 30°C and stable at temperature below 30°C. The substrate specificity study showed that *p*-nitrophenyl butyrate is a preference of the enzyme. Recombinant lipase is diverse in its sensitivity to solvents but more stable in non-polar than polar organic solvents. The obtained lipase was shown to be activated by the metal ions Ca^{2+} whereas Zn^{2+}, Hg^{2+}, Ag^{+} and Co^{2+} inhibited the enzyme activity. In addition, inhibition was also observed with SDS, EDTA and Tween-80. The purified recombinant lipase could hydrolyze only emulsion of coconut oil as a substrate. However, no detectable fatty acid methyl ester was found in transesterification reaction. This gehWA lipase seems to be proper for application as a biocatalyst in food, pharmaceutical industries and for bioremediation of some pesticides.

Field of Study : Biotechnology Student's Signature Kaewjai Sangkhaha
Academic Year : 2009 Advisor's Signature Pakorn Winayanuwattikun

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LIST OF ABBREVIATIONS

A	adenine
Amp	ampicillin
bp	basepair
C	cytosine
°C	degree of celcius
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
et al.	et alibi (latin), and others
g	gram
G	guanosine
hr	hour
IPTG	isopropyl-β-D- thiogalactopyranoside
kb	kilobase
kDa	kilodalton
L	liter
LB	Luria-Bertini medium
M	Molar
mg	milligram
min	minute
ml	milliliter
mol	mole
mM	millimolar
nm	nanometer
OD	optical density
p	para
PAGE	polyacrylamide gel electrophoresis

rpm	revolution per minute
SDS	sodium dodecyl sulfate
sec	second
SOB	Super Optimal Broth
T	thymine
TAE buffer	Tris-Acetate-EDTA buffer
TB buffer	transformation buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	microgram
µl	microliter

CHAPTER I
INTRODUCTION

1.1 Statement of purpose

Nowadays, the demand of fuel oils is increasing but the availability is quite limited. So, searching alternative energy is necessary for the future. Biodiesel, which is one of alternative fuels has been likely to serve as potential replacement of petrodiesel because it is renewable, non-toxic, environmentally safe and biodegradable.

In addition, Thailand is agricultural country that grows many oil plants such as cotton, peanut, sesame, castor bean, physic nut, coconut and palm. Such raw materials can be used to produce biodiesel. It has the same properties as fossil diesel fuel that can be used in diesel engines without any modifications. There are many methods to produce biodiesel such as blending, microemulsion, pyrolysis and transesterification but the most popular method of producing biodiesel is transesterification of vegetable oil or animal fat with a short chain alcohol; methanol and ethanol in the presence of a catalyst. Catalyst normally used in biodiesel production is acid or base. At present, scientists are increasingly interested in using the lipase as biocatalyst in transesterification reaction. Since the synthesis of biodiesel catalyzed by chemical catalyst has several drawbacks for instance; it is energy intensive requirement, recovery of glycerol is difficult, the acid or alkaline catalyst has to be removed from the product and waste-water requires treatment. However, using of enzyme can overcome these problems since the reaction can occur under mild conditions, the glycerol can be easily recovered, the purification of fatty acid methyl esters and glycerol is simple to accomplish and environment pollution can be eliminated. (Ranganathan *et al.* 2008).

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) catalyze the hydrolysis of triglycerides to glycerol and free fatty acid. Lipase can be found in plants, animals and microorganism. Microbial lipase is the most attractive because it can grow quickly, easy to screen for desired optimal properties corresponding to the process such as organic solvent tolerance, pH and thermal stability (Abdel-Fattah and Gaballa 2008). Lipase is the most important lipolytic enzyme, which can be applied in a variety of

biotechnological process as biocatalyst. Nevertheless, natural microbial lipases can be produced in very small quantity which is insufficient for the industrial applications. Utilizing gene technology, cloning of lipase gene is applied in order to produce large volume of the enzyme (Yang *et al*. 2007).

From the previous studies, lipase producing bacteria were isolated and screened on tributyrin and rhodamine B agar plates. After that, the hydrolytic activities of lipase with *ρ*-nitrophenyl palmitate were compared. The result showed that lipase from *Staphylococcus warneri* has the highest specific activity (1.29 ± 0.23 µmole/min/mg protein) (Chutima Kaewpiboon, 2007). Hence, lipase from *Staphylococcus warneri* was investigated in this work.

1.2 Objectives of this research

The aim of the present study was to clone, express and characterize lipase from *S. warneri* as catalyst in transesterification for the production of biodiesel.

1.3 Scopes of the investigation

1.3.1 To identify the lipase producing bacterium

1.3.2 To clone lipase gene

1.3.3 To express lipase gene in *E. coil* host

1.3.4 To purify recombinant lipase

1.3.5 To characterize recombinant lipase

1.4 Expected results

Lipase can be produced from *S. warneri* in order to catalyze transesterification for biodiesel production.

1.5 Thesis organization

This thesis consists of five chapters as follows: Chapter 1 is the introduction. Chapter 2 gives the theoretical and literature reviews. Chapter 3 comprises material and

methods. The results can be found in Chapter 4 and the final chapter contains the discussion and conclusion.

CHAPTER II
THEORETICAL BACKGROUND AND LITERATURE REVIEWS

The background information about biodiesel, lipase, lipase gene cloning and properties of recombinant lipase are reviewed in this chapter.

2.1 Biodiesel

Biodiesel is made from biomass oils. Biodiesel appears to be an attractive energy resource for several reasons. Firstly, biodiesel is a renewable resource of energy that can be sustainably supplied with the petroleum fuel which is going to be depleted in less than 50 years at the present rate of consumption (Sheehan *et al*., 1998). Secondly, biodiesel appears to have several favorable environmental properties resulting in no net increased release of carbon dioxide and very low sulfur content (Antolin *et al*., 2002 and Vicente *et al*., 2004). The release of sulfur content and carbon monoxide will be cut down by 30% and 10%, respectively, by using biodiesel as energy source. Moreover, biodiesel contains no aromatic compounds and other chemical substances which are harmful to the environment. Recent investigation has indicated that the use of biodiesel can decrease 90% of air toxicity and 95% of cancers compared to common diesel source (Sharp, 1996). Thirdly, biodiesel appears to have significant economic potential because a non-renewable fuel that is fossil fuel the price will increase inescapability further in the future (Cadenas *et al*., 1998). Finally, biodiesel is better than diesel fuel in terms of flash point and biodegradability (Ma and Hanna, 1999). Biodiesel is a mixture of mono-alkyl esters obtained from vegetable oils like soybean oil (Bunyakia *et al*., 2006), jatropha oil (Berchmans and Hirata, 2008), rapeseed oil (Azcan and Danisman, 2008), palm oil (Singh and Singh, 2009), sunflower oil (Winayanuwattikun *et al*., 2008), corn oil (Saraf and Thomas, 2007), peanut oil (Goering *et al*., 1982), canola oil (Patil and Deng, 2009) and cottonseed oil (Saraf and Thomas, 2007). Apart from vegetable oils, biodiesel can also be produced from other sources like animal fat (beef tallow, lard) (Bhatti *et al*., 2008), waste cooking oil, greases (trap grease, float grease) (Issariyakul *et al*., 2008) and

algae (Huang *et al*., 2010). There are many methods to produce biodiesel as shown in Table 2-1.

Table 2-1 Different methods of biodiesel production.

Methods	Definition	Advantage	Disadvantage	Problems of using in engines
Direct use and blending	Direct use as diesel fuel or blend with diesel fuel	Liquid nature-portability Heat content (80% of diesel fuel) Readily available; renewability	Higher viscosity Lower volatility Reactivity of unsaturated hydrocarbon chains	Coking and trumpet Formation Carbon deposits Oil ring sticking; thickening and gelling of the lubricating oil
Microemulsions	A colloidal equilibrium dispersion of optically isotropic fluid microstructures with dimensions generally in the 1–150 nm range formed spontaneously from two immiscible liquids and one or more ionic or non-ionic amphiphiles	Better spray patterns during Combustion Lower fuel viscosities	Lower cetane number Lower energy content	Irregular injector needle sticking; incomplete combustion Heavy carbon deposits; increase lubrication oil viscosity
Pyrolysis	The conversion of long-chain and saturated substance (biomass basis) to biodiesel by means of heat	Chemically similar to petroleumderived gasoline and diesel fuel	Energy intensive and hence higher cost	-
Transesterification	The reaction of a fat or oil with an alcohol in the presence of catalyst to form esters and glycerol	Renewability; higher cetane number; lower emissions; higher combustion efficiency	Disposal of byproduct (glycerol and waste water)	-

From Leung *et al*., 2010

The most popular method of producing biodiesel is transesterification of vegetable oil or animal fat with an alcohol in the presence of a catalyst. Catalyst that is normally used in biodiesel production is chemical catalyst such as acid or base. At present, there are increasing interest in using the lipase as biocatalyst in transesterification reaction. Synthesis of biodiesel catalyzed by chemical catalyst have several drawbacks for instance; it is energy intensive requirement, recovery of glycerol is difficult, the acidic or alkaline catalyst has to be removed from the product and waste-water requires treatment. However using of enzyme can overcome these problems. Since the reaction can occur under mild conditions, the glycerol can be easily recovered, the purification of fatty acid methyl esters and glycerol is simple to accomplish and eliminate environment pollution (Ranganathan *et al.* 2008).

2.2 Lipase

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) catalyze the hydrolysis of triglycerides to glycerol and free fatty acid at a lipid–water interface, which facilitates the catalysis of several unnatural reactions such as esterification, interesterification and transesterification (Horchani *et al*., 2009).

2.2.1 Source of lipase

Lipase can be found in plants, animals and microorganisms. Microbial lipase is often more useful than enzyme derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Jaeger *et al*., 1999). Only about 2% of the world's microorganisms have been tested as enzyme sources. Bacterial strains are generally more used as they offer higher activities compared to yeasts (Frost and Moss, 2001) and tend to have neutral or alkaline pH optima and are often thermostable. Genetic and environmental manipulation to increase the yield of

cells, to increase the enzyme activity of the cells by making the enzyme of interest constitutive, or by inducing it, or to produce altered enzymes (Jaeger *et al*., 1997), may be employed easily using microbial cells because of their short generation times, their relatively simple nutritional requirements, and screening procedures for the desired characteristic are easier. Some of the lipase producing bacteria are listed in Table 2-1.

Table 2-2 Some of the lipase producing bacteria

Source	Genus	Species	Reference(s)
Bacteria (Gram-positive)	*Bacillus*	*B. megaterium*	Godtfredsen, 1990
		B. cereus	El-Shafei and Rezkallah, 1997
		B. stearothermophilus	Gowland *et al*., 1987; Kim *et al*., 1998
		B. subtilis	Kennedy and Rennarz, 1979
		Recombinant *B. subtilis* 168	Lesuisse *et al*., 1993
		B. brevis	Hou, 1994
		B. thermocatenulatus	Rua *et al*., 1998
		Bacillus sp. IHI-91	Becker *et al*., 1997
		Bacillus strain WAI 28A5	Janssen *et al*., 1994
		Bacillus sp.	Helisto and Korpela, 1998
		B. coagulans	El-Shafei and Rezkallah, 1997
		B. acidocaldarius	Manco *et al*., 1998
		Bacillus sp. RS-12	Sidhu *et al*., 1998a,b
		B. thermoleovorans ID-1	Lee *et al*., 1999
		Bacillus sp. J 33	Nawani and Kaur, 2000
	Staphylococcus	*S. canosus*	Tahoun *et al*., 1985
		S. aureus	Lee and Yandolo, 1986
		S. hyicus	Van Oort *et al*., 1989; Meens *et al*., 1997; van Kampen *et al*., 1998
		S. epidermidis	Farrell *et al*., 1993; Simons *et al*., 1998
		S. warneri	Talon *et al*., 1995
	Lactobacillus	*Lactobacillus delbruckii* sub sp. *Bulgaricus*	El-Sawah *et al*., 1995
		Lactobacillus sp.	Meyers *et al*., 1996
	Streptococcus	*Streptococcus lactis*	Sztajer *et al*., 1988
	Micrococcus	*Micrococcus freudenreichii*	Hou, 1994
		M. luteus	Hou, 1994
	Propionibacterium	*Propionibacterium acne*	Sztajer *et al*., 1988
		Pr. granulosum	Sztajer *et al*., 1988
	Burkholderia	*Burkholderia* sp.	Yeo *et al*., 1998

Table 2-2 (continued)

Source	Genus	Species	Reference(s)
		Bu. glumae	El Khattabi *et al*., 2000
Bacteria (Gram-negative)	*Pseudomonas*	*P. aeruginosa*	Aoyama *et al*., 1988; Hou, 1994; Ito *et al*., 2001
		P. fragi	Mencher and Alford, 1967
		P. mendocina	Jaeger and Reetz, 1998
		P. putida 3SK	Lee and Rhee, 1993
		P. glumae	Frenken *et al*., 1993; Noble *et al*., 1994
		P. cepacia	Penereac'h and Baratti, 1996; Lang *et al*., 1998; Hsu *et al*., 2000
		P. fluorescens	Maragoni, 1994; Lacointe *et al*., 1996
		P. aeruginosa KKA-5	Sharon *et al*., 1998
		P. pseudoalcaligenes F-111	Lin *et al*., 1995, 1996
		Pseudomonas sp.	Sin *et al*., 1998; Miyazawa *et al*., 1998; Reetz and Jaeger, 1998; Dong *et al*., 1999
		P. fluorescens MF0	Guillou *et al*., 1995
		Pseudomonas sp. KWI56	Yang *et al*., 2000
	Chromobacterium	*Ch. viscosum*	Rees and Robinson, 1995; Helisto and Korpela, 1998; Jaeger and Reetz, 1998; Diogo *et al*., 1999
	Acinetobacter	*Aci. pseudoalcaligenes*	Sztajer *et al*., 1988
		Aci. radioresistens	Chen *et al*., 1999
	Aeromonas	*Ae. hydrophila*	Anguita *et al*., 1993
		Ae. sorbia LP004	Lotrakul and Dharmsthiti, 1997

From Sharma *et al*., 2001

2.2.2 Applications of microbial lipases

Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. Microbial lipases are widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications as shown in Table 2-3. Lipases are used in two distinct fashions. They are used as biological catalysts to manufacture other products

(such as food ingredients) and by their application as such (in making fine chemicals).

Table 2-3 Industrial applications of microbial lipases

Industry	Action	Product or application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonaise, dressings, and whippings
Health foods	Transesterification	Health food
Meat and fish	Flavor development	Meat and fish products; fat removal
Fats and oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipids, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats

From Vulfson, 1994

2.2.3 DNA technology

The utilization of gene technology has 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 of animal and plant origin, the realization of enhanced efficiency and high product purity, and also the production of enzymes with improved stability and activity (Falch, 1991). Genetic engineering of lipase involves modification of the gene encoding the enzyme. Features of this technology include the ability to isolate and express genes of interest and the ability to change the amino acid occupying a single, or multiple, sites in a protein. This technology also allows the insertion or deletion of single or

multiple amino acids, and the fusion of segments from different genes and different organisms (Villeneuve *et al*., 2000).

2.2.4 Purification

Fusion of segments or several tags not only aim for improvement of enzyme activity or stability but also facilitate the protein purification. Several cloning vectors containing a C- or N-terminal in-frame sequence for His-tag have been engineered to enable the expression of recombinant proteins in prokaryotic and eukaryotic expression systems (Murphy and Doyle, 2005). For large scale purification of recombinant proteins, the use of affinity tags is usually preferred due to the specificity of the process and the relatively easy purification schemes. In previous studies, the genes encoding the two extracellular lipases of *Staphylococcus simulans* (SSL) and *Staphylococcus xylosus* (SXL) were subcloned in the pET-14b expression vector and expressed in *Esherichia coli* BL21 (DE3) (Sayari *et al*., 2007and Mosbah *et al*., 2006). The two recombinant lipases (rSSL and rSXL) were expressed as amino terminal His6-tagged recombinant proteins. One-step purification of the recombinant lipases was achieved with nickel metal affinity column. Using emulsified system, the results showed that the N-terminal extension that contained six successive histidine residues did not alter the biochemical properties and the catalytic efficiencies of rSSL or rSXL, compared, respectively, to the native SSL (Sayari *et al*., 2001) or SXL (Mosbah *et al*., 2005).

From previous studies of our research group, lipase producing bacteria were isolated and screened on tributyrin and rhodamine B agar plates. After the hydrolytic activities were compared, lipase from *Staphylococcus warneri* has showed the highest specific activity. Hence, lipase from *Staphylococcus warneri* was cloned, expressed and characterized as catalyst in transesterification for the production of biodiesel.

CHAPTER III
MATERIALS AND METHODS

3.1 Equipments

Agarose gel electrophoresis	(BioRad, U.S.A)
Autoclave	(Ta Chang Medical instrument, Taiwan)
Balance	(Sartorius, Germany)
Centrifugal Filter Devices	(Millipore, U.S.A.)
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)
Incubator shaker	(New Brunswick Scientific Co., Inc., USA)
Laminar flow	(Thermo electron corporation, USA)
Magnetic stirrer	(KIKA Labortechnik, Malaysia)
Magnetic bar	(Lio Lab Limited Partnership)
Microcentrifuge	(Satorious, Germany)
Microplate spectrometer	(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., Inc., USA)
Slab gel electrophoresis equipment	(BioRad, USA)
Thermal Cycler	(BioRad, USA)
ThermoE	(Bioer technology Co., Ltd, China)
Ultrasonic homogenizer	(SONOPULS, USA)
UV Transilluminator	(UVP, UK)
UV-VIS spectrophotometer	(Thermo scientific, UK)

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

3.2 Chemicals and reagents

All chemicals and reagents used throughout this study were analytical grade, and/or molecular biological grade and were purchased from various manufacturers (Sigma (USA), Merk (Germany), Fluka (Switzerland), Carlo Erba (Italy), Ajax Finechem (Australia), Difco (USA), Usb (USA), Biorad (USA) and Invitrogen (Canada)).

3.3 Bacterial stain

Staphylococcus warneri was used as the donor of chromosomal DNA.

Escherichia coli strain JM109 carried pET-17b (plasmid).

E.coli strain XL-1Blue carried pET-28a (plasmid).

E.coli strain 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.

E.coli strain BL21(DE3) (F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])) was used as host for expression of the gene constructed in pET- and pT7-vector series

3.4 Enzymes

*Bam*H I	New England Biolabs (USA)
*Eco*R I	New England Biolabs (USA)
*Hin*d III	New England Biolabs (USA)
Nde I	New England Biolabs (USA)
Nhe I	New England Biolabs (USA)
Xho I	New England Biolabs (USA)
Taq DNA polymerase	New England Biolabs (USA)
T4 DNA ligase	New England Biolabs (USA)

3.5 Plasmids

pGEM®-T Easy vector (Figure 3-1)	Promega (USA)
pET-17b vector (Figure 3-2)	Novagen (Germany)
pET-28a vector (Figure 3-3)	Novagen (Germany)

3.6 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 a primer, were purchased from Bio Basic Inc. (Canada). The primers information was shown in Table 3-1.

