การแสดงออกเกินปกติของไลเพส จาก *Aureobasidium pullulans* var. *melanogenum* SRY14-3 และ *Fusarium solani* ใน *Pichia pastoris* และการประยุกต์สำหรับการบำบัดน้ำเสียขั้นต้น

นางสาวชินพร วงศ์วัฒนไพบูลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

OVEREXPRESSION OF LIPASE FROM *Aureobasidium pullulans* var. *melanogenum* SRY14-3 AND *Fusarium solani* IN *Pichia pastoris* AND APPLICATION FOR WASTEWATER PRETREATMENT

Miss Jinaporn Wongwatanapaiboon

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2014

Thesis Title	OVEREXPRESSION OF LIPASE FROM *Aureobasidium pullulans* var. *melanogenum* SRY14-3 AND *Fusarium solani* IN *Pichia pastoris* AND APPLICATION FOR WASTEWATER PRETREATMENT
By	Miss Jinaporn Wongwatanapaiboon
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Warawut Chulalaksananukul, Ph.D.
Thesis Co-Advisor	Professor Alain Marty, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Tosak Seelanan, Ph.D.)

.....Thesis Advisor
(Associate Professor Warawut Chulalaksananukul, Ph.D.)

.....Thesis Co-Advisor
(Professor Alain Marty, Ph.D.)

.....Examiner
(Assistant Professor Chompunuch Glinwong, Ph.D.)

.....Examiner
(Assistant Professor Kittinan Komolpis, Ph.D.)

.....External Examiner
(Chalermchai Ruangchainikom, Ph.D.)

.....External Examiner
(Rungtiwa Piamtongkam, Ph.D.)

ชินพร วงศ์วัฒนไพบูลย์ : การแสดงออกเกินปกติของไลเพสจาก *Aureobasidium pullulans* var. *melanogenum* SRY14-3 และ *Fusarium solani* ใน *Pichia pastoris* และการประยุกต์สำหรับการบำบัดน้ำเสียขั้นต้น (OVEREXPRESSION OF LIPASE FROM *Aureobasidium pullulans* var. *melanogenum* SRY14-3 AND *Fusarium solani* IN *Pichia pastoris* AND APPLICATION FOR WASTEWATER PRETREATMENT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วรวุฒิ จุฬาลักษณานุกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร.อแลง มาร์ตี้, 91 หน้า.