Table 3-1 List of primers

Primer	Sequence	T_m°C
16S_F	5’ -GGG**GGATCC**GCTCAGATTGAACGCTGGCG- 3’ (*Bam*H I)	76.2
16S_R	5’ -CCC**AAGCTT**ACATTTCACAACACGAGCTG-3’ (*Hin*d III)	65.2
gehWA_F	5’-GGGG**GCTAGC**AATCATGACCCTATTATACTTGTACATGG–3’ (*Nhe* I)	70.0
gehWA_R	5’-GGGG**CTCGAG**TTATTGATAAGTCACTTTTTCGTTTCTTACAGC-3’ (*Xho* I)	71.6
lipWY_F	5’ -GGGAATTC**CATATG**AAGGCAAAACATAAATGGTTG- 3’ (*Nde* I)	67.7
lipWY_R	5’ -C**GAATTC**TTATTCACTAAATGGGTCTAATTGTACATC-3’ (*Eco*R I)	62.3

3.8 Columns for purification

HiTrap affinity column	GE Healthcare Bio-Science AB (Sweden)
HiTrap Desalting column	GE Healthcare Bio-Science AB (Sweden)

3.9 Oil

Coconut oil	(Pumedin natural products, Thailand)
Olive oil	(Rafael Salgado, Spain)
Palm oil	(Morakot industry, Thailand)
Safflower oil	(OHIO, Maxico)

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)

3.11 Data analysis

Sequences 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 bacterium

3.12.2 Lipase genes cloning

3.12.3 Expression of lipase genes in *E.coil* host

3.12.4 Purification of recombinant lipase

3.12.5 Characterization of recombinant lipase

3.12.1 Identification of the lipase producing bacterium

3.12.1.1 Based on biochemical characteristics

The bacteria were biochemically classified by Department of Medical Sciences, Ministry of Public Health, Thailand

3.12.1.2 Based on 16S rRNA sequence

3.12.1.2.1 DNA preparation

A single colony of *Staphylococcus warneri* was inoculated to NB medium and then incubated at 30^{o} C, 250 rpm for 24 hr. The genomic DNA was extracted using Qiagen genomic kit. The 16S rRNA gene was amplified from genomic DNA of *S. warneri* using universal primers (16S_F: 5'GGGGGATCCGCTCAGATTGAAC GCTGGCG-3' and 16S_R: 5'-CCCAAGCTTACATTTCACAAC ACGAGCTG-3'). The 50 µl PCR mixture contained 0.2 µmol of each primer, 0.1 µm dNTP, approximately 2 µmol genomic DNA as template, 1X ThermolPol Buffer and 1.5 U Taq DNA polymerase. The PCR was carried out for 32 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. After that the PCR product was analyzed by 1% agarose gel electrophoresis in 1X TAE buffer. The PCR product mixed with 6X DNA loading dye was loaded onto agarose gel and run at constant voltage of 100 volts for 1 hr. The gel was stained in 10 µg/ml ethidium bromide (EtBr) solution for 5 min and destained in water for 15-20 min. The stained gel was visualized under UV light and photographed. The PCR product was extracted and purified from 1% agarose gel with Qiagen gel extraction kit according to the manufacturer's instructions.

3.12.1.2.2 Ligation of purified PCR product into vector

The purified PCR product was ligated into pGEM-T Easy vector at lacZ gene. In general, the molar ratio of insert to vector of 3:1 was used. A 10X T4 DNA ligase buffer and steriled distilled water was added to make final concentration of 1X before addition of 3 unit of T4 DNA ligase and incubated at 4°C for overnight.

3.12.1.2.3 Preparation of competent *E.coli* cells

3.12.1.2.3.1 Preparation of competent cells by Simple and efficient method (SEM)

A single colony of E.coli strain DH5α was inoculated to 25 ml of SOB media (starter) and then incubated at 18°C, 250 rpm for overnight. Twenty five millilitres were inoculated to new SOB media (250 ml) and incubated at 18°C, 250 rpm until the absorbance at 600 nm was approximately 0.6. The *E.coli* culture was incubated on ice for 10 min and centrifuged at 2,500 g at 4°C for10 min. The cell pellets were resuspended in 80 µl of ice-cold TB buffer incubated on ice for 10 min and centrifuged at 2,500 at 4°C for 10 min. The cell pellets were collected and resuspended in 20 ml of ice-cold TB buffer. Then dimethyl sulfoxide (DMSO) was slowly added and gently mixed to give a 7% final concentration and the mixture was incubated on ice for 10 min. The competent cells of 200 µl were divided into microcentrifuge tubes and frozen immediately with liquid nitrogen and stored in -80°C until use for transformation.

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

A single colony of *E.coli* strain BL21(DE3) was inoculated to 5 ml of LB broth, and then incubated at 37°C, 250 rpm for overnight. Five hundred microlitres of culture cells were inoculated to new LB broth (50 ml) and incubated at 37°C, 250 rpm until the absorbance at 600 nm was 0.3 to 0.4 and then the culture was cooled on ice for 10 min and harvested by centrifugation (4,000 rpm, 4°C for 10

min). The supernatant was removed and the pellet was resuspended with chilled 10 ml 0.1 M $CaCl_2$ and stored on ice for 10 min. The resuspended pellet was centrifuged at 4,000 rpm, 4°C for 10 min. The supernatant was removed and the pellet was resuspended again with chilled 10 ml 0.1 M $CaCl_2$, stored on ice for 10 min, centrifuged at 4,000 rpm, 4°C for 10 min. The supernatant was removed and the pellet was resuspended with chilled 2 ml 0.1 M $CaCl_2$ and steriled glycerol was added to make final concentration of 30% (900 µl). The solution was gently mixed and stored on ice for 15 min. The competent cells of 100 µl were divided into microcentrifuge tubes and stored in -80°C until use for transformation.

3.12.1.2.4 Transformation into *E.coli* DH5α competent cell by heat shock method

Ten microlitres ligation reaction were mixed with 200 µl of competent cells *E.coli* DH5α. The mixture was incubated on ice for 30 min and immediately heat shocked at 42°C for 90 sec. The reaction was quickly chilled on ice for 5 min. 1 ml LB broth was added and incubated at 37°C with shaking at 250 rpm for 1 hr. The transformed cells were spread on LB agar plate containing 100 µg/ml ampicillin. Agar plate must be prespread with 50 µl of 20 mg/ml X-gal and 10 µl of 100 mM IPTG at least 30 min before spreading transformed cell. The agar plate was further incubated at 37°C for overnight.

3.12.1.2.5 Screening for recombinant clones using rapid size screening method

Bacteria containing recombinant plasmids display white colonies on LB agar containing 100 µg/ml ampicillin, 50 µl of 20 mg/ml X-gal and 10 µl of 100 mM IPTG. A single colony of each recombinant clone was picked and lysed in 30 µl of prewarmed lysis buffer (100 mM NaOH, 60 mM KCl, 5 mM EDTA, 10% (w/v) sucrose, 0.25% (w/v) SDS and 0.05% (w/v) bromphenol blue). The reaction 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. The aqueous phase was loaded on agarose gel electrophoresis. The clones, containing plasmid DNA with larger size than control plasmid, were selected for plasmid extraction.

3.12.1.2.6 Plasmid DNA extraction

The selected clone was inoculated to 5 ml LB broth containing 100 µg/ml ampicillin and incubated at 37°C with shaking 250 rpm for 14-16 hours. The cells were collected by centrifugation at 5,000 rpm for 10 min at 4°C and supernatant was discarded. The recombinant plasmid was extracted using Qiagen spin miniprep kit.

3.12.1.2.7 Restriction analysis

The recombinant plasmid was digested by 3 units of *Bam*H I and *Hin*d III restriction enzymes in 10 µl of appropriate reaction buffer (Table 3-2) and incubated at 37°C for 3 hr. The digested recombinant plasmid was analyzed by agarose gel electrophoresis and visualized under UV light and photographed.

Table 3-2 Restriction enzymes

Restriction enzymes	Recognition sequence (5'-3')	Reagents Supplied
*Bam*H I	G´GATCC	NEBuffer 3 + BSA
*Eco*R I	G´AATTC	NEBuffer EcoRI
*Hin*d III	A´AGCTT	NEBuffer 2
Nde I	CA´TATG	NEBuffer 4
Nhe I	G´CTAGC	NEBuffer 4 + BSA
Xho I	C´TCGAG	NEBuffer 4 + BSA

3.12.1.2.8 DNA sequencing and sequence analysis

The expected recombinant clones which contained 16S rRNA fragment were verified by nucleotide sequencing using automated DNA sequencer (1st BASE, Malaysia). Sequences were checked by using Chromas Lite and BioEdit program and aligned in GenBank database using the Blast program to determine the closest relatives of the 16S rRNA gene.

3.12.2 Lipase genes cloning

3.12.2.1 Preparation of lipase genes

3.12.2.1.1 The lipWY gene

The lipase gene was amplified using genomic DNA of *S. warneri* as the template. Two primers lipWY_F (5' –GGGAATTC **CATATG**AAGGCAAAACATAAATGGTTG- 3') and lipWY_R (5' –C**GAATTC**TTATTCACTAAATGGGTCTAATTGTACATC -3') were designed on the basis of the completed lipWY gene. The restriction sites *Nde* I and *Eco*R I were incorporated into the forward and reverse primer sequence, respectively. The PCR was carried out by a 32-cycle reaction with steps at 94 °C for 3 min, 45-60 °C for 30 s and 72 °C for 1:30 min per cycle. The PCR product was analyzed by agarose gel electrophoresis and visualized

under UV light and photographed. The PCR product was purified from 1% agarose gel with Qiagen gel extraction kit.

3.12.2.1.2 The gehWA gene

The primers were designed on basis of the *S.warneri* mature lipase gene and fused in frame with C-terminal tag which encodes 6 tandem of histidine for protein purification, that was introduced restriction site for *Nhe* I and *Xho* I at 5' end for direct cloning into pET28a plasmid. The sequences of the forward and reverse primers used to amplify gehWA were: 5'GGGG**GCTAGC**AATCATGACCCTATTATACTTGTACATG G–3' and 5'-GGGG**CTCGAG**TTATTGATAAGTCACTTTTTCG TTTCTTACAGC-3'. Amplification was achieved by using chromosomal DNA from *S.warneri* as template. The PCR was performed with 1 cycle at 94 °C for 3 min for initial denaturation; 32 cycles at 94 °C, 3 min; 45-60 °C, 30 s; 72 °C, 1:30 min for amplification, and a final elongation cycle at 72 °C for 7 min. The PCR product was analyzed by agarose gel electrophoresis and visualized under UV light and photographed. The PCR product was purified from 1% agarose gel with Qiagen gel extraction kit.

3.12.2.2 Preparation of plasmids

3.12.2.2.1 The pET-17b plasmid

A single colony of *E.coli* strain JM109 carried pET-17b plasmid was inoculated to LB medium containing 100 µg/ml ampicillin and incubated at 37°C with shaking 250 rpm overnight. The cells were collected by centrifugation at 5,000 rpm for 10 min at 4°C and supernatant was discarded. The pET-17b plasmid was extracted using Qiagen spin miniprep kit and analyzed by agarose

gel electrophoresis and visualized under UV light and photographed.

3.12.2.2.2 The pET-28a plasmid

A single colony of *E.coli* strain XL-1Blue carried pET-28a plasmid was inoculated to LB medium containing 50 µg/ml kanamycin and incubated at 37°C with shaking 250 rpm overnight. The cells were collected by centrifugation at 5,000 rpm for 10 min at 4°C and supernatant was discarded. The pET-28a plasmid was extracted using Qiagen spin miniprep kit and analyzed by agarose gel electrophoresis and visualized under UV light and photographed.

3.12.2.3 Double digestion

3.12.2.3.1 Double digestion lipWY PCR and pET-17b plasmid

Both pET-17b plasmid and lipWY gene were double digested with 6 units of *Nde* I and *Eco*R I restriction enzymes in 30 µl of appropriate reaction buffer (Table 3-2) and incubated at 37°C for 3 hr. Both digested pET-17b plasmid and digested lipWY gene were analyzed by agarose gel electrophoresis and visualized under UV light and photographed.

3.12.2.3.2 Double digestion gehWA PCR and pET-28a plasmid

Both pET-28a plasmid and gehWA gene were double digested with 6 units of *Nhe* I and *Xho* I restriction enzymes in 30 µl of appropriate reaction buffer (Table 3-2) and incubated at 37°C for 3 hr. Both digested pET-28a plasmid and digested gehWA gene were analyzed by agarose gel electrophoresis and visualized under UV light and photographed.

3.12.2.4 Ligation of digested lipase genes into vectors

The digested lipWY gene was ligated into digested pET-17b vector and the digested gehWA gene was ligated into digested pET-28a vector. In general, the molar ratio of insert to vector of 3:1 was used. A 10X T4 DNA ligase buffer and steriled distilled water was added to make final concentration of 1X before addition of 3 units of T4 DNA ligase and incubated at 16°C overnight.

3.12.2.5 Transformation into *E.coli* DH5α competent cells

Ten microlitres ligation reaction of pET17b-lipWY and pET28a-gehWA were transformed into *E.coli* DH5α by heat shock method as mentioned above. The transformed cells of pET17b-lipWY were spread on LB agar plate containing 100 µg/ml ampicillin and the transformed cells of pET28a-gehWA were spread on LB agar plate containing 50 µg/ml kanamycin. The plates were further incubated at 37°C overnight.

3.12.2.6 Screening for recombinant clones

Both recombinant clones of pET17b-lipWY and pET28a-gehWA were primarily checked by rapid size screening method and verified by restriction analysis (*Nde* I and *Eco*R I for pET17b-lipWY and *Nhe* I and *Xho* I for pET28a-gehWA).

3.12.2.7 DNA sequencing and sequence analysis

The expected recombinant clones which contained lipWY fragment and expected recombinant clones which contained gehWA fragment were verified by nucleotide sequencing using automate DNA sequencer (1st BASE, Singapore). Nucleotide sequences were checked by using Chromas Lite and BioEdit program and compared to the data from the BLAST (http://blast.ncbi.nlm.nih.gov).

3.12.3 Expression of lipase genes in *E.coil* host

3.12.3.1 Optimized concentration of isopropyl-thiogalactoside (IPTG)

The pET17b-lipWY plasmid was transformed to *E.coli* strain BL21(DE3) by heat shock method and then was grown in LB medium with 100 µg/ml ampicillin. The pET28a-gehWA plasmid was transformed to *E.coli* strain BL21(DE3) by heat shock method and then was grown in LB medium with 50 µg/ml kanamycin. The samples were then later incubated at 37°C, 250 rpm until the absorbance at 600 nm was 0.5 to 0.6 and then 0.1, 0.25, 0.5, 0.75, 1.0, 1.25 mM of IPTG were added. The cells were collected after 3 hr of induction by centrifugation and were later analyzed by SDS-PAGE.

3.12.3.2 Optimized post-induction time

E.coli transformants were grown in LB broth containing appropriate antibiotic and incubated at 37°C with shaking at 250 rpm until the absorbance at 600 nm was 0.5 to 0.6 and then 0.1 mM of IPTG was added. Next, the sample was chilled on ice for 30 min and then it was incubated at 18°C and cells were collected at 0, 1, 2, 3, 4, 5, 6, 7 and 8 hr after induction. Cell samples were later analyzed by SDS-PAGE.

3.12.3.3 SDS-PAGE

The sample was prepared by mixing with 5X sample buffer to final concentration of 1X sample buffer. The reaction was heated at 95°C for 5 min and centrifuged at 13,000 rpm for 5 min. The protein sample was run on SDS-PAGE gels (Separating gel 12%, Stacking gel 4%)

3.12.4 Purification of recombinant lipase

3.12.4.1 Cell lysis and purification

After induction, cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C. The bacterial cell pellet was thawed on ice for 15

min and resuspended in buffer A (20mM Tris-HCl, pH 8 with 50 mM NaCl), and lysed by sonication on an ice bath (30 s pulse with 2 min). The lysate was centrifuged for 20 min at 10,000x g, 4°C. The resulted supernatant containing soluble protein fraction was separated and filtered by using a 0.2 µM nylon membrane to eliminate small particles. The purification procedure was performed at room temperature. HiTrap affinity column (5ml; $NiSO_4$) was equilibrated with 10 column volumes (CV) of buffer A. Next, filtered supernatant was loaded on column at a flow-rate of 1ml/min followed by washing with 10 CV of the buffer A (Wash A), washed again by 10 CV washing buffer (Wash I) (20 mM imidazole in buffer A) and eluted with elution buffer (100 mM imidazole in buffer A). Eluted fractions (5 ml) were collected and lipase activities were analyzed. The active fractions were collected and concentrated to 1.5 ml by a Centricon. The concentrated sample was loaded on a HiTrap desalting column and eluted with 20 mM Tris-HCl, pH 8.5.The active fractions were pooled, concentrated, and analyzed for purity by using SDS–PAGE.

3.12.4.2 Lipase assay

Lipase activity was determined spectrophotometrically using two solutions. Solution A contained 90 mg *p*-nitrophenyl palmitate dissolved in 30 ml 2-propanol. Solution B contained 2 g TritonX-100 and 0.5 g gum arabic dissolved in 450 ml buffer (Tris-HCl, 50 mM, pH 8.5). The assay reagent was prepared by adding 1 ml of solution A to 9 ml of solution B drop wisely to get an emulsion that remained stable for 2 h. The assay mixture contained 180 µl of the emulsion and 20 µl appropriately diluted enzyme solution. The liberated *p*-nitrophenol was measured at 410 nm, room temperature. One unit of enzyme was defined as the amount of enzyme that releases 1 µmol *p*-nitrophenol from the substrate per minute.

3.12.4.3 Protein determination

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

3.12.5 Characterization of recombinant lipase

3.12.5.1 Effect of pH and temperature on enzyme activity

The effects of pH and temperature on the lipase activity were determined spectrophotometrically using *p*-nitrophenyl palmitate as the substrate. The optimum pH for enzyme activity was determined at room temperature from pH 5.0 to 11.0 in various buffers: in 50mM acetate/Na-acetate buffer (pH 5.0), 50mM K_2HPO_4/KH_2PO_4 buffer (pH 6.0-7.0), 50mM Tris–HCl buffer (pH 8.0-10.0) and 50mM $NaCO_3$/HCl (pH 11.0). The optimum temperature for lipase activity was determined by measuring the rate of reaction at temperatures ranging from 20 to 60°C under the standard assay conditions.

3.12.5.2 Effect of pH and temperature on enzyme stability

The effect of pH on lipase stability was determined by incubating the purified lipase (0.5mg/ml) in the buffers having different pH values (pH 5.0-11.0) for 20 hr at 4°C, and residual activity was assayed spectrophotometrically at room temperature. To determine the influence of temperature on the enzyme stability, the purified lipase (1.0 mg/ml) was preincubated for 20 hr at temperature range of 20–60°C in 50mM Tris–HCl buffer, pH 8.5. Subsequently, the residual activity was analyzed at room temperature.

3.12.5.3 Substrate specificity

Substrate specificities of the purified lipase, towards different *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 analysed spectrophotometrically.

3.12.5.4 Effect of organic solvents on lipase

The purified lipase (2.0 mg/ml) was mixed with the same volume of organic solvent to prepare the 50% organic solution, and then the mixtures were incubated at room temperature on the rotator at 300 rpm for 1 and 6 hr. The residual activity was measured at room temperature and the organic solution tolerance of enzymes was calculated by comparing the residual activity with the control (purified lipase (2.0 mg/ml) was mixed with the same volume of 20mM Tris–HCl buffer, pH 8.5).

3.12.5.5 Effect of metal ions and detergents on lipase

The effect of metal ions on the lipase activity was determined by incubating purified lipase (1.0 mg/ml) with various metal ions (K^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+},Co^{2+}, Na^{+}, Zn^{2+}, Fe^{3+}, Hg^{2+}, Ag^{2+}, Mn^{2+} and Li^{2+} (1 or 10 mM)) in 50 mM Tris–HCl buffer, pH 8.5, at room temperature for 1 hr. The effect of a various detergents on enzyme activity was analyzed by incubating purified lipase (1.0 mg/ml) for 1 hr at room temperature in 50 mM Tris–HCl buffer, pH 8.5, containing 1% (v/v) of DMSO, β-mercaptoethanol, DTT, Triton X-100, Tween-80, 1 mM EDTA and 1% (w/v) of SDS. The activity assayed in the absence of metal ions was defined as control. The remaining activity was measured using the spectrophotometric assay as above.

3.12.5.6 Hydrolysis of oil

Hydrolysis of oil was preliminary checked by plate assay. The purified lipase was incubated in plate consisting of 0.001% (w/v) rhodamine B, 2% (w/v) agar and 1% (w/v) various kinds of oil; coconut, olive, palm and safflower oil. The hydrolysis activity was detected as orange fluorescence under UV light. After that, the activity was confirmed by titration of lipase-hydrolyzed oils. The substrate reaction mixture; 5 ml of 50 mM Tris–HCl buffer, pH 8.5 and 4 ml of 50% oil emulsion (2 ml of 2.0% polyvinyl alcohol mixed with the same volume of various oils) was sonicated and kept at room temperature for 5 min. 1 ml of the purified lipase was later added to the reaction mixture. The reaction were stirred at 300 rpm, room temperature for 5 hr. 10 ml of 95% ethanol was immediately added to stop the reaction. Liberated free fatty acids were titrated with 50 mM NaOH using phenolphthalein as indicator. One unit of hydrolytic activity of the lipase was defined as the amount of enzyme which catalyzes the release of 1 µmol of free fatty acids per min under the above conditions.

3.12.5.7 Transesterification

Transesterification reactions were carried out in 20 ml screw-capped vials containing 0.5 g of coconut oil and 7.8 mg of the purified lipase and later mixed with 1:3 molar ratio of methanol. The stepwise reaction was conducted by three addition steps of methanol at 0, 3 and 6 hours. The reaction was carried out by stirring the mixtures with magnetic stirrer at 250 rpm for 24 hr at room temperature. Samples were taken from the reaction mixture and later analyzed for the products by high-performance liquid chromatography (HPLC).

3.12.5.8 HPLC analysis

Samples were taken from the reaction mixture at specified time and centrifuged at 12,000 rpm 30 min to obtain the upper layer. The 10 µl 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 its 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 G.

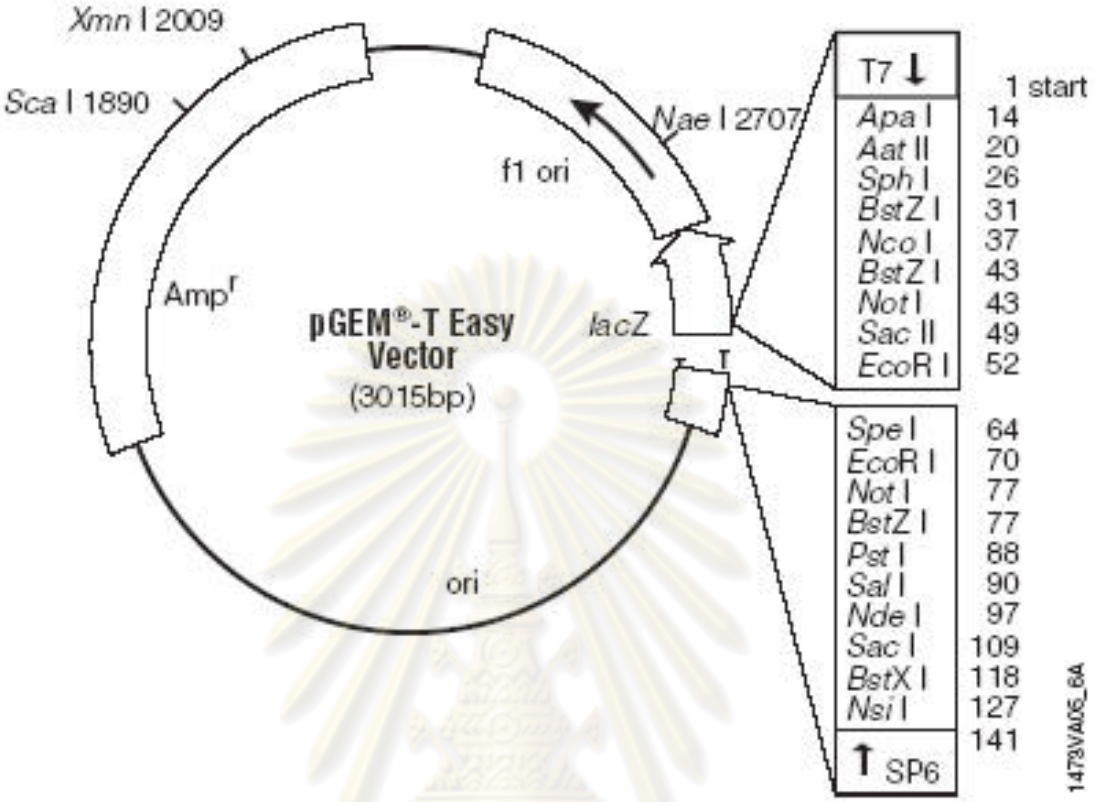

Figure 3-1 Physical map of pGEMT® - T Easy vector

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

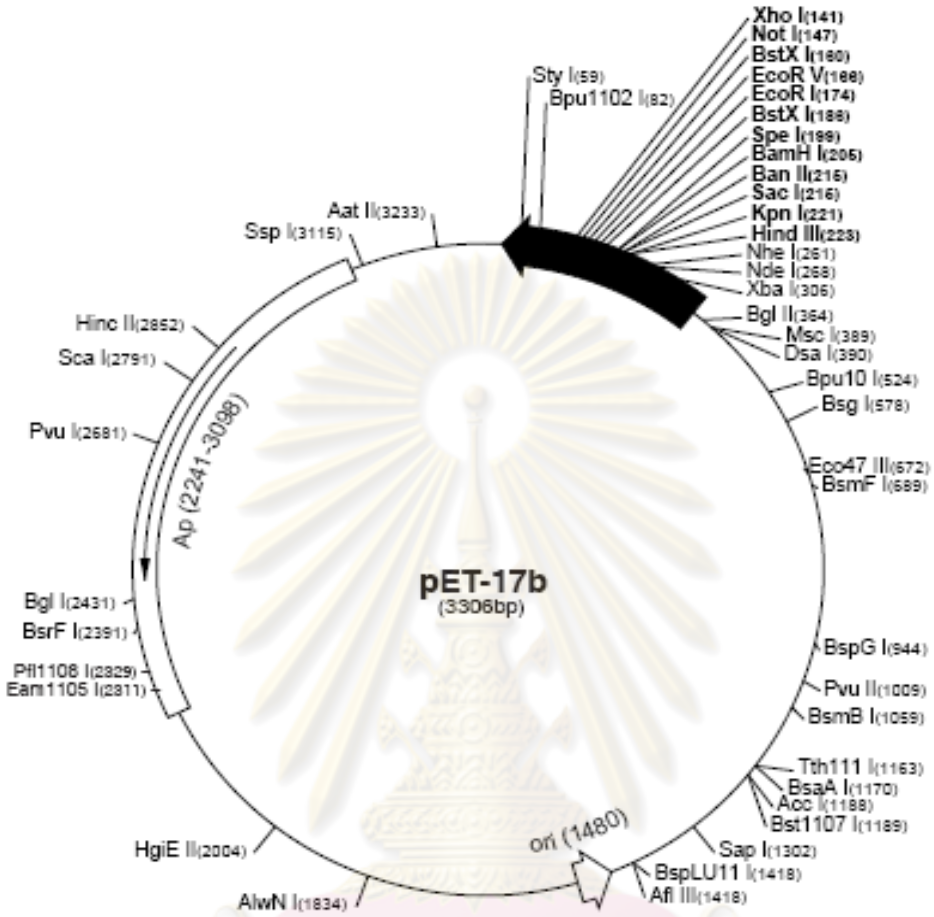

Figure 3-2 Physical map of pET-17b vector

T7 promoter	333-349
T7 transcription start	332
T7•Tag coding sequence	237-269
Multiple cloning sites (*Hin*d III - *Xho* I)	141-228
T7 terminator	28-74
pBR322 origin	1480
bla coding sequence	2241-3098

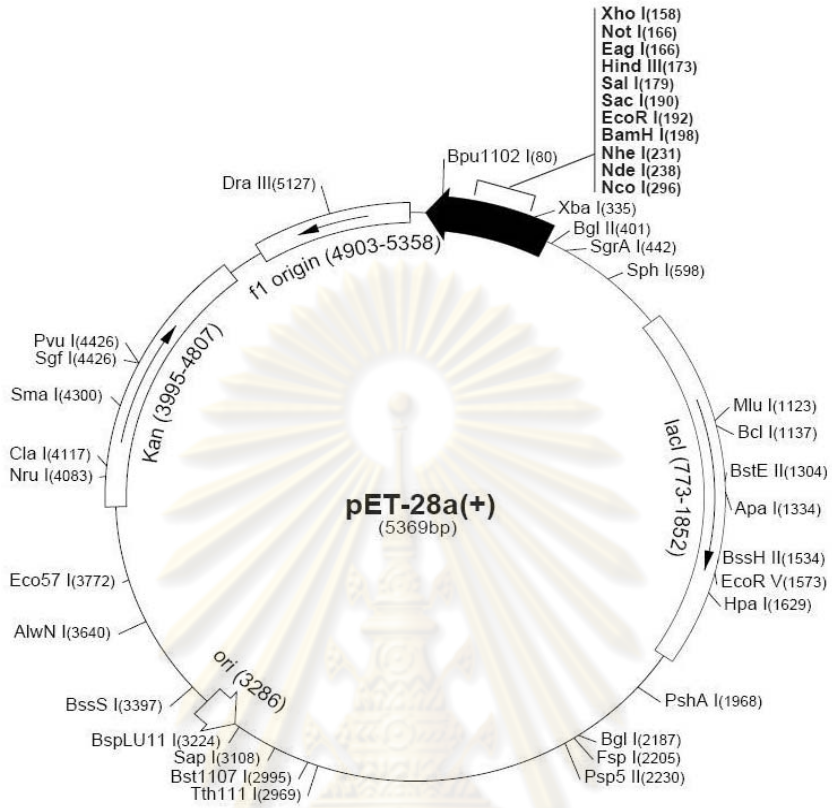

Figure 3-3 Physical map of pET-28a vector

T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites (*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

CHAPTER IV
RESULTS

4.1 Identification of the lipase producing bacterium

4.1.1 Based on biochemical characteristics

The bacteria were identified by Department of Medical Sciences, Ministry of Public Health. Biochemical characteristics showed that the lipase producing bacterium was Gram-positive, facultatively anaerobic, furazolidone and lysostaphin-sensitive cocci. These characteristics indicated that the sample was member of the genus *Staphylococcus*. As shown in Table 4-1, the production of yellow pigment and acetylmethylcarbinol; fermentation of sucrose, maltose, mannitol, trehalose, and fructose; weak reduction or none of nitrate to nitrite; demonstration of arginase, urease, and β-glucosidase activities; and sensitivity to novobiocin indicated that the sample could be strain of *Staphylococcus warneri.*

Table 4-1. Biochemical characteristics of the lipase producing bacterium

Characteristics	Lipase producing bacterium
Pigment	yellow
Anaerobic growth	+
Acetylmethylcarbinol	-
Xylose	-
L-Arabinose	-
Cellobiose	-
Raffinose	-
Salicin	-
Sucrose	+
Maltose	+
Mannitol	v
Mannose	-

Table 4-1. (continued)

Characteristics	Lipase producing bacterium
Trehalose	v
Lactose	v
Galactose	v
Fructose	+
Ribose	-
Nitrate reduction	w
Phosphatase	-
Arginase	v
Urease	+
β-Glucosidase	-
β-Galactosidase	-
Novobiocin resistance	-

v, variable; w, weak reaction; +, positive reaction; -, negative reaction

4.1.2 Based on 16S rRNA sequence

Genomic DNA of *S.warneri* was extracted and the 16S rRNA gene was amplified (Figure4-1) purified and ligated into pGEM-T Easy vector. The expected recombinant clones which contained 16S rRNA fragment were verified by nucleotide sequencing using automated DNA sequencer (1st BASE, Malaysia). Nucleotide sequences were checked by using Chromas Lite and BioEdit program. The sequence was aligned in GeneBank database by using the Blast program to determine the closest relatives of the 16S rRNA gene and deposited in the GenBank with the accession number L37603.1.The closest known relative of the submitted sequence, with 99% identity, was *S.warneri* (Figure4-2).

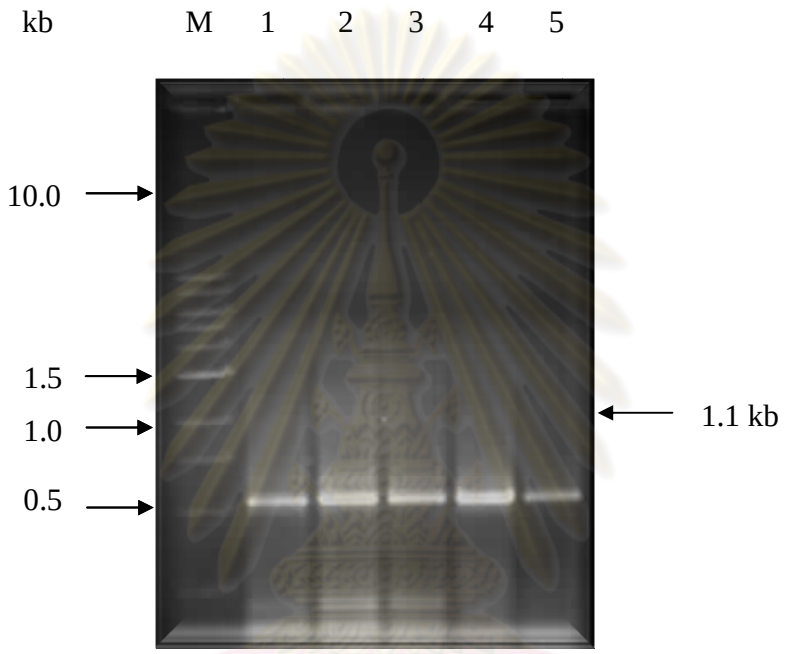

Figure 4-1 Analysis of PCR products (16S rRNA gene) by 1.0% agarose gel electrophoresis

Lane M : TriDye ™ 1 kb DNA Ladder

Lane 1-5 : PCR products (16S rRNA gene)

gb|L37603.1|STARGDH Staphylococcus warneri 16S ribosomal RNA (16S rRNA) gene
Length=1470

 Score = 1962 bits (1062), Expect = 0.0
 Identities = 1066/1068 (99%), Gaps = 0/1068 (0%)
 Strand=Plus/Plus

```
Sample 1     GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGATAAGGAGCTTGCT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1     GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGATAAGGAGCTTGCT  60

Sample 61    CCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  61    CCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGG  120

Sample 121   ATAACTTCGGGAAACCGGAGCTAATACCGGATAACATATTGAACCGCATGGTTCAATAGT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  121   ATAACTTCGGGAAACCGGAGCTAATACCGGATAACATATTGAACCGCATGGTTCAATAGT  180

Sample 181   GAAAGGCGGCTTTGCTGTCACTTATAGATGGATCCGCGCCGTATTAGCTAGTTGGTAAGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  181   GAAAGGCGGCTTTGCTGTCACTTATAGATGGATCCGCGCCGTATTAGCTAGTTGGTAAGG  240

Sample 241   TAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  241   TAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGA  300

Sample 301   ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  301   ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCG  360

Sample 361   AAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  361   AAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTT  420

Sample 421   ATCAGGGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTGATCAGAAAG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  421   ATCAGGGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTGATCAGAAAG  480

Sample 481   CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  481   CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAA  540

Sample 541   TTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  541   TTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTC  600

Sample 601   AACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCC  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  601   AACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCC  660

Sample 661   ATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  661   ATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCT  720

Sample 721   GGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  721   GGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGG  780

Sample 781   TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  781   TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGC  840
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Sample 841   AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  841   AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA  900

Sample 901   TTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  901   TTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC  960

Sample 961   CTTACCAAATCTTGACATCCTTTGACCGCTCTAGAGATAGAGTCTTCCCCTTCGGGGGAC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  961   CTTACCAAATCTTGACATCCTTTGACCGCTCTAGAGATAGAGTCTTCCCCTTCGGGGGAC  1020

Sample 1021  AAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTTGTGAAATGT  1068
             |||||||||||||||||||||||||||||||||||| |||| ||||
Refer  1021  AAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT  1068
```

Figure 4-2 Alignment of nucleotide sequence of 16S rRNA gene and sequence data available from GenBank

4.2 Lipase genes cloning

4.2.1 The lipWY gene

The fragment containing the lipase operon was amplified by PCR with the primer lipWY_F and lipWY_R. The size of PCR product was 1,100 bp (Figure 4-3). The purified lipWY gene was ligated into pET-17b vector and then recombinant plasmid pET17b-lipWY was successfully constructed. The recombinant plasmid size was checked by restriction analysis. The two bands were observed on gel; 1,100 bp of lipWY and 3,306 bp of cloning vector (Figure 4-4). The recombinant plasmid was subjected for sequencing. Obtained sequence was checked with Chromas Lite and BioEdit program and compared to sequence data available from GenBank by using the BLAST program. It showed 99% identity with *S.warneri* lipWY gene (Figure 4-5). The open reading frame of lipWY was composed of 1,053 bp, which encoded 350 amino acids and molecular weight of approximately 40.234 kDa (Figure 4-6).

4.2.2 The gehWA gene

In order to clone the lipase gene of *S.warneri*, the gehWA gene was amplified by PCR. The size of PCR product was 1,100 bp (Figure 4-7). The purified gehWA gene was ligated into pET28a vector and then recombinant plasmid pET28a-gehWA was successfully constructed. The recombinant plasmid size was checked by restriction analysis. The two bands were observed on gel; 1,100 bp of gehWA and 5,369 bp of cloning vector (Figure 4-8). The recombinant plasmid was subjected for sequencing. Obtained sequence was checked with Chromas Lite and BioEdit program and compared to sequence data available from GenBank using the BLAST program. It showed 99% identity with *S.warneri* gehWA gene (Figure 4-9). From obtained nucleotide sequence, it was shown that gehWA was composed of 1,140 bp, encoding 379 amino acids and molecular weight of approximately 42.415 kDa (Figure 4-10).

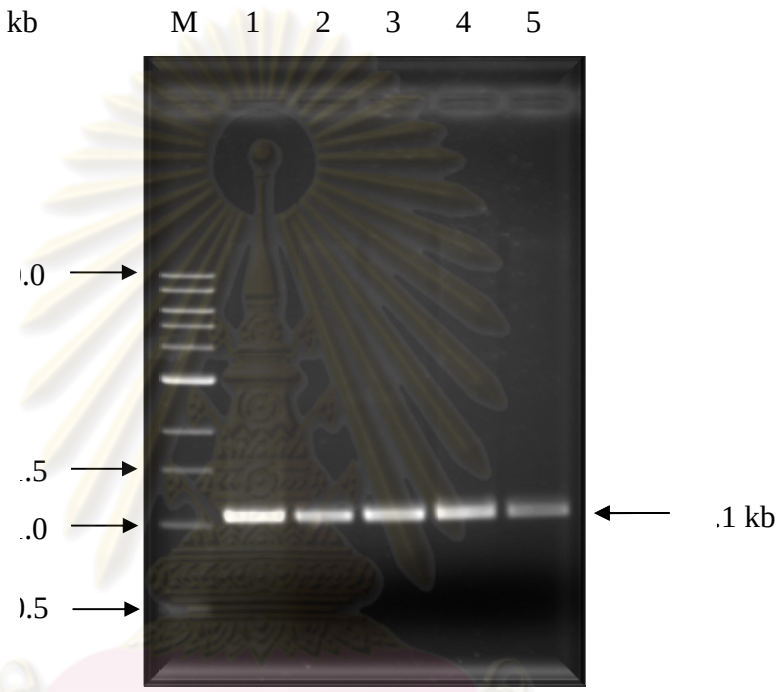

Figure 4-3 Analysis of PCR products (lipWY gene) by 1.0% agarose gel electrophoresis

Lane M : TriDye ™ 1 kb DNA Ladder

Lane 1-5 : PCR products (lipWY gene)

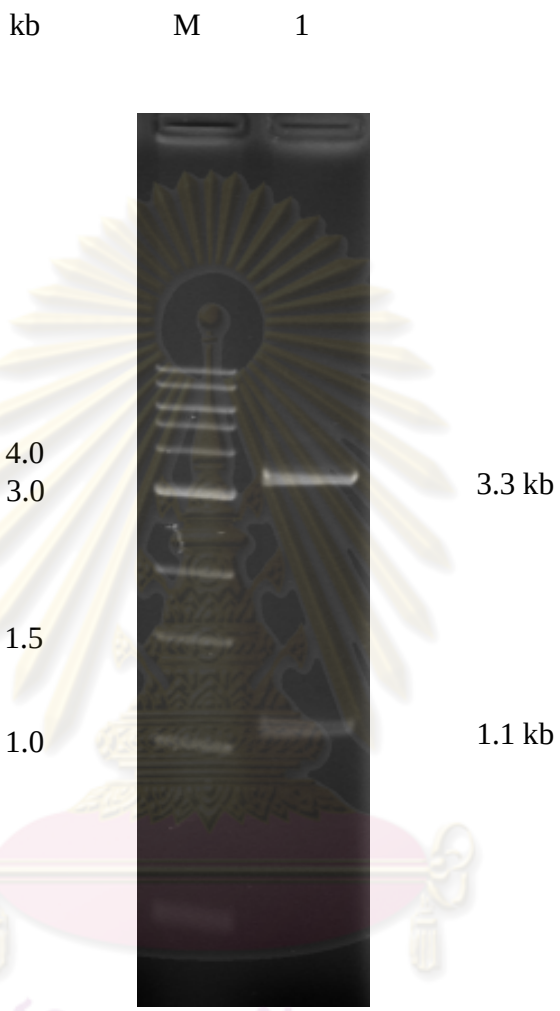

Figure 4-4 Restriction enzymes digest of pET17b-lipWY were analyzed on 1.0% agarose gel electrophoresis.

Lane M : TriDye ™ 1 kb DNA Ladder

Lane 1 : Double digestion pET17b-lipWY with *Nde* I and *Eco*R I

dbj|AB189473.1| Staphylococcus warneri lipWY gene for lipase, complete cds
Length=1765

 Score = 1934 bits (1047), Expect = 0.0
 Identities = 1051/1053 (99%), Gaps = 0/1053 (0%)
 Strand=Plus/Plus

```
Sample 1     ATGAAGGCAAAACATAAATGGTTGTTGATAACTGTCGTTATTATTTTAATAGTAGGTACT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  480   ATGAAGGCAAAACATAAATGGTTGTTGATAACTGTCGTTATTATTTTAATAGTAGGTACT  539

Sample 61    AGTACTGGTCTGTTATTGAAAAAATATTATGATCATGAACACCGACAACAACAAAATAAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  540   AGTACTGGTCTGTTATTGAAAAAATATTATGATCATGAACACCGACAACAACAAAATAAA  599

Sample 121   GAAAAAGTCCAAATAAATAATAAAAACGTGAAAGTCTTACAGAATATTTCTTATGGTCAA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  600   GAAAAAGTCCAAATAAATAATAAAAACGTGAAAGTCTTACAGAATATTTCTTATGGTCAA  659

Sample 181   GGCATACCTAATAGTAAATTAGATATTATTATGCCATCTGATATGAATAAAGATAGTAAG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  660   GGCATACCTAATAGTAAATTAGATATTATTATGCCATCTGATATGAATAAAGATAGTAAG  719

Sample 241   TTACCAGTCATCTTTTGGATGCATGGTGGTGGTTTTATTGCTGGTGATAAACAGTATAAA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  720   TTACCAGTCATCTTTTGGATGCATGGTGGTGGTTTTATTGCTGGTGATAAACAGTATAAA  779

Sample 301   AATCCTTTACTATCTAAAATAGCTGAGCAAGGTTACATTGTGGTAAACGTCAACTATGCG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  780   AATCCTTTACTATCTAAAATAGCTGAGCAAGGTTACATTGTGGTAAACGTCAACTATGCG  839

Sample 361   TTAGCGCCTCAATACAAGTATCCTACACCTATTGAACAAATGAACAAAGCTGTCAAATTT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  840   TTAGCGCCTCAATACAAGTATCCTACACCTATTGAACAAATGAACAAAGCTGTCAAATTT  899

Sample 421   ATAAAAACGAATGAACATGATTTGCCTATTGATTTTGATCAAGTCATTATAGGTGGTGAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  900   ATAAAAACGAATGAACATGATTTGCCTATTGATTTTGATCAAGTCATTATAGGTGGTGAT  959

Sample 481   TCTGCTGGCGCACAATTAACCAGTCAATATGTTGCTATGCAAACAAATCAATCGTTACGT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  960   TCTGCTGGCGCACAATTAACCAGTCAATATGTTGCTATGCAAACAAATCAATCGTTACGT  1019

Sample 541   GATGAAATGAAATTTGAACCCGAATTTAAACCTTCTCAAATTAAAGCAGCGATATTCTTT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1020  GATGAAATGAAATTTGAACCCGAATTTAAACCTTCTCAAATTAAAGCAGCGATATTCTTT  1079

Sample 601   GGTGGTTTCTATGATATGAAGACAGTTAAAGCTACAGAGTTTCCTAGGATCCAATTATTT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1080  GGTGGTTTCTATGATATGAAGACAGTTAAAGCTACAGAGTTTCCTAGGATCCAATTATTT  1139

Sample 661   ATGAGAAGTTATACAGGTACAACAAATTGGGAGAGTAATTTTAAAAACTTATCTCAAATG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1140  ATGAGAAGTTATACAGGTACAACAAATTGGGAGAGTAATTTTAAAAACTTATCTCAAATG  1199

Sample 721   TCTACGATAAATCAAATAACTAAAGATTATCCGCCTACATTTTTATCAGTGGGTGATGCG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1200  TCTACGATAAATCAAATAACTAAAGATTATCCGCCTACATTTTTATCAGTGGGTGATGCG  1259

Sample 781   GATCCATTTTATAGTCAGAATATAGATTTTTATAAAAAATTAAAAGAGAAAGATGTGCCA  840
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Refer  1260  GATCCATTTTATAGTCAGAATATAGATTTTTATAAAAAATTAAAAGAAAAAGATGTGCCA  1319
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Sample 841   GCTGAAACATTACTTTATGATGGTTCACATCACTTACACCATCAATATCAATTTCATTTA  900
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Refer  1320  GCTGAAACATTATTTTATGATGGTTCACATCACTTACACCATCAATATCAATTTCATTTA  1379

Sample 901   AATAAACCAGAATCCAAAGAGAATATTAAACGGGTACTCTTATTCTTAAGTAGAAACACA  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1380  AATAAACCAGAATCCAAAGAGAATATTAAACGGGTACTCTTATTCTTAAGTAGAAACACA  1439

Sample 961   TCTTCTTCAGGTGTTGAACGAAACAATCAATCTAATCACAATGAAAACAATAATTTGAAT  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1440  TCTTCTTCAGGTGTTGAACGAAACAATCAATCTAATCACAATGAAAACAATAATTTGAAT  1499

Sample 1021  CAAGATGTACAATTAGACCCATTTAGTGAATAA  1053
             ||||||||||||||||||||||||||||||||
Refer  1500  CAAGATGTACAATTAGACCCATTTAGTGAATAA  1532
```

Figure 4-5 Alignment of nucleotide sequence of lipWY gene and sequence data available from GenBank

```
1       ATGAAGGCAAAACATAAATGGTTGTTGATAACTGTCGTTATTATTTTAATAGTAGGTACT  60
          M  K  A  K  H  K  W  L  L  I  T  V  V  I  I  L  I  V  G  T
61      AGTACTGGTCTGTTATTGAAAAAATATTATGATCATGAACACCGACAACAACAAAATAAA  120
          S  T  G  L  L  L  K  K  Y  Y  D  H  E  H  R  Q  Q  Q  N  K
121     GAAAAAGTCCAAATAAATAATAAAAACGTGAAAGTCTTACAGAATATTTCTTATGGTCAA  180
          E  K  V  Q  I  N  N  K  N  V  K  V  L  Q  N  I  S  Y  G  Q
181     GGCATACCTAATAGTAAATTAGATATTATTATGCCATCTGATATGAATAAAGATAGTAAG  240
          G  I  P  N  S  K  L  D  I  I  M  P  S  D  M  N  K  D  S  K
241     TTACCAGTCATCTTTTGGATGCATGGTGGTGGTTTTATTGCTGGTGATAAACAGTATAAA  300
          L  P  V  I  F  W  M  H  G  G  G  F  I  A  G  D  K  Q  Y  K
301     AATCCTTTACTATCTAAAATAGCTGAGCAAGGTTACATTGTGGTAAACGTCAACTATGCG  360
          N  P  L  L  S  K  I  A  E  Q  G  Y  I  V  V  N  V  N  Y  A
361     TTAGCGCCTCAATACAAGTATCCTACACCTATTGAACAAATGAACAAAGCTGTCAAATTT  420
          L  A  P  Q  Y  K  Y  P  T  P  I  E  Q  M  N  K  A  V  K  F
421     ATAAAAACGAATGAACATGATTTGCCTATTGATTTTGATCAAGTCATTATAGGTGGTGAT  480
          I  K  T  N  E  H  D  L  P  I  D  F  D  Q  V  I  I  G  G  D
481     TCTGCTGGCGCACAATTAACCAGTCAATATGTTGCTATGCAAACAAATCAATCGTTACGT  540
          S  A  G  A  Q  L  T  S  Q  Y  V  A  M  Q  T  N  Q  S  L  R
541     GATGAAATGAAATTTGAACCCGAATTTAAACCTTCTCAAATTAAAGCAGCGATATTCTTT  600
          D  E  M  K  F  E  P  E  F  K  P  S  Q  I  K  A  A  I  F  F
601     GGTGGTTTCTATGATATGAAGACAGTTAAAGCTACAGAGTTTCCTAGGATCCAATTATTT  660
          G  G  F  Y  D  M  K  T  V  K  A  T  E  F  P  R  I  Q  L  F
661     ATGAGAAGTTATACAGGTACAACAAATTGGGAGAGTAATTTTAAAAACTTATCTCAAATG  720
          M  R  S  Y  T  G  T  T  N  W  E  S  N  F  K  N  L  S  Q  M
721     TCTACGATAAATCAAATAACTAAAGATTATCCGCCTACATTTTTATCAGTGGGTGATGCG  780
          S  T  I  N  Q  I  T  K  D  Y  P  P  T  F  L  S  V  G  D  A
781     GATCCATTTTATAGTCAGAATATAGATTTTTATAAAAAATTAAAAGAGAAAGATGTGCCA  840
          D  P  F  Y  S  Q  N  I  D  F  Y  K  K  L  K  E  K  D  V  P
841     GCTGAAACATTACTTTATGATGGTTCACATCACTTACACCATCAATATCAATTTCATTTA  900
          A  E  T  L  L  Y  D  G  S  H  H  L  H  H  Q  Y  Q  F  H  L
901     AATAAACCAGAATCCAAAGAGAATATTAAACGGGTACTCTTATTCTTAAGTAGAAACACA  960
          N  K  P  E  S  K  E  N  I  K  R  V  L  L  F  L  S  R  N  T
961     TCTTCTTCAGGTGTTGAACGAAACAATCAATCTAATCACAATGAAAACAATAATTTGAAT  1020
          S  S  S  G  V  E  R  N  N  Q  S  N  H  N  E  N  N  N  L  N
1021    CAAGATGTACAATTAGACCCATTTAGTGAATAA  1053
          Q  D  V  Q  L  D  P  F  S  E  *
```

Figure 4-6 Nucleotide sequence and deduced amino acid sequence of lipWY gene

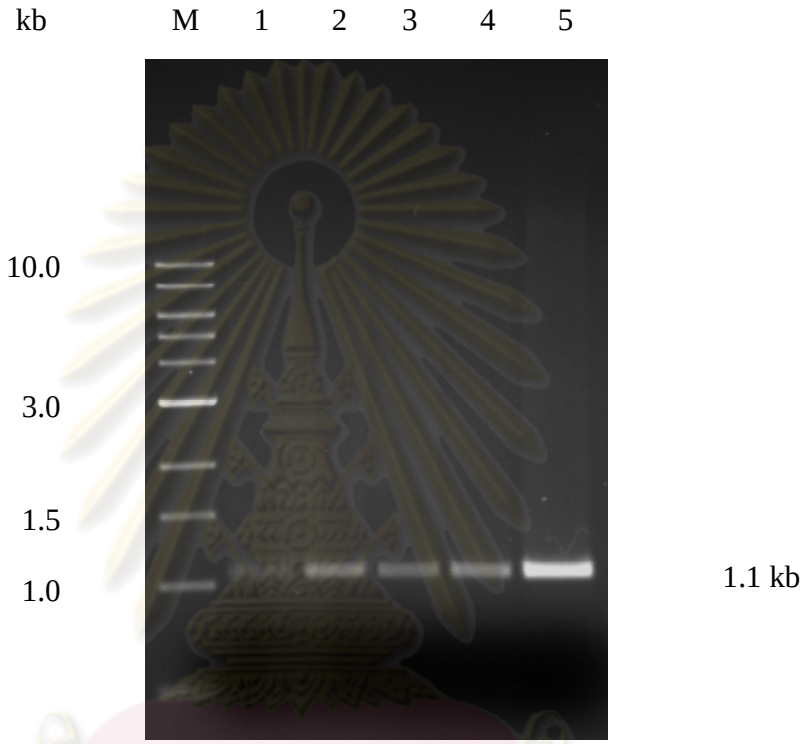

Figure 4-7 Analysis of PCR products (gehWA gene) by 1.0% agarose gel electrophoresis
Lane M : TriDye ™ 1 kb DNA Ladder
Lane 1-5 : PCR products (gehWA gene)

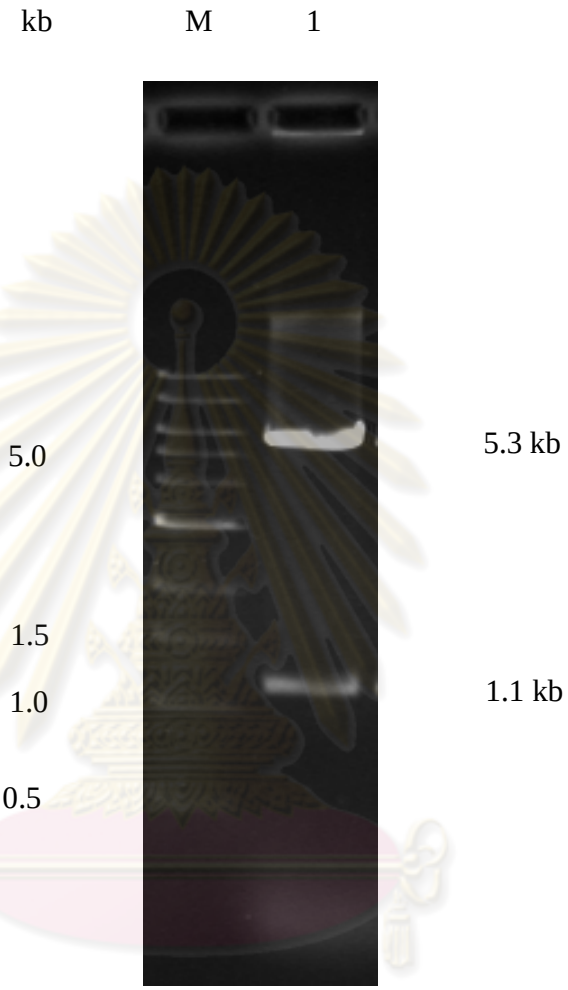

Figure 4-8 Restriction enzymes digest of pET28a-gehWA were analyzed on 1.0% agarose gel electrophoresis.

Lane M : TriDye ™ 1 kb DNA Ladder

Lane 1 : Double digestion pET28a-gehWA with *Xho* I and *Nhe* I

dbj|AB189476.1| Staphylococcus warneri SWM2196, gehWA genes for hypothetical protein, lipase, complete cds
Length=3816

Score = 2078 bits (1125), Expect = 0.0
Identities = 1136/1141 (99%), Gaps = 2/1141 (0%)
Strand=Plus/Plus

```
Sample 1     AATCATGACCCTATTATACTTGTACATGGATTTAATGGTTTTACGGCTGATAATGGACCT  60
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Refer  1618  AATCATGACCCTATTATACTTGTACATGGATTTAATGGTTTTACAGCTGATAATGGACCT  1677

Sample 61    GGTTTAGGAGATAGTAATTATTGGGGTGGCGAACGTCTTAACATCACACAAGAAGCACGT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1678  GGTTTAGGAGATAGTAATTATTGGGGTGGCGAACGTCTTAACATCACACAAGAAGCACGT  1737

Sample 121   GCAAAAGGTTATAACGTGAGTGAAGCTAGTGTAAGTGCATTAGGTAGTAATTATGATCGT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1738  GCAAAAGGTTATAACGTGAGTGAAGCTAGTGTAAGTGCATTAGGTAGTAATTATGATCGT  1797

Sample 181   GCGGTTGAATTGTACTACTATATCAAAGGTGGCACTGTAGATTATGGTGCGGCACATGCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1798  GCGGTTGAATTGTACTACTATATCAAAGGTGGCACTGTAGATTATGGTGCGGCACATGCA  1857

Sample 241   GCTAAATATGGTCATGAACGTTATGGCAAGTCTTATGCAGGGGCCTATAGAGATTGGAAA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1858  GCTAAATATGGTCATGAACGTTATGGCAAGTCTTATGCAGGGGCCTATAGAGATTGGAAA  1917

Sample 301   CCAGGCCAGAAAATTCATTTAATCGGCCATAGTATGGGTGGACAAACTGTTCGTTTATTA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1918  CCAGGCCAGAAAATTCATTTAATCGGCCATAGTATGGGTGGACAAACTGTTCGTTTATTA  1977

Sample 361   GAAGAAATGTTACGTAATGGAAATCCTGAAGAAATCGAATATCAAAAGCAACATGGTGGT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  1978  GAAGAAATGTTACGTAATGGAAATCCTGAAGAAATCGAATATCAAAAGCAACATGGTGGT  2037

Sample 421   GAGATTTCACCATTATATAAAGGTGGACAAGATAACATGATTTCTTCCATTACTACGTTA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  2038  GAGATTTCACCATTATATAAAGGTGGACAAGATAACATGATTTCTTCCATTACTACGTTA  2097

Sample 481   GCGTCACCTCATAACGGTACACACGCCTCTGATTTACTAGGTGATGAAGCGATTGTAAGA  540
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Refer  2098  GCGTCACCTCATAACGGTACACACGCCTCTGATTTACTAGGTAATGAAGCGATTGTAAGA  2157

Sample 541   CAAGCTGTTTATGATTTTGCGAAATCACAAGGTAATAAATTCTCTCGTGCCGATCTTGGT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  2158  CAAGCTGTTTATGATTTTGCGAAATCACAAGGTAATAAATTCTCTCGTGCCGATCTTGGT  2217

Sample 601   TTAACTCAATGGGGCTTAAAACAGAGACCTGATGAAACCTATATCGACTACGTGAAACGC  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  2218  TTAACTCAATGGGGCTTAAAACAGAGACCTGATGAAACCTATATCGACTACGTGAAACGC  2277

Sample 661   GTTGAAAATAGTAGCTTATGGAAGACAAAAGATAATGGATTCTATGATTTAACAACTGAA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  2278  GTTGAAAATAGTAGCTTATGGAAGACAAAAGATAATGGATTCTATGATTTAACAACTGAA  2337

Sample 721   GGTGCACAAGAGTTGAATAATCATACGTCACTCAATCCAAAAATAGTTTATAAAACATAT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  2338  GGTGCACAAGAGTTGAATAATCATACGTCACTCAATCCAAAAATAGTTTATAAAACATAT  2397
```

```
Sample 781   ACTGGCGAATCATCTGACCCAGATAAAAATGGTATCCATCATCGTAATAGTCATATGAAT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  2398  ACTGGCGAATCATCTGACCCAGATAAAAATGGTATCCATCATCGTAATAGTCATATGAAT  2457

Sample 841   ATTAAATATTTACCAACAACAAATGTGATTGGTAAATTAGACGATCAAGCTTGGCGA-GA  899
             |||||||||||||||||||||||||||||||||||||||||||||||||||| || ||
Refer  2458  ATTAAATATTTACCAACAACAAATGTGATTGGTAAATTAGACGATCAAGCTTGG-GATGA  2516

Sample 900   AAATGACGGCCTAGTATCAGTAGTTTCGGCACAACATCCATCTAATCAAAAATATGTAGA  959
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  2517  AAATGACGGCCTAGTATCAGTAGTTTCGGCACAACATCCATCTAATCAAAAATATGTAGA  2576

Sample 960   TGCTACAGATCAAATTCAAAAAGGCGTATGGCAAGTCACACCTGTCCAACACGATTGGGA  1019
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  2577  TGCTACAGATCAAATTCAAAAAGGCGTATGGCAAGTCACACCTGTCCAACACGATTGGGA  2636

Sample 1020  CCATGGCGACTTTGTCGGAACACAAAAAGATGAAAACGGCATCTCAATCGAGCAATTCCA  1079
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Refer  2637  CCATGGCGACTTTGTCGGAACACAAAAAGATGAAAACGGCATCTCAATCGAGCAATTCCA  2696

Sample 1080  AGGCTTCTGGGATAACCTATTAAACGATGCTGTAAGAAACGAAAAAGTGACTTATCAATA  1139
             ||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Refer  2697  AGGCTTCTGGGATAACCTATTAAACGATGCTGTAAGAAACGAAAAAGTGACTGATCAATA  2756

Sample 1140  A  1140
             |
Refer  2757  A  2757
```

Figure 4-9 Alignment of nucleotide sequence of gehWA gene and sequence data available from GenBank

```
1       AATCATGACCCTATTATACTTGTACATGGATTTAATGGTTTTACGGCTGATAATGGACCT   60
          N  H  D  P  I  I  L  V  H  G  F  N  G  F  T  A  D  N  G  P
61      GGTTTAGGAGATAGTAATTATTGGGGTGGCGAACGTCTTAACATCACACAAGAAGCACGT   120
          G  L  G  D  S  N  Y  W  G  G  E  R  L  N  I  T  Q  E  A  R
121     GCAAAAGGTTATAACGTGAGTGAAGCTAGTGTAAGTGCATTAGGTAGTAATTATGATCGT   180
          A  K  G  Y  N  V  S  E  A  S  V  S  A  L  G  S  N  Y  D  R
181     GCGGTTGAATTGTACTACTATATCAAAGGTGGCACTGTAGATTATGGTGCGGCACATGCA   240
          A  V  E  L  Y  Y  Y  I  K  G  G  T  V  D  Y  G  A  A  H  A
241     GCTAAATATGGTCATGAACGTTATGGCAAGTCTTATGCAGGGGCCTATAGAGATTGGAAA   300
          A  K  Y  G  H  E  R  Y  G  K  S  Y  A  G  A  Y  R  D  W  K
301     CCAGGCCAGAAAATTCATTTAATCGGCCATAGTATGGGTGGACAAACTGTTCGTTTATTA   360
          P  G  Q  K  I  H  L  I  G  H  S  M  G  G  Q  T  V  R  L  L
361     GAAGAAATGTTACGTAATGGAAATCCTGAAGAAATCGAATATCAAAAGCAACATGGTGGT   420
          E  E  M  L  R  N  G  N  P  E  E  I  E  Y  Q  K  Q  H  G  G
421     GAGATTTCACCATTATATAAAGGTGGACAAGATAACATGATTTCTTCCATTACTACGTTA   480
          E  I  S  P  L  Y  K  G  G  Q  D  N  M  I  S  S  I  T  T  L
481     GCGTCACCTCATAACGGTACACACGCCTCTGATTTACTAGGTGATGAAGCGATTGTAAGA   540
          A  S  P  H  N  G  T  H  A  S  D  L  L  G  D  E  A  I  V  R
541     CAAGCTGTTTATGATTTTGCGAAATCACAAGGTAATAAATTCTCTCGTGCCGATCTTGGT   600
          Q  A  V  Y  D  F  A  K  S  Q  G  N  K  F  S  R  A  D  L  G
601     TTAACTCAATGGGGCTTAAAACAGAGACCTGATGAAACCTATATCGACTACGTGAAACGC   660
          L  T  Q  W  G  L  K  Q  R  P  D  E  T  Y  I  D  Y  V  K  R
661     GTTGAAAATAGTAGCTTATGGAAGACAAAAGATAATGGATTCTATGATTTAACAACTGAA   720
          V  E  N  S  S  L  W  K  T  K  D  N  G  F  Y  D  L  T  T  E
721     GGTGCACAAGAGTTGAATAATCATACGTCACTCAATCCAAAAATAGTTTATAAAACATAT   780
          G  A  Q  E  L  N  N  H  T  S  L  N  P  K  I  V  Y  K  T  Y
781     ACTGGCGAATCATCTGACCCAGATAAAAATGGTATCCATCATCGTAATAGTCATATGAAT   840
          T  G  E  S  S  D  P  D  K  N  G  I  H  H  R  N  S  H  M  N
841     ATTAAATATTTACCAACAACAAATGTGATTGGTAAATTAGACGATCAAGCTTGGCGAGAA   900
          I  K  Y  L  P  T  T  N  V  I  G  K  L  D  D  Q  A  W  R  E
901     AATGACGGCCTAGTATCAGTAGTTTCGGCACAACATCCATCTAATCAAAAATATGTAGAT   960
          N  D  G  L  V  S  V  V  S  A  Q  H  P  S  N  Q  K  Y  V  D
961     GCTACAGATCAAATTCAAAAAGGCGTATGGCAAGTCACACCTGTCCAACACGATTGGGAC   1020
          A  T  D  Q  I  Q  K  G  V  W  Q  V  T  P  V  Q  H  D  W  D
1021    CATGGCGACTTTGTCGGAACACAAAAAGATGAAAACGGCATCTCAATCGAGCAATTCCAA   1080
          H  G  D  F  V  G  T  Q  K  D  E  N  G  I  S  I  E  Q  F  Q
1081    GGCTTCTGGGATAACCTATTAAACGATGCTGTAAGAAACGAAAAAGTGACTTATCAATAA   1140
          G  F  W  D  N  L  L  N  D  A  V  R  N  E  K  V  T  Y  Q  *
```

Figure 4-10 Nucleotide sequence and deduced amino acid sequence of gehWA gene

4.3 Expression of lipase genes in *E.coil* host

For the study of expression of pET17b-lipWY, the plasmid was transformed into the compatible host, *E.coli* BL21(DE3). After induction with 1mM IPTG, the 0.5 OD_{600} culture was harvested by centrifugation and resuspended in 60 µl of distilled water and mixed with 5X sample buffer to final concentration of 1X sample buffer. The lysate-sample mixtures were analyzed by SDS-PAGE, no expression of protein band approximately 40 kDa was detected even after IPTG induction for 3 and 6 hr as shown in Figure 4-11.

For the study of expression of pET28a-gehWA, the plasmid was transformed into the compatible host, *E.coli* BL21(DE3). After induction with 1mM IPTG, the 0.5 OD_{600} culture was harvested by centrifugation and resuspended in 60 µl of distilled water and mixed with 5X sample buffer to final concentration of 1X sample buffer. The lysate-sample mixtures were analyzed by SDS-PAGE. The expression of protein band, approximately 40 kDa, was detected after IPTG induction for 3 and 6 hr as shown in Figure 4-12. The culture was harvested at 3 hr post-induction by centrifugation. The cell pellet was resuspended in 200 µl buffer A and lysed by sonication on an ice. The lysate was centrifuged at 10,000x g, 4°C for 20 min. Both supernatant and pellet were checked on SDS-PAGE to verify solubility as shown in Figure 4-13. The result showed that recombinant protein was expressed as insoluble protein. So, the protein expression was comparatively performed at 18, 25 and 37°C. The lowering temperature expression was successful to obtain soluble protein that the yields were clearly decreased with increasing induction temperature. This probably reflects that the overexpression might influence the initial folding of enzyme resulting in the failure of the molecule to reach the native state at 37°C. Therefore, proteins expressed at the more permissive temperature of 18°C were utilized in this study (Figure 4-14).

4.3.1 Optimized concentration of IPTG

E.coli transformants were grown in LB broth containing 50 µg/ml kanamycin and incubated at 37°C with shaking at 250 rpm until the absorbance at 600 nm was 0.5 to 0.6. The sample was chilled on ice for 30 min before 0.1, 0.25,

0.5, 0.75, 1.0, 1.25 mM of IPTG were added. Next, the culture was further incubated at 18°C and cells were collected at 0 and 3 hr after induction with IPTG. Cell samples were later analyzed by SDS-PAGE. Expression of protein band approximately 40 kDa was detected after all concentrations of IPTG induction as shown in Figure 4-15.

4.3.2 Optimized post-induction time

E.coli transformants were grown in LB broth containing 50 µg/ml kanamycin and incubated at 37°C with shaking at 250 rpm until the absorbance at 600 nm was 0.5 to 0.6 and then sample was chilled on ice for 30 min before 0.1 mM of IPTG was added. Next, the culture was further incubated at 18°C and cells were collected at 0, 1, 2, 3, 4, 5, 6, 7 and 8 hr after induction. Cell samples were analyzed by SDS-PAGE. Expression of protein band approximately 40 kDa was detected as shown in Figure 4-16 that showed crude extract fraction and Figure 4-17 showed supernatant fraction.

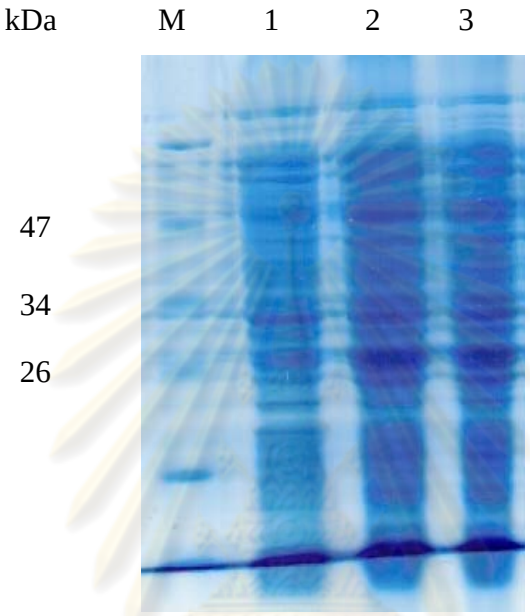

Figure 4-11 SDS-PAGE of expressed protein in the presence of 1 mM IPTG as inducer (crude extract of *E.coli* BL21(DE3) cells harboring pET17b-lipWY)

Lane M : Prestained Protein Ladder

Lane 1 : Crude extract from uninduced *E.coli* BL21(DE3)/ pET17b-lipWY

Lane 2 : Crude extract from induced *E.coli* BL21(DE3)/ pET17b-lipWY for 3 hr

Lane 3 : Crude extract from induced *E.coli* BL21(DE3)/ pET17b-lipWY for 6 hr

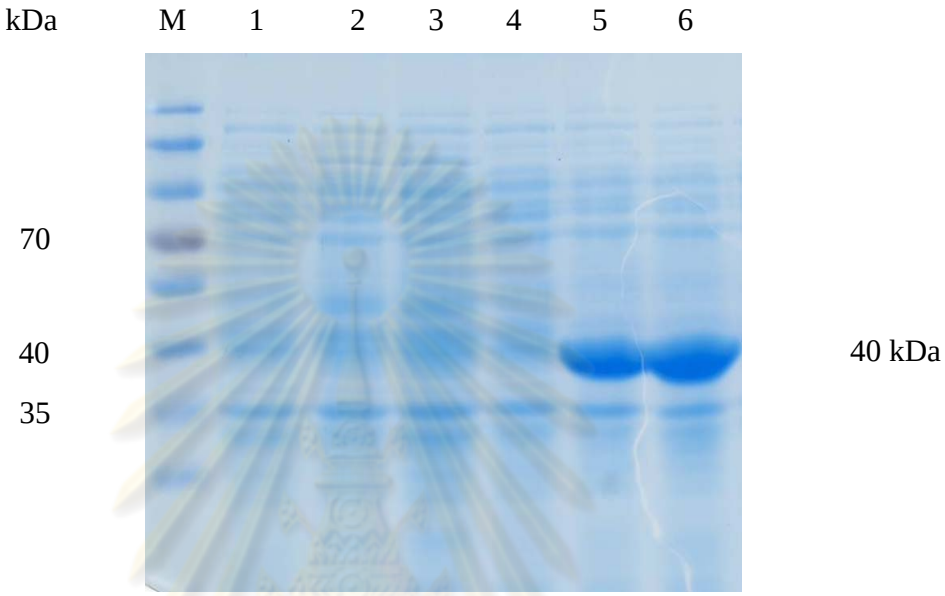

Figure 4-12 SDS-PAGE of expressed protein in the presence of 1 mM IPTG as inducer (crude extract of *E.coli* BL21(DE3) cells harboring pET28a-gehWA)

Lane M : Prestained Protein Ladder

Lane 1 : Crude extract from uninduced *E.coli* BL21(DE3)

Lane 2 : Crude extract from induced *E.coli* BL21(DE3) for 3 hr

Lane 3 : Crude extract from induced *E.coli* BL21(DE3) for 6 hr

Lane 4 : Crude extract from uninduced *E.coli* BL21(DE3)/pET28a-gehWA

Lane 5 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 3 hr

Lane 6 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 6 hr

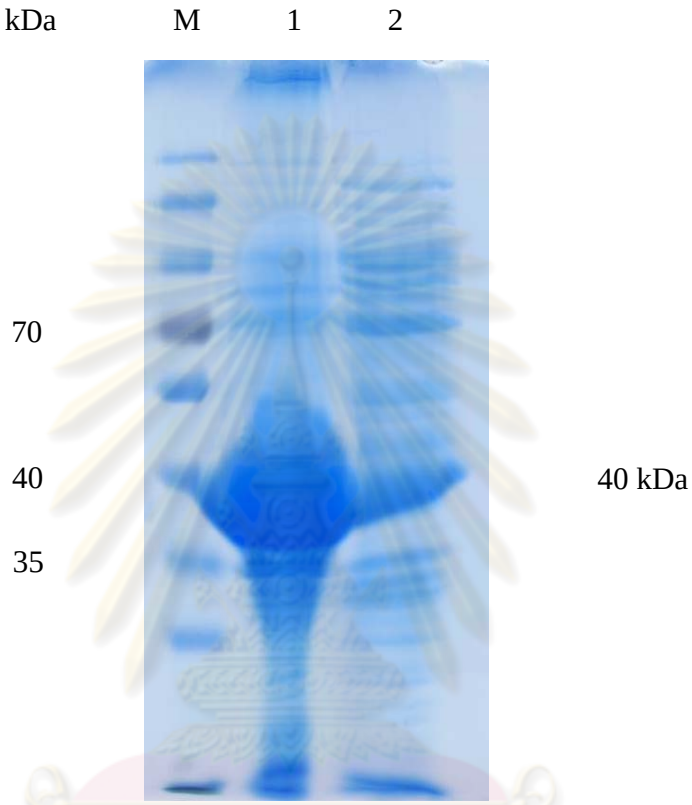

Figure 4-13 SDS-PAGE of expressed protein in the presence of 1 mM IPTG as inducer, expression at 37°C

Lane M : Prestained Protein Ladder

Lane 1 : Pellet from induced *E.coli* BL21(DE3)/pET28a-gehWA for 3 hr

Lane 2 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 3 hr

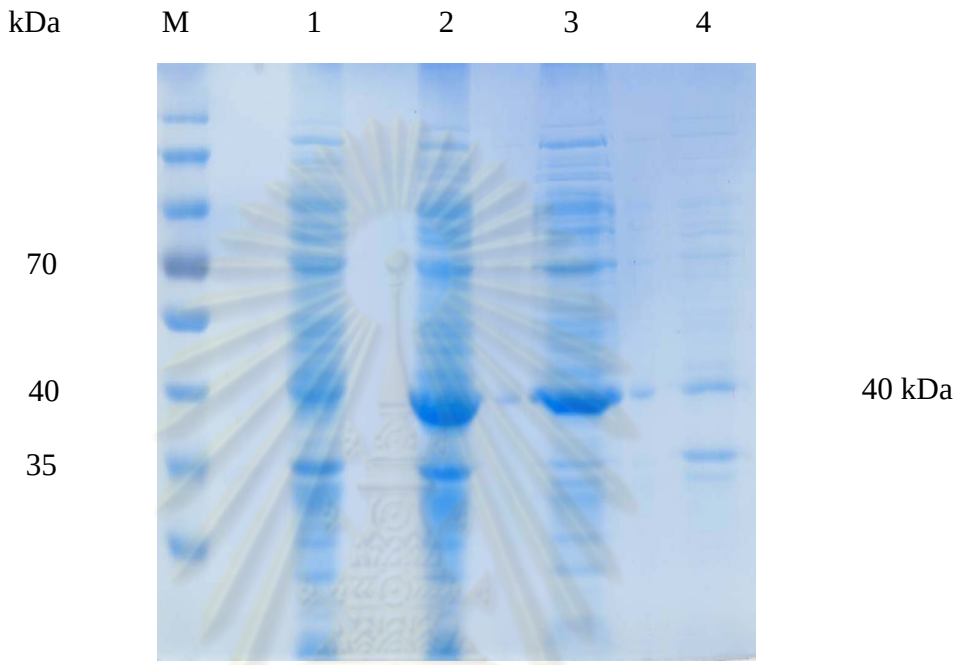

Figure 4-14 SDS-PAGE of expressed protein in the presence of 1 mM IPTG as inducer, expression at 18°C

Lane M : Prestained Protein Ladder

Lane 1 : Crude extract from uninduced *E.coli* BL21(DE3)/pET28a-gehWA

Lane 2 : Crude extract from induced *E.coli* BL21(DE3) for 3 hr

Lane 3 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 3 hr

Lane 4 : Pellet from induced *E.coli* BL21(DE3)/pET28a-gehWA for 3 hr

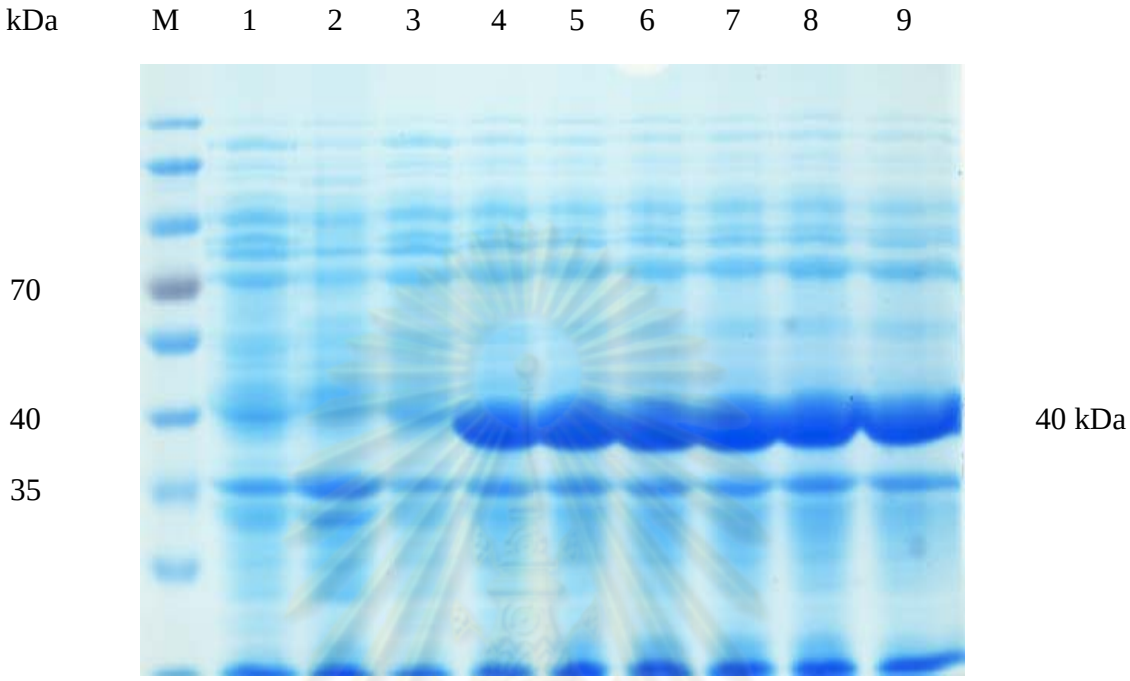

Figure 4-15 SDS-PAGE of expressed protein in the presence of variable concentration of IPTG (0 – 1.25 mM) as inducer for 3 hr post-induction

Lane M : Prestained Protein Ladder

Lane 1 : Crude extract from uninduced *E.coli* BL21(DE3)

Lane 2 : Crude extract from induced *E.coli* BL21(DE3) with 1.25 mM

Lane 3 : Crude extract from uninduced *E.coli* BL21(DE3)/pET28a-gehWA

Lane 4 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA with 0.1 mM of IPTG

Lane 5 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA with 0.25 mM of IPTG

Lane 6 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA with 0.5 mM of IPTG

Lane 7 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA with 0.75 mM of IPTG

Lane 8 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA with 1.0 mM of IPTG

Lane 9 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA with 1.25 mM of IPTG

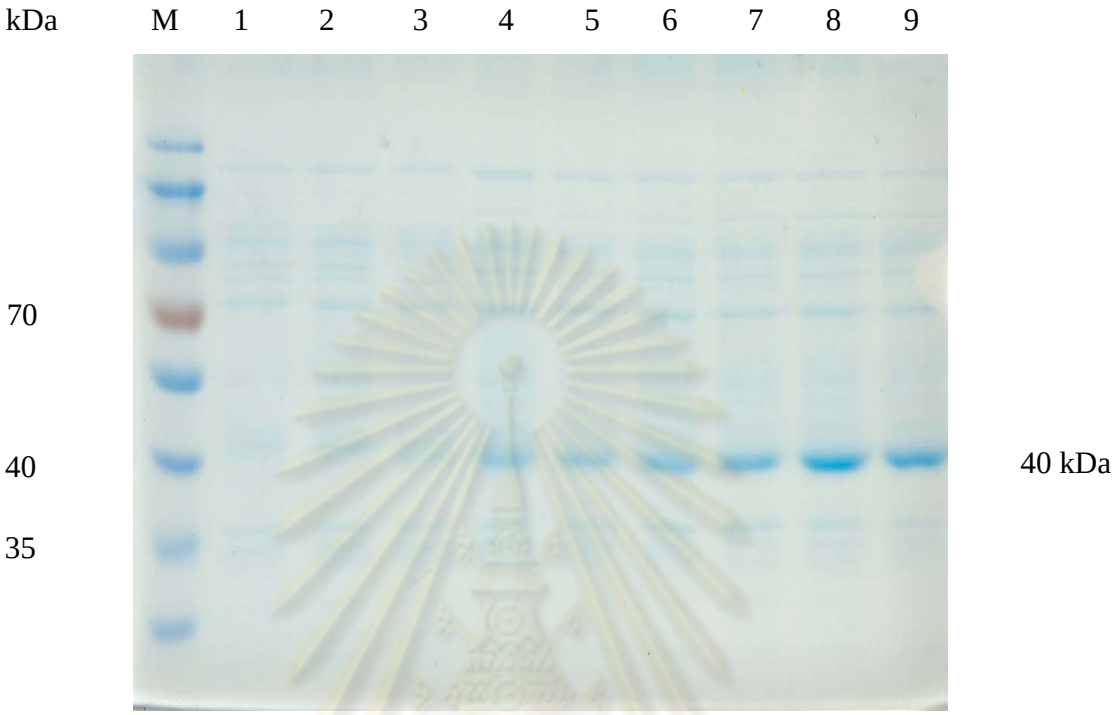

Figure 4-16 SDS-PAGE of crude extract fraction from induced *E.coli* BL21(DE3)/pET28a-gehWA with variable post-induction time (0-8 hr)

Lane M : Prestained Protein Ladder

Lane 1 : Crude extract from uninduced *E.coli* BL21(DE3)/pET28a-gehWA

Lane 2 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 1 hr

Lane 3 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 2 hr

Lane 4 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 3 hr

Lane 5 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 4 hr

Lane 6 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 5 hr

Lane 7 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 6 hr

Lane 8 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 7 hr

Lane 9 : Crude extract from induced *E.coli* BL21(DE3)/pET28a-gehWA for 8 hr

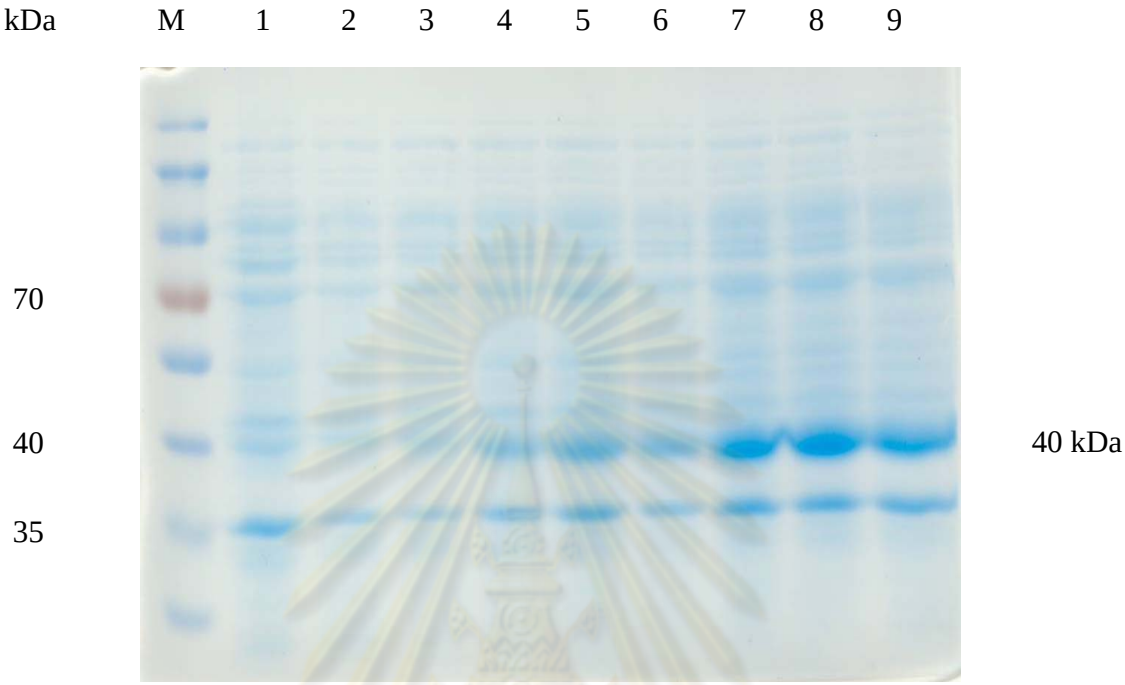

Figure 4-17 SDS-PAGE of supernatant fraction from induced *E.coli* BL21(DE3)/pET28a-gehWA with variable post-induction time (0-8 hr)

Lane M : Prestained Protein Ladder

Lane 1 : Supernatant from uninduced *E.coli* BL21(DE3)/pET28a-gehWA

Lane 2 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 1 hr

Lane 3 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 2 hr

Lane 4 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 3 hr

Lane 5 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 4 hr

Lane 6 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 5 hr

Lane 7 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 6 hr

Lane 8 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 7 hr

Lane 9 : Supernatant from induced *E.coli* BL21(DE3)/pET28a-gehWA for 8 hr

4.4 Purification of recombinant lipase

E.coli cells harboring pET28a-gehWA modified construct containing six c-terminal histidine residues were grown in 200 ml culture at 18°C. 0.1 mM IPTG was added when the absorbance at 600 nm reached 0.5-0.6. After 5 hr of induction, cells were collected, suspended in buffer A and sonicated. After centrifugation, lipase present in the supernatant that was filtered by using a 0.2 μm nylon membrane to eliminate small particles. Filtered supernatant was purified by HiTrap affinity column and then imidazole from the elution process was removed by gel filtration using a HiTrap desalting column. Tables 4-2 describe specific activity and the purification fold. The purity of the lipase after the purification increased approximately 38 folds over the crude extract. The purified lipase showed a single band on SDS–PAGE (Figure 4-18). Its molecular weight was estimated to be approximately 40 kDa by SDS–PAGE, which is consistent with the predicted molecular weight of lipase (42. 415 kDa).

Table 4-2 Activity and yield of the recombinant lipase in purification procedure

Fraction	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg protein)	Purification (fold)	Yield (%)
Crude extract	153	0.7	0.004	1	100
Flow through	96.5	0.02	0.0002	-	3
Wash A	46	0	0	-	0
Wash I	11.3	0.05	0.004	-	7
Elution	23	0.9	0.039	10	129
Desalting	21.8	3.3	0.151	38	471

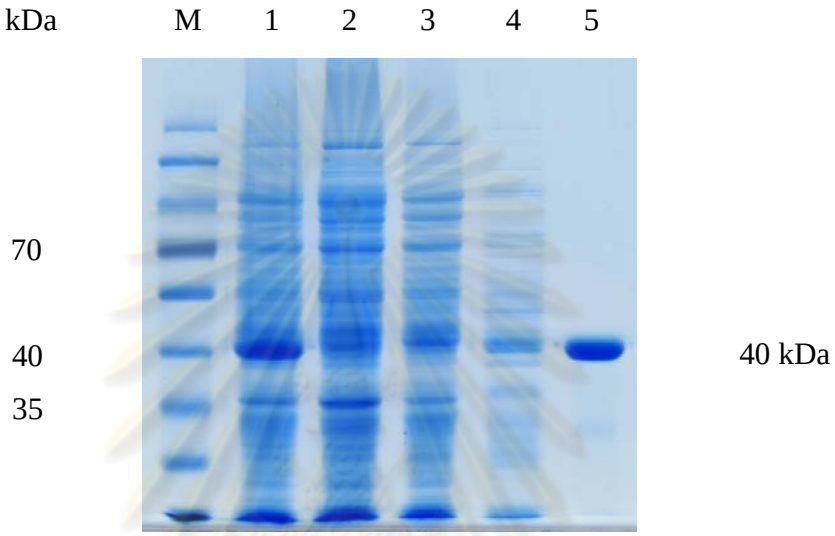

Figure 4-18 SDS-PAGE of the purified recombinant lipase after Ni HiTrap affinity column

Lane M : Prestained Protein Ladder

Lane 1 : Crude extract (20 mg protein) after centrifugation

Lane 2 : Flow through (20 mg protein)

Lane 3 : Wash A (20 mg protein)

Lane 4 : Wash I (20 mg protein)

Lane 5 : Purified lipase (5 mg protein) after Ni HiTrap affinity column

4.5 Characterization of recombinant lipase

4.5.1 Effect of pH and temperature on enzyme activity

The effects of pH and temperature on the lipase activity were determined spectrophotometrically using *p*-nitrophenyl palmitate as the substrate. The optimum pH for enzyme activity was determined in triplicate at room temperature from pH 5.0 to 11.0. The pH profile showed that the lipase was active at slightly alkaline pH range (pH 8–9.5) and the optimum pH was 8.5 (Figure 4-19). The optimum temperature for lipase activity was determined by measuring the rate of reaction at temperatures ranging from 20 to 60°C under the standard assay conditions. The effect of temperature on the activity of the recombinant lipase was shown in Figure 4-20. The optimal temperature of lipase was 30°C. The enzyme exhibited high activities at temperatures range of 25–35°C.

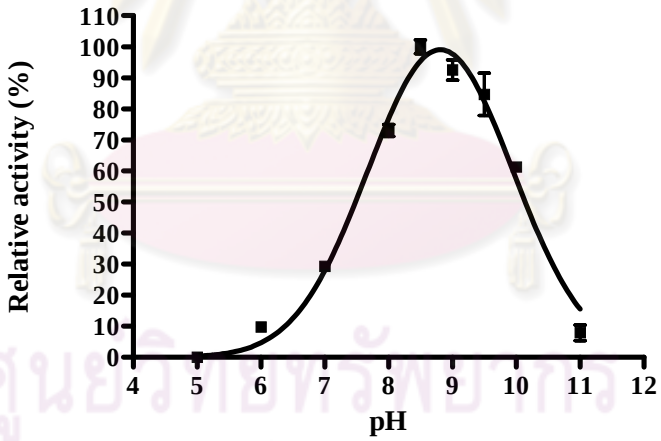

Figure 4-19 Effect of pH on enzyme activity of the purified recombinant lipase.

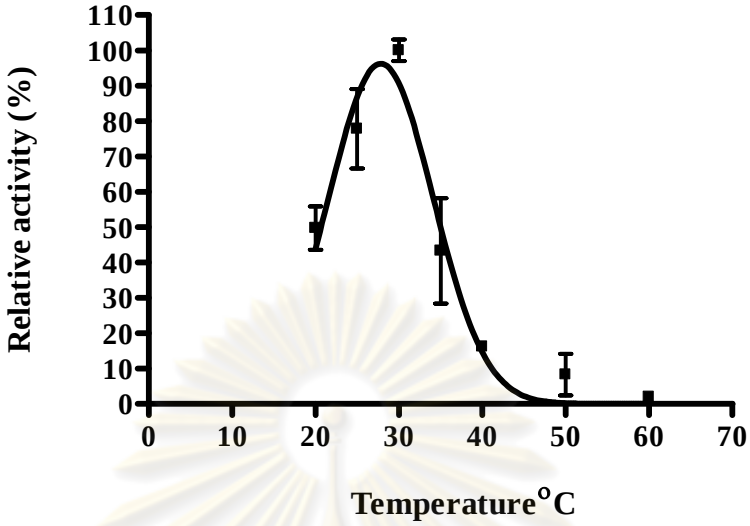

Figure 4-20 Effect of temperature on enzyme activity of the purified recombinant lipase.

4.5.2 Effect of pH and temperature on enzyme stability

The effect of pH on lipase stability was determined by incubating the purified lipase (0.5mg/ml) in the buffers having different pH values (pH 5.0-11.0) for 20 hr at 4°C, and residual activity was assayed spectrophotometrically at room temperature. The pH stability curve showed that the lipase activity remained over 80% in the buffers ranging from pH 8.0 to 10.0 after incubated at 4°C for 20 hr (Figure 4-21). It indicates that the recombinant enzyme is stable in a weak alkaline condition. To determine the influence of temperature on the enzyme stability, the purified lipase (1.0 mg/ml) was preincubated for 20 hr at temperature range of 20–60°C in 50mM Tris–HCl buffer, pH 8.5. Subsequently, the residual activity was analyzed at room temperature. The enzyme retained above 80% activity at temperatures below 30°C. However, the stability of the enzyme decreased sharply when the temperature was over 35°C (Figure 4-22). These data demonstrated that the recombinant lipase is reasonably stable below 30°C.

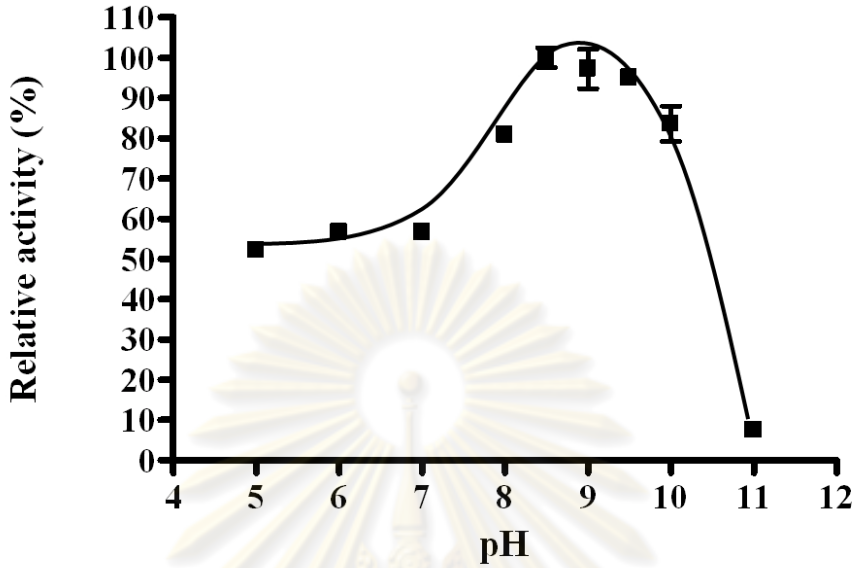

Figure 4-21 Effect of pH on enzyme stability of the purified recombinant lipase at 20 hr hours.

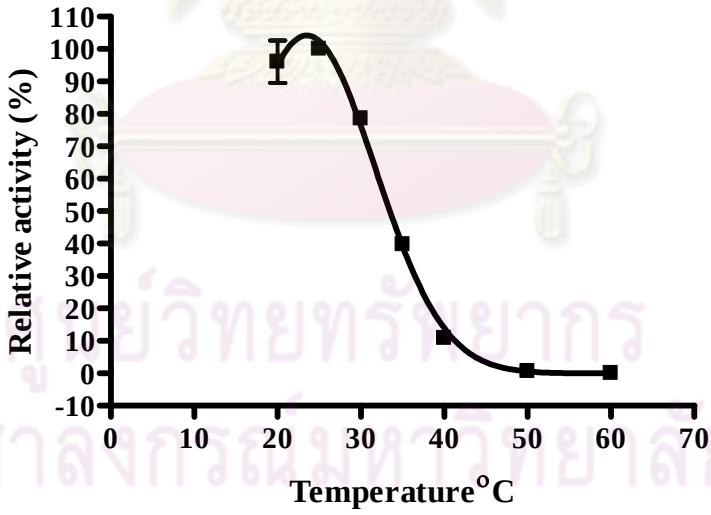

Figure 4-22 Effect of temperature on enzyme stability of the purified recombinant lipase at 20 hours.

4.5.3 Substrate specificity

Recombinant lipase substrate specificity was studied using *p*-nitrophenyl esters with varying chain length as substrates. To avoid artifacts due to physicochemical changes of the substrate that appear when chain lengths are varied, the selectivity studies were performed in an inert micellar system containing a low molar fraction of substrate. The results are shown in Figure 4-23. Obviously, recombinant lipase activity strongly depends on the chain length of the substrate. The lipase is mainly active on short chain *p*-nitrophenyl esters and enzymatic activity dramatically decreased with longer chain length. The low activity on long chain substrates raises the question of whether this enzyme should be called a lipase or an esterase. Because of the sequence similarity with Staphylococcal lipase and ill defined character of the name esterase, lipase was more preferential in this study.

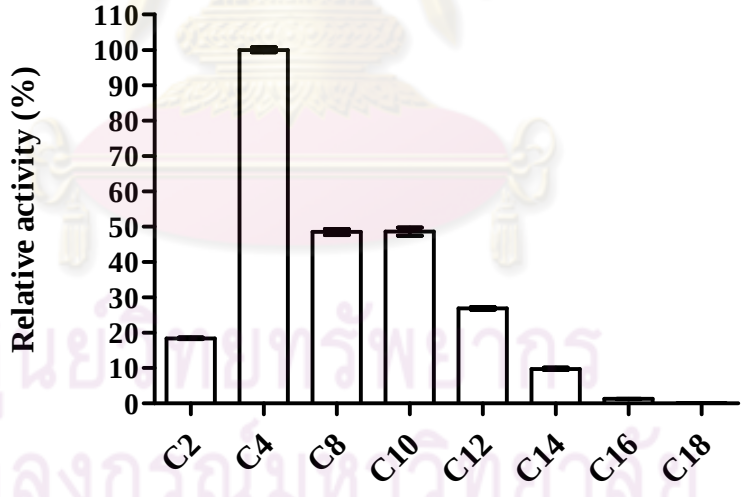

Figure 4-23 Substrate specificities of the purified recombinant lipase towards different *p*-nitrophenyl esters

4.5.4 Effect of organic solvents on lipase

The effect of various organic solvents on the stability of the purified recombinant lipase was studied by incubating enzyme solution in 50% various polar and non-polar organic solutions at room temperature with shaking for 1 and 6 hr. The residual activity was measured at room temperature and the organic solvent tolerance of enzymes was calculated by comparing the residual activity with the control. The preserved activities of lipases after incubation for 1 and 6 hr were shown in Figure 4-24. Recombinant lipase is diverse in its sensitivity to solvents but more stable in non-polar than polar organic solvents.

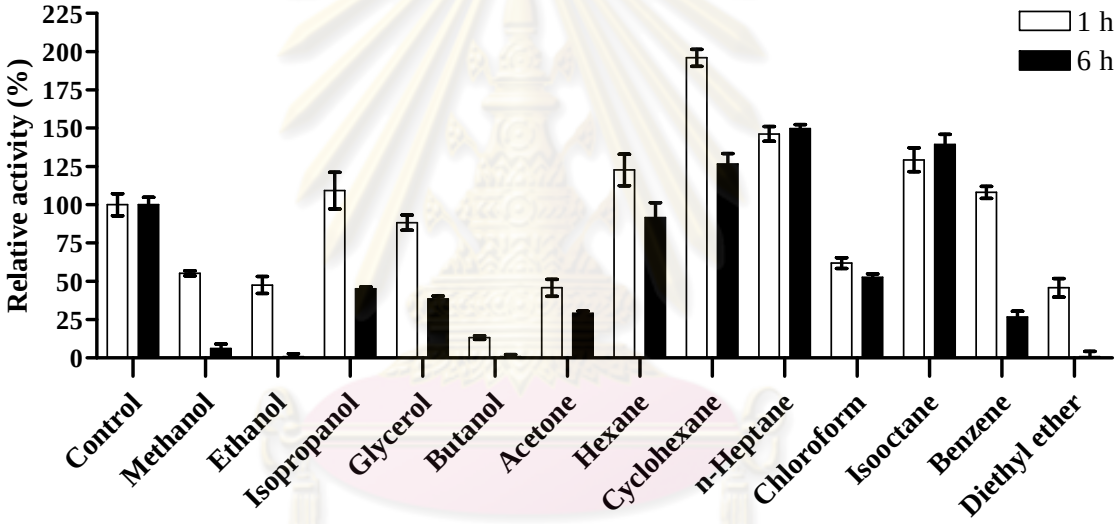

Figure 4-24 Effect of organic solvents on enzyme stability of the purified recombinant lipase.

4.5.5 Effect of metal ions and detergents on lipase

The effect of various metal ions and detergents on the stability of the purified recombinant lipase was studied by mixing enzyme solution with various kinds of metal ions and detergents and incubating at room temperature for 1 hr. The residual activity was measured at room temperature and the effect of various metal ions and detergents of enzymes was calculated by comparing the residual activity with the control. The remaining activity of purified recombinant lipase after incubated for 1 hr was shown in Figure 4-25 and Figure 4-26. The Figure 4-25 indicated that 1 and 10 mM Ca^{2+} could significantly activate the lipase; especially, 10 mM Ca^{2+} could increase 39% of its activity. The lipase activity was strongly inhibited in the presence of 1 and 10 mM Zn^{2+}, Hg^{2+}, Ag^{+}, $Co^{2+}$and 10 mM Fe^{3+}, Cu^{2+} and Fe^{2+}. 1 and 10 mM Na^{+}, Mg^{2+}, K^{+}, Mn^{2+}, Li^{2+} and 1mM Fe^{3+}, Cu^{2+} and Fe^{2+} had no significant effect on the enzyme activity. Figure 4-26 showed β- mercaptoethanol and DTT no effect on the activity whereas DMSO and Triton X-100 showed slightly decrease. The lipase activities were reduced in the presence of SDS, EDTA and Tween-80.

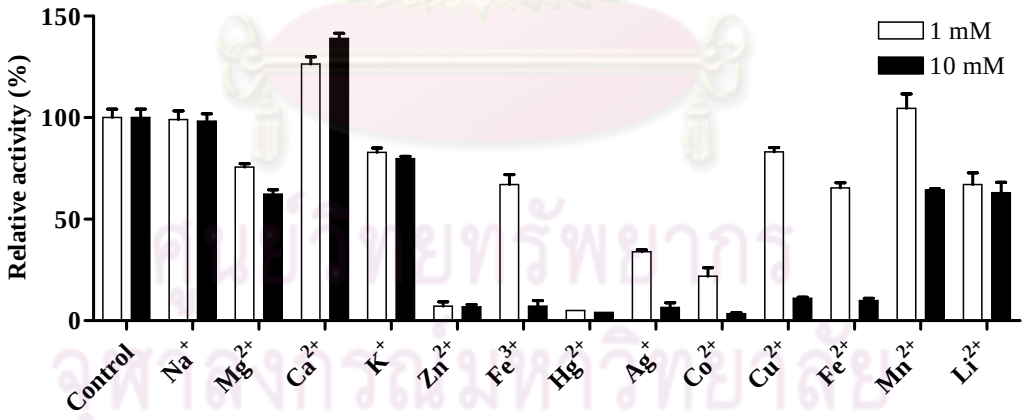

Figure 4-25 Effect of metal ions on enzyme stability of the purified recombinant lipase.

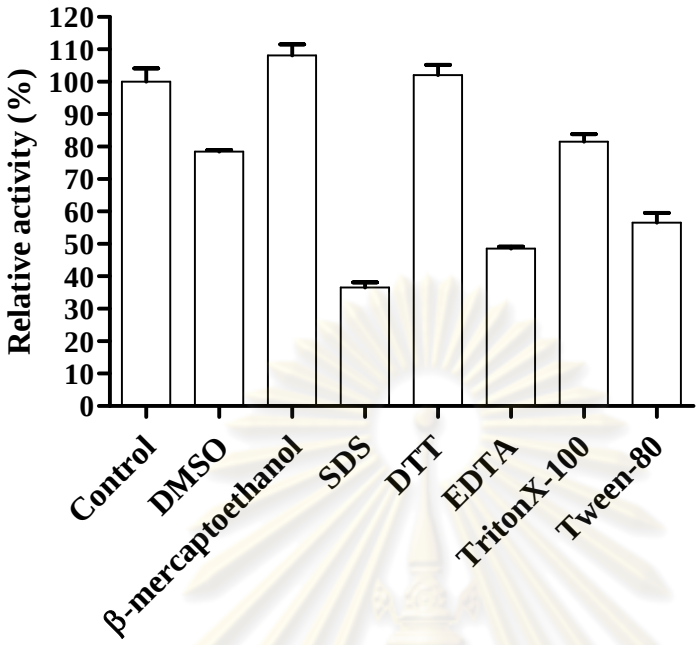

Figure 4-26 Effect of detergents on enzyme stability of the purified recombinant lipase.

4.5.6 Hydrolysis of oil

Aliquots of the purified recombinant lipase were incubated on agar plates containing rhodamine B and various kinds of oil; coconut, olive, palm and safflower oil, for preliminary screening of hydrolysis activity. The fluorescence halo was clearly observed on agar plate supplemented with coconut oil but no halo was detected on agar plates supplemented with other types of oils as shown in Figure 4-27. Supportively, the purified recombinant lipase can hydrolyze only emulsion of coconut oil as a substrate (Table 4-3). The specific activity of hydrolytic reaction toward coconut oil was 0.157 μmol/min/mg protein.

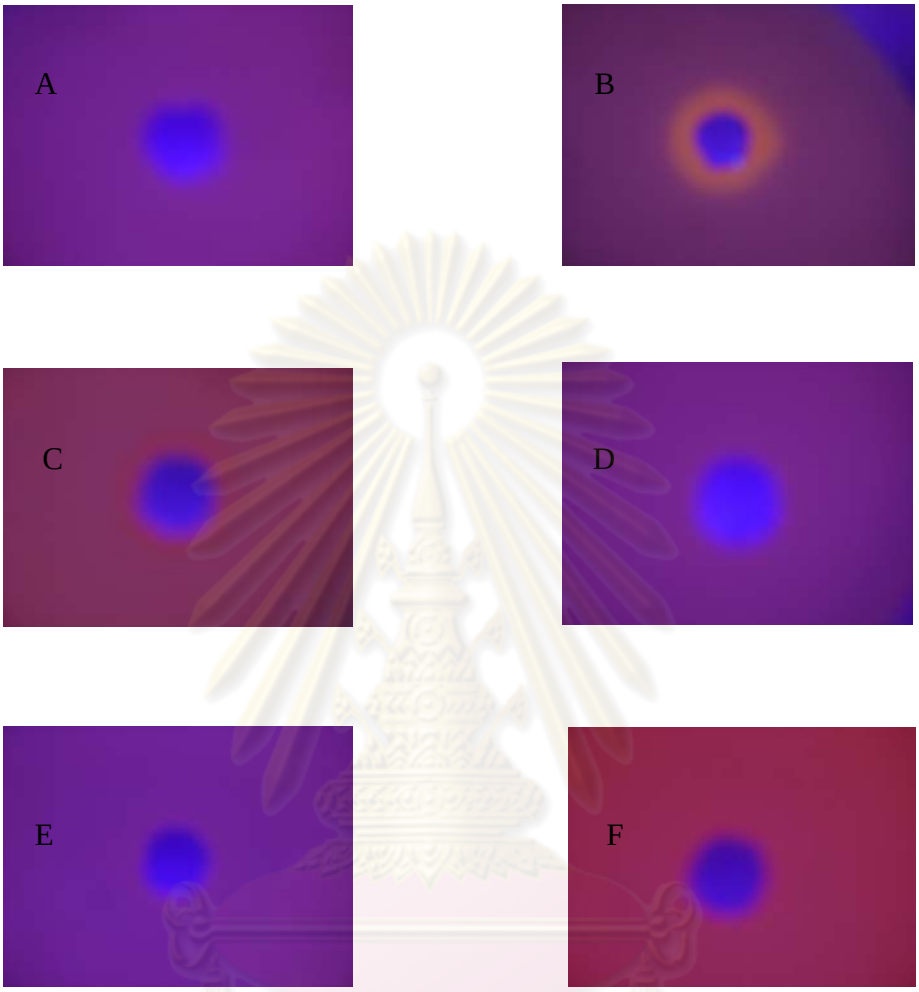

Figure 4-27 The purified recombinant lipase screened by agar plates supplemented with rhodamine B and various kinds of oil (observation under UV light).

A : Negative control (50 mM Tris–HCl buffer, pH 8.5)

B : Positive control (Crude lipase from *Candida rugosa*)

C : Fluorescent halo on agar plate supplemented with coconut oil

D : Fluorescent halo on agar plate supplemented with olive oil

E : Fluorescent halo on agar plate supplemented with palm oil

F : Fluorescent halo on agar plate supplemented with safflower oil

Table 4-3 Hydrolysis of oil

Oil	50 mM NaOH (ml)		Specific activity µmol/min/mg protein
	Control	Reaction	
Coconut	7.2 ± 0.1	10.8 ± 0.1	0.157
Olive	1.1 ± 0.1	1.2 ± 0.1	-
Palm	0.9 ± 0.1	1.1 ± 0.1	-
Safflower	1.0 ± 0.1	1.1 ± 0.1	-

Calculation of the lipase activity was described in Appendix F.

4.5.7 Transesterification

To evaluate the potential of *S.warnari* lipase for biodiesel production, the lipase was conducted under preliminary reaction condition, which may not be the optimum condition. Figure 4-28 showed no peak at retention time was 5 min indicated that no detectable of fatty acid methyl ester was found in transesterification reaction even when the coconut oil was used as the substrate.

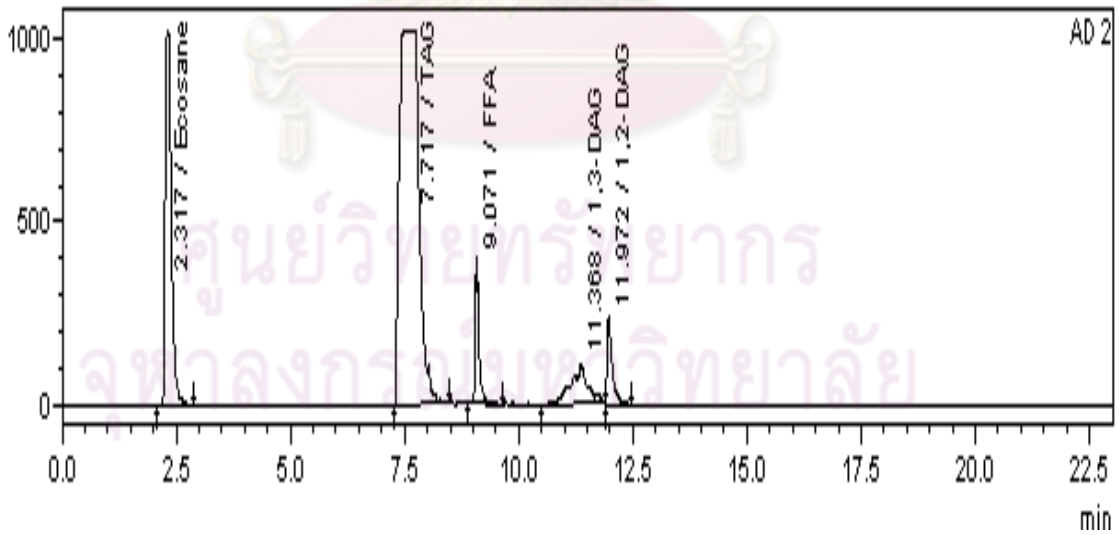

Figure 4-28 Chromatogram of transesterification reaction catalyzed by the purified recombinant lipase using coconut oil and methanol as substrate.

CHAPTER V

DISCUSSION

5.1 Identification of the lipase producing bacterium

Biochemical characteristics showed that the lipase producing bacterium was Gram-positive, facultatively anaerobic, furazolidone and lysostaphin-sensitive cocci. These characteristics indicated that the sample was member of the genus *Staphylococcus* (Lanser, 1993). The production of yellow pigment and acetylmethylcarbinol; fermentation of sucrose, maltose, mannitol, trehalose, and fructose; weak reduction or none of nitrate to nitrite; demonstration of arginase, urease, and β-glucosidase activities; and sensitivity to novobiocin indicated that the sample could be strain of *Staphylococcus warneri.* 16S rRNA sequencing has become the reference method for bacterial taxonomy and identification (Mignard and Flandrois, 2006). DNA sequencing of 16S rRNA genes has been used previously for identification of lipase producing bacteria (Abdel-Fattah and Gaballa, 2008). In this study, a 1,100 bp region of the highly conserved 16S rRNA gene was PCR-amplified and sequenced. The closest known relative of the submitted sequence, with 99% identity, was *S.warneri.* These results suggest that 16S gene primers are suitable for distinguishing between such closely related species.

5.2 Lipase gene cloning

The lipase of *S.warneri* is secreted in the supernatant as a pre-pro-lipase with an apparent molecular weight of 90 kDa. Similarly, staphylococcal lipases are primary synthesised as pre-pro-enzymes and have molecular masses of approximately 70-95 kDa (Kampen *et al*., 2001). While the function of the leader peptide in secretion is obvious, the role for the pro-peptide remained unclear. One hypothesis proposed a function in masking the enzyme activity until the secretion process is completed in order to protect the producing cell from detrimental effects of the lipase activity. A database comparison using this amino acid sequence revealed a significant similarity with various staphylococcal lipases. It was found that *S.warneri* lipase is translated as a 732-amino-

acid precursor protein with a signal peptide of 35 amino acids and a pro-peptide of 313 amino acids, which is processed to the mature lipase of 379 amino acids. This lipase was secreted into the supernatant as a pro-lipase and processed during purification to the mature form with an apparent molecular weight of 45 kDa. In order to perform this study, the fragment containing the lipase operon was amplified by PCR with the primer lipWY_F and lipWY_R. The size of PCR product was 1,100 bp. The purified lipWY gene was ligated into pET-17b vector and then recombinant plasmid pET17b-lipWY was successfully constructed. The recombinant plasmid was subjected for sequencing. Obtained sequence was checked with Chromas Lite and BioEdit program and compared to sequence data available from GenBank by using the BLAST program. It showed 99% identity with *S.warneri* lipWY gene. The open reading frame of lipWY was composed of 1,053 bp, which encoded 350 amino acids and molecular weight of approximately 40.234 kDa. Moreover, he primers were designed on basis of the *S.warneri* mature lipase gene (gehWA). The lipase gene of *S.warneri* was amplified by PCR, the size of PCR product was 1,100 bp. The purified gehWA gene was ligated into pET28a vector. The recombinant plasmid was subjected for sequencing. Obtained sequence was checked and compared to sequence data available from GenBank. It showed 99% identity with *S.warneri* gehWA gene. From obtained nucleotide sequence, it was shown that gehWA was composed of 1,140 bp, encoding 379 amino acids and molecular weight approximately 42.415 kDa. Lipases produced by other Staphylococcal species have similar molecular masses approximately 43-46 kDa.

5.3 Expression of lipase gene in *E.coil* host

The *E.coli* is commonly used in genetic engineering for preparation of recombinant proteins because of its superior characteristics, such as low-cost culture medium, simple culture conditions, fast cell proliferation and high target gene expression (Terpe, 2006). The recombinant plasmid was transformed into *E.coli* BL21(DE3). The expressions of pET17b-lipWY in *E.coli* BL21(DE3) were carried out in presence of 1 mM IPTG. It was not expression in *E.coli* BL21(DE3) that might be possible caused from the overexpression of recombinant protein might be toxic to the cell. In contrast, the

expressions of pET28a-gehWA in *E.coli* BL21(DE3) were carried out in presence of different IPTG concentration. It was found that the protein expressed all concentrations of IPTG at 37°C for 3 hr after IPTG induction. However, the enzyme was expressed as insoluble protein. The expression of eukaryotic proteins in *E.coli* often yields insoluble aggregates that are known as inclusion bodies. The insoluble proteins are in general misfolded that might be possible caused from the overexpression of recombinant protein might be toxic to the cell or influence the initial folding of enzyme resulting molecule fail to reach the native state resulting for biologically inactive. To overcome this problem, especially, in folding process, the expression conditions were improved by choosing an alternative *E.coli* strains, induction at low temperature or fusion to soluble proteins (Esposito *et al*., 2006). The lowering temperature expression was successful to obtain soluble protein. This probably reflects that the overexpression might influence the initial folding of enzyme resulting in the failure of the molecule to reach the native state at 37°C.

5.4 Purification of recombinant lipase

Purification of the recombinant proteins was facilitated by the presence of six histidine residues at the C-terminus of the enzymes. This allowed us to use only one step to purify the lipases using HiTrap affinity column. The results of SDS–PAGE analysis of the pooled fraction of the HiTrap affinity column are given in Figure 4-18. The enzyme exhibits one band corresponding to a molecular mass of about 40 kDa nearly mature lipase (SAL3) from *S.aureus* (45kDa) (Horchani *et al*., 2009). The purity of the lipase after the purification increased approximately 38 folds over the crude extract.

5.5 Characterization of recombinant lipase

The purified recombinant lipase showed the temperature and pH optimum of 30°C and pH 8.5, respectively. This result is similar to native extracellular *S.warneri*, *S.hyicus*, *S.haemolyticus* and *S.epiderminis* lipase (Ma *et al*., 2006). In the pH stability study, the purified recombinant lipase exhibited its stability at a broad range of pH values between pH 8.0 and pH 10.0. It indicates that the recombinant enzyme is stable in a weak

alkaline condition. This is in accordance to native *S.warneri* lipase and *S.aureus* lipase (SAL3) (Horchani *et al*., 2009). This enzyme has a great potential for application in the detergent industry (Chen *et al*., 1998). In the temperature stability study, the enzyme retained activity over 80% at temperatures below 30◦C. However, the stability of the enzyme decreased sharply when the temperature was over 35◦C (Figure 4-22). This data demonstrated that the recombinant lipase is reasonably stable below 30◦C. Unlike the native *S.warneri* lipase, the enzyme activity can be maintained over 80% when it was incubated at 50◦C. It is possible that the histidine-tag added to facilitate enzyme purification might affect thermal stability by changing the charge at the surface molecule or the packing of the enzyme. The substrate specificity of the recombinant lipase was studied with *p*-nitrophenyl esters of various chain lengths (C2-C18). The highest activity was with short chain length substrate; *p*NP-C4 with a relative activity of 100%. The recombinant lipase showed a significant decrease in the hydrolysis of *p*NP-C16 and *p*NP-C18. These results demonstrate that the recombinant lipase has the strong catalytic ability to the substrates with short or medium size chain lengths and has low activity to the long chain ester substrates. This is in accordance to mature lipase (gehSE1) from *S. epidermidis* (Simons *et al*., 1998). It is believed that there is a relation between the ability of lipases to hydrolyse different carbon chain lengths of *p*-nitrophenyl esters and the presence of the so-called lid on the enzyme (Santarossa *et al*., 2005). The recombinant lipase has potentials to produce esters and fatty acids with short chain lengths, since those products are important for application in aromatics and fine chemistries, the recombinant lipase could have bright commercial future. Recombinant lipase is diverse in its sensitivity to solvents but more stable in non-polar than polar organic solvents. Stability of this lipase in organic solvents suggests it could be used as a biocatalyst in non-aqueous medium (Du *et al*., 2004). Metal cations, particularly Ca^{2+}, play important roles in influencing the structure and function of enzyme, and calcium-stimulated lipases have been reported (Gupta *et al*., 2004). Therefore, the effect of some metal ions on the recombinant lipase activity was assessed. The purified recombinant lipase was mixed with various kinds of metal ions and incubated at room temperature for 1 hr. The Figure

4-25 indicated that 1 and 10 mM Ca^{2+} could significantly activate the lipase; especially, 10 mM Ca^{2+} could increase 39% of its activity. This effect has been suggested to be due to the formation of long chain fatty acid calcium salts (Kim *et al*., 2005). The lipase activity was strongly inhibited in the presence of 1 and 10 mM Zn^{2+}, Hg^{2+}, Ag^{+}, $Co^{2+}$and 10 mM Fe^{3+}, Cu^{2+} and Fe^{2+}. 1 and 10 mM Na^{+}, Mg^{2+}, K^{+}, Mn^{2+}, Li^{2+} and 1mM Fe^{3+}, Cu^{2+} and Fe^{2+} had no significant effect on the enzyme activity. Like many lipases, the enzyme was severely inhibited by Hg^{2+} (Sugihara *et al*., 1991 and Sugihara *et al*., 1992). The effect of various detergents on the stability of the purified recombinant lipase was also studied by mixing enzyme solution with various kinds of detergents and incubating at room temperature for 1 hr. Figure 4-26 showed β- mercaptoethanol and DTT no effect on the activity whereas DMSO and Triton X-100 showed slightly decrease. The lipase activities were reduced in the presence of SDS, EDTA and Tween-80. In contrast with native *S.warneri* lipase, a low residual activity of 3% was found in the presence of 1 mM EDTA. The recombinant *S.warneri* lipase has a higher affinity for Ca^{2+} resulting in the high residual activity was found relatively of 50% in the presence of 1 mM EDTA. In addition, the lipase activities reduce in the presence of strong detergent; SDS which approximately 35% was retained, as similar as *Streptococcus* lipase (Tripathi *et al*, 2004). Aliquots of the purified recombinant lipase were incubated on agar plates containing rhodamine B and various kinds of oil; coconut, olive, palm and safflower oil, for preliminary screening of hydrolysis activity. The fluorescent halo was clearly observed on agar plate supplemented with coconut oil but no halo was detected on agar plates supplemented with other types of oils as shown in Figure 4-27. Supportively, the purified recombinant lipase can hydrolyze only emulsion of coconut oil as a substrate (Table 4-3). The specific activity of hydrolytic reaction toward coconut oil was 0.157 μmol/min/mg protein. According to the fatty acid composition of coconut oil; 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) as shown in table G-1, the result strongly confirm that the recombinant lipase prefer short chain hydrocarbon. To evaluate the potential of *S.warnari* lipase for biodiesel production, the transesterification by lipase was conducted under preliminary reaction condition, which may not be the optimum condition. However, no detectable of fatty acid methyl

ester was found in transesterification reaction even was used coconut as substrate that might be possible that the recombinant *S.warneri* lipase (gehWA) was not tolerated to methanol as shown in Figure 4-24.

Although *S.warneri* lipase gene was cloned and expression of lipase gene in heterologous strain prouded an alternative method for large scale production, the characteristics of recombinant *S.warneri* lipase (gehWA), at low optimum temperature, less stable in polar organic solvent and short chain preference substrate specificity, seemed to be non-suitable for biodiesel production. Nevertheless, it still can be applied as a biocatalyst in other prospective fields as food and pharmaceutical industries including bioremediation in some of pesticides.

CONCLUSION

Both biochemical and genetic tests confirmed that lipase producing bacterium was *S.warneri*. The lipWY gene of *S.warneri* was cloned into the plasmid pET-17b producing recombinant plasmid, pET17b-lipWY. The open reading frame of lipWY was composed of 1,053 bp, which encoded 350 amino acids, but the pET17b-lipWY wasn't expression in *E. coli* BL21(DE3). A mature lipase gene (gehWA) from *S.warneri* was cloned into the plasmid pET-28a producing recombinant plasmid, pET28a-gehWA. The mature gehWA gene was composed of 1,140 bp, encoding 379 amino acids. The recombinant plasmid was transformed to *E.coli* for overexpression. The optimum condition for protein expression was obtained by inducing with 0.1 M IPTG for 5 hr at 18°C. The recombinant lipase was purified with one step Ni-NTA HiTrap affinity column to 38 folds purity. The purified recombinant lipase revealed homogeneity on SDS-PAGE with the molecular mass approximately 40 kDa. The optimum pH for the purified recombinant lipase was 8.5 and its stability was maintained at a range of pH values between pH 8.0 to 10.0. The purified recombinant lipase exhibited an optimum temperature of 30°C and stable below 30°C. The substrate specificity study showed that *p*-nitrophenyl butyrate is a preference of the enzyme. Recombinant lipase is diverse in its sensitivity to solvents but more stable in non-polar than polar organic solvents. The metal ions Ca^{2+} could activate lipase, whereas Zn^{2+}, Hg^{2+}, Ag^{+} and Co^{2+} inhibited the activity. β- mercaptoethanol and DTT displayed no effect on its activity. Moreover, inhibitory effect was also observed from SDS, EDTA and Tween-80. The purified recombinant lipase was able to hydrolyze only emulsion of coconut oil as a substrate. The specific activity of hydrolytic reaction toward coconut oil was 0.157 μmol/min/mg protein. However, no detectable fatty acid methyl ester was found in transesterification reaction even when the coconut oil was used as substrate. The characteristics of recombinant *S.warneri* lipase (gehWA) such as low optimum temperature, less stable in polar organic solvent and short chain preference substrate specificity, seem to be proper for application as the biocatalyst in food, pharmaceutical industries and for bioremediation of some pesticides.

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APPENDICES

APPENDIX A

Preparation for media

Luria-Bertini (LB) broth 1 L

Bactotryptone	10	g
Yeast extract	5	g
NaCl	10	g

Dissolve all components with 1,000 ml distilled water. Autoclave at 121° C, 15 1b/in^2 for 15 min.

LB agar 1 L

Bactotryptone	10	g
Yeast extract	5	g
NaCl	10	g
agar	15	g

Dissolve all components with 1,000 ml distilled water. Autoclave at 121° C, 15 1b/in^2 for 15 min.

APPENDIX B

Reagent for heat shock transformation

Super Optimal Broth (SOB) 1 L

Bacto tryptone	20	g
Yeast extract	5	g
NaCl	0.5	g
250 mM KCl	10	ml

Adjust the pH to 7.0 with 5 N NaOH and make to 1 liter with distilled water then autoclave. After autoclaving, cool down, add sterile 100 mM $MgCl_2$ 100 ml and store at 4° C.

Transformation buffer (TB) 500 ml

10 ml Pipes	1.512	g
15 mM $CaCl_2$	1.103	g
250 mM KCl	7.320	g

Adjust the pH to 6.7 with 5 N KOH and add 56 mM $MnCl_2$ (~5.443 g). Filter-sterile with 0.45 μm filter membrane and store at 4° C.

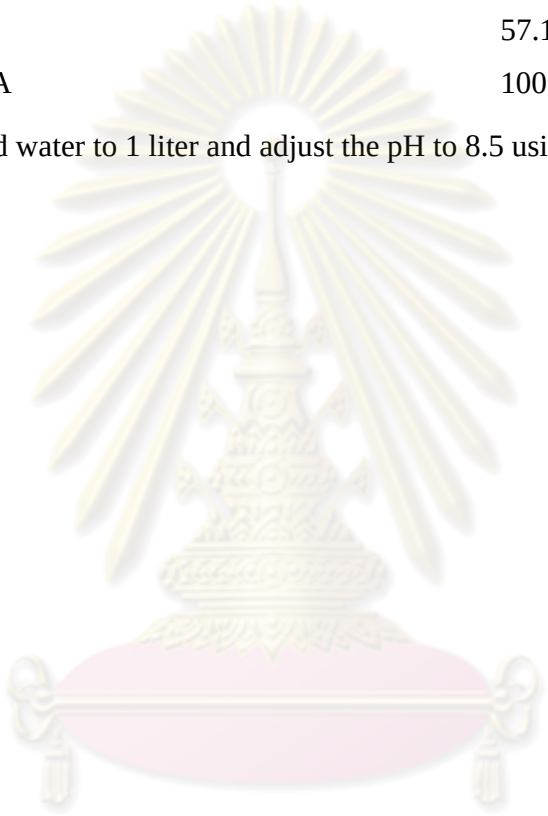
APPENDIX C

Reagent for DNA agarose gel electrophoresis

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

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

Add distilled water to 1 liter and adjust the pH to 8.5 using KOH.



APPENDIX D

Preparation for polyacrylamide gel electrophoresis

Stock reagents

30 % Acrylamide, 0.8 % bis- acrylamide 100 ml

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

1.5 M Tris-HCl, pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

1 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

10 % $(NH_4)_2S_2O_8$

$(NH_4)_2S_2O_8$	0.1	mg
Distilled water	1	ml

10 % SDS

SDS	0.1	mg
Distilled water	1	ml

5X Sample buffer for SDS-PAGE

1 M Tris-HCl, pH 6.8	0.6	ml
Glycerol	2.5	ml
10 % SDS	2.0	ml

2-mercaptoethanol	0.5	ml
1 % bromophenol blue	1.0	ml
Distilled water	3.4	ml

One part of 5X sample buffer is added to four parts of sample. The mixture is heated at 95°C for 5 min and centrifuged at 10,000 rpm for 5 min before loading to the gel.

SDS-PAGE

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 % $(NH_4)_2S_2O_8$	0.1	ml
TEMED	0.004	ml

4.0 % stacking gel

Distilled water	2.1	ml
1. M Tris-HCl, pH 8.8	0.38	ml
30 % acrylamide solution	0.5	ml
10 % SDS	0.3	ml
10 % $(NH_4)_2S_2O_8$	0.3	ml
TEMED	0.003	ml

10X Electrophoresis buffer for SDS-PAGE 1 L

Tris (hydroxymethyl)-aminomethane	30.3	g
Glycine	144	g
SDS	10	g

Adjust volume to 1 liter with distilled water.

Staining solution

Coomassie brilliant blue R-250	0.1	g
Methanol	40	ml
Glacial acetic acid	10	ml

Add distilled water to 100 ml and mix.

Destaining solution

Methanol	40	ml
Glacial acetic acid	10	ml

Add distilled water to 100 ml and mix.

APPENDIX E

Protein determination

1. Preparation of solutions for protein assays

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

2. Standard curve of BSA

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

1. Prepare stock bovine serum albumin with concentration 20 mg/ml.
2. 20 mg/ml BSA was diluted with distilled water as 0.1-0.6 mg/ml (Table E-1)

Table E-1 Composition for standard BSA

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

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

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

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

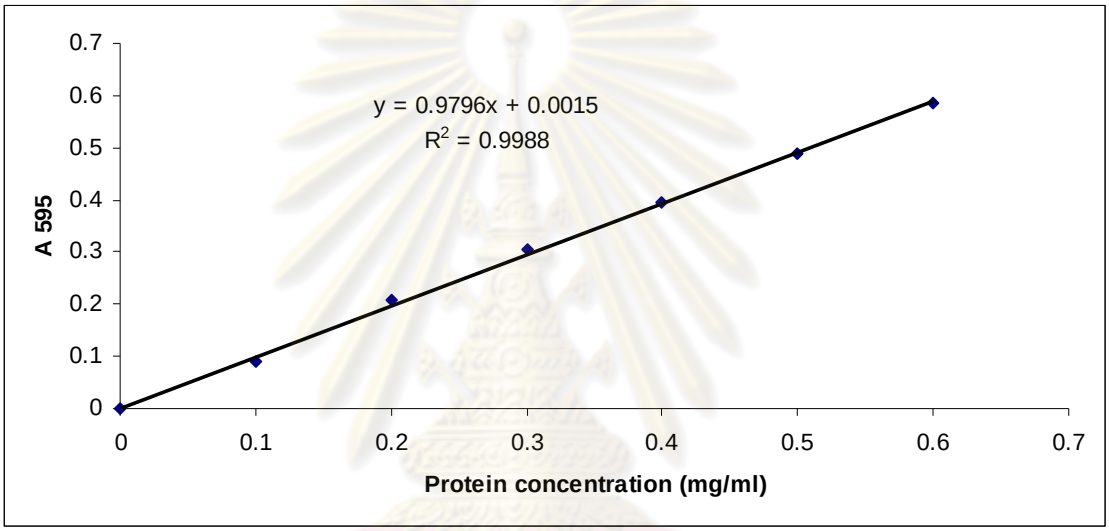


Figure E-1 Calibration curve for protein determination by Bradford's method

3. Calculation of total protein

The absorbance value at 595 nm was calculated by:

$$Y = aX + b$$

Where

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

Value Y axis = Absorbance at 595 nm

APPENDIX F

Calculation of the lipase activity

1. *p*-nitrophenyl palmitate as substrate

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

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

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

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

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. Various kinds of oil as substrate

Table F-1 Fatty acid composition of oils

Common name	Abbreviation	% Fatty acid			
		Coconut	olive	palm	safflower
Lauric acid	12 : 0	34.37	0.03	0.59	1.09
Myristic acid	14 : 0	13.75	0.02	0.96	2.99
Palmitic acid	16 : 0	9.29	11.37	38.67	8.19
Palmitoleic acid	16 : 1	4.83	0.63	0.11	1.72
Stearic acid	18 : 0	10.53	2.58	3.32	15.56
Oleic acid	18 : 1	12.34	80.46	45.45	22.88
Linoleic acid	18 : 2	6.46	4.17	10.87	33.10
Linolenic acid	18 : 3	0.72	0.56	0.20	3.70
Arachidic acid	20 : 0	7.72	0.21	0.23	5.10
Benenic acid	22 : 0	-	0.01	0.02	5.67

Table F-2 The volume of 50 mM NaOH was used as titrant in hydrolysis of oil

Oil	**50 mM NaOH (ml)**	
	Control	**Reaction**
Coconut	7.2 ± 0.1	10.8 ± 0.1
Olive	1.1 ± 0.1	1.2 ± 0.1
Palm	0.9 ± 0.1	1.1 ± 0.1
Safflower	1.0 ± 0.1	1.1 ± 0.1

Different volume of 50 mM NaOH was used in neutralization reaction and control of hydrolysis of oil.

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

Mole of NaOH = Mole of Oil/3

Change to μmole

Specific activity = μMole of oil/min/mg protein

APPENDIX G

Calculation of transesterification reaction

1. Molecular weight of coconut oil

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

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

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

Where,

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

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

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

MW_n = Molecular weight of three fatty acid – COOH

= $MW_{FA} - 45$

Table G-1 Fatty acid composition of coconut oil

Common name	Abbreviation	% Fatty acid
Lauric acid ($C_{12}H_{24}O_2$)	12 : 0	34.37
Myristic acid ($C_{14}H_{28}O_2$)	14 : 0	13.75
Palmitic acid ($C_{16}H_{32}O_2$)	16 : 0	9.29
Palmitoleic acid ($C_{16}H_{30}O_2$)	16 : 1	4.83
Stearic acid ($C_{18}H_{36}O_2$)	18 : 0	10.53
Oleic acid ($C_{18}H_{34}O_2$)	18 : 1	12.34
Linoleic acid ($C_{18}H_{32}O_2$)	18 : 2	6.46
Linolenic acid ($C_{18}H_{30}O_2$)	18 : 3	0.72
Arachidic acid ($C_{20}H_{40}O_2$)	20 : 0	7.72

The example of triglyceride in used coconut oil calculation

$$R_{Ave} = \left(\frac{34.37}{100}\times 155\right)+\left(\frac{13.75}{100}\times 183\right)+\left(\frac{9.29}{100}\times 211\right)+\left(\frac{4.83}{100}\times 209\right)+\left(\frac{10.53}{100}\times 239\right)$$
$$+\left(\frac{12.34}{100}\times 237\right)+\left(\frac{6.46}{100}\times 235\right)+\left(\frac{0.72}{100}\times 233\right)+\left(\frac{7.72}{100}\times 267\right)$$

$$= 53.276 + 25.163 + 19.602 + 10.095 + 25.167 + 29.246 + 15.181 + 1.678 + 20.612$$

$$= 200.016$$

$$MW_{TG} = (3 \times 200.016) + 173$$

$$= 673.05$$

Therefore molecular weight of coconut oil is equal to 673.05

2. Volume of methanol

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

Molecular weight of coconut oil = 673.05

So, coconut oil 0.5 g = 0.5/673.05

= 7.43×10^{-4} mole

Thus, the volume of methanol = $3\times7.43\times10^{-4}$

= 2.23×10^{-3} mole

Since molecular weight of methanol = 32

So, methanol 2.21×10^{-3} mole = $2.23\times10^{-3}\times32 = 0.071$ g

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

D = Density of methanol (0.79)

M = Mass of methanol (0.071 g)

V = Volume of methanol (ml)

$$V = \frac{0.071}{0.79}$$

So, the volume of methanol is equal to 0.09 ml.

3. %conversion yield from HPLC analysis

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

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

FAME = Concentration of methyl ester

FFA = Concentration of free fatty acid

TAG = Concentration of triglyceride

DAG = Concentration of diglyceride

MAG = Concentration of monoglyceride

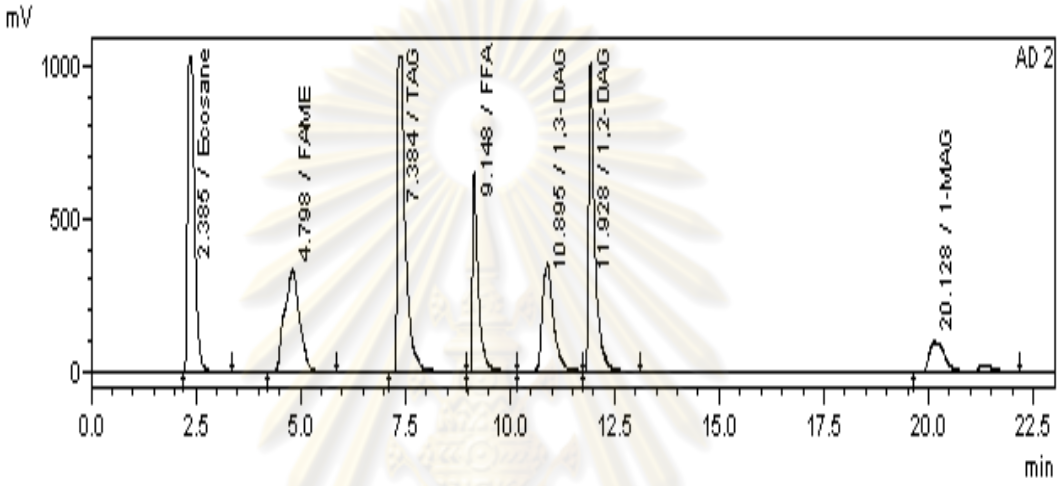


Figure G-1 Chromatogram of methyl ester from transesterification catalyzed by microbial lipase and analyzed by high performance liquid chromatography (Chutima Kaewpiboon, 2007).

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)

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