วัตถุประสงค์ของงานวิจัยนี้คือ เพื่อที่จะโคลนยีนไลเพสจาก *Aureobasidium melanogenum* และ *Fusarium solani* เพื่อเพิ่มการผลิตไลเพสผ่านระบบการแสดงออกของ *Pichia pastoris*, ตรวจสอบคุณสมบัติของไลเพส และประยุกต์ใช้รีคอมบิแนนท์ *P. pastoris* ที่มีการแสดงออกของไลเพสในการบำบัดน้ำเสียขั้นต้นที่มีไขมันเป็นองค์ประกอบ ยีนไลเพสของ *A. melanogenum* (*AML*) และ *F. solani* (*FSL*) สามารถแสดงออกได้อย่างสำเร็จใน *P. pastoris* โดยใช้ระบบการแสดงออกแบบเหนี่ยวนำ และระบบการแสดงออกแบบตลอดเวลา ภายใต้การควบคุมของโพรโมเตอร์แอลกอฮอล์ออกซิเดส1 (p*AOX1*) และโพรโมเตอร์กลีเซอรอลดีไฮด์-3-ฟอสเฟต ดีไฮโรจีเนส (p*GAP*) ตามลำดับ ทั้งสองระบบสามารถผลิตไลเพสได้มากกว่าสายพันธุ์ดั้งเดิม รีคอมบิแนนท์ *Pichia* ที่มีการแสดงออกของ *AML* ซึ่งได้มาจากโพรโมเตอร์ที่มีการแสดงออกแบบเหนี่ยวนำ (*P. pastoris*/pPICZαA-*AML*) พบว่า มีค่าแอกทิวิทีจำเพาะของไลเพสสูงที่สุดเท่ากับ 9.9 ยูนิตต่อมิลลิกรัมโปรตีน ที่เวลา 6 วัน เมื่อมีการเหนี่ยวนำด้วยเมทานอล 2 เปอร์เซ็นต์โดยปริมาตร ค่าแอกทิวิทีที่เหมาะสม คือ ที่อุณหภูมิ 35 – 37 องศาเซลเซียส และพีเอช 7.0 โดยใช้พารา-ไนโตรฟีนิลลอเรตเป็นสารตั้งต้น ค่าแอกทิวิทีของไลเพสสามารถเพิ่มได้โดยการเติม Mg^{2+}, Mn^{2+}, Li^{+}, Ca^{2+} และ Ni^{2+} (1 มิลลิโมลาร์), DTT และ EDTA (5 มิลลิโมลาร์) แต่จะถูกยับยั้งโดย Hg^{2+}, Ag^{+}, SDS, Tween 20 และ Triton X-100 รีคอมบิแนนท์ *Pichia* ที่มีการแสดงออกของ *FSL* ซึ่งได้มาจากโพรโมเตอร์ที่มีการแสดงออกแบบตลอดเวลา (*P. pastoris*/pGAPZαA-*FSL*) พบว่า มีค่าแอกทิวิทีจำเพาะของไลเพสสูงที่สุดเท่ากับ 18.8 ยูนิตต่อมิลลิกรัมโปรตีน ที่เวลา 3 วัน ค่าแอกทิวิทีที่เหมาะสม คือ ที่อุณหภูมิ 35 องศาเซลเซียส และพีเอช 7.0 โดยใช้พารา-ไนโตรฟีนิลลอเรตเป็นสารตั้งต้น ค่าแอกทิวิทีของไลเพสสามารถเพิ่มได้โดยการเติม Mn^{2+}, Ba^{2+}, Li^{+}, Ca^{2+}, Ni^{2+}, CHAPS (1 มิลลิโมลาร์) และ Triton X-100 (0.1 และ 1 เปอร์เซ็นต์โดยปริมาตร) แต่จะถูกยับยั้งโดย Hg^{2+}, Ag^{+} และ SDS รีคอมบิแนนท์ไลเพสจาก *P. pastoris*/pPICZαA-*AML* และ *P. pastoris*/pGAPZαA-*FSL* มีความเสถียรทั้งในตัวทำละลายอินทรีย์ที่ละลายน้ำได้และละลายไม่ได้ การเติม octanol และ *p*-xylene ที่ความเข้มข้น 10 เปอร์เซ็นต์โดยปริมาตร สามารถเพิ่มแอกทิวิทีของไลเพสได้ทั้งคู่ ในการประยุกต์สำหรับการบำบัดน้ำเสียขั้นต้น พบว่า เมื่อเลี้ยง *P. pastoris*/pGAPZαA-*FSL* ในน้ำเสียสังเคราะห์ที่มีน้ำมันปาล์ม 1 เปอร์เซ็นต์โดยน้ำหนักต่อปริมาตร จะส่งผลให้เกิดการย่อยสลายน้ำมันได้ 87 เปอร์เซ็นต์ ภายใน 72 ชั่วโมง ค่า K_M และ V_{max} ของ *P. pastoris*/pGAPZαA-*FSL* สำหรับการย่อยสลายน้ำมันปาล์ม เท่ากับ 1 เปอร์เซ็นต์โดยน้ำหนักต่อปริมาตร และ 38.4 ไมโครโมลาร์ของไตรกลีเซอไรด์ที่ถูกย่อยสลายต่อชั่วโมงต่อค่าการดูดกลืนแสงที่ 600 นาโนเมตร ตามลำดับ จากผลการทดลองที่ได้ แสดงให้เห็นว่า *P. pastoris* ที่มีการแสดงออกของ *FSL* ในระบบการแสดงออกแบบตลอดเวลา สามารถใช้เป็นจุลินทรีย์ทางเลือกในการบำบัดน้ำเสียขั้นต้นได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5273882823 : MAJOR BIOTECHNOLOGY
KEYWORDS: AUREOBASIDIUM PULLULANS / FUSARIUM SOLANI / HETEROLOGOUS EXPRESSION / LIPASE / LIPID-CONTAINING WASTEWATER PRETREATMENT / PICHIA PASTORIS

JINAPORN WONGWATANAPAIBOON: OVEREXPRESSION OF LIPASE FROM *Aureobasidium pullulans* var. *melanogenum* SRY14-3 AND *Fusarium solani* IN *Pichia pastoris* AND APPLICATION FOR WASTEWATER PRETREATMENT. ADVISOR: ASSOC. PROF. WARAWUT CHULALAKSANANUKUL, Ph.D., CO-ADVISOR: PROF. ALAIN MARTY, Ph.D., 91 pp.

The purpose of this study was to clone a lipase gene from *Aureobasidium melanogenum* and *Fusarium solani* for increasing lipase production via *Pichia pastoris* expression system, determine the properties of lipase and apply the recombinant *P. pastoris* expressing lipase for lipid-containing wastewater pretreatment. The genes of *A. melanogenum* lipase (*AML*) and *F. solani* lipase (*FSL*) were successfully expressed in *P. pastoris* using inducible expression system and constitutive expression system under the control of alcohol oxidase 1 promoter (p*AOX1*) and glyceraldehyde-3-phosphate dehydrogenase promoter (p*GAP*), respectively. Both of them produced more lipase than the wild-type. The recombinant *Pichia* expressing *AML* obtained using the inducible promoter (*P. pastoris*/pPICZαA-*AML*) showed specific lipase activity of 9.9 U/mg after 6 days of 2% v/v methanol induction. Optimal lipase activity was observed at 35–37 °C and pH 7.0 using *p*-nitrophenyl laurate as the substrate. Lipase activity was enhanced by Mg^{2+}, Mn^{2+}, Li^{+}, Ca^{2+} and Ni^{2+} (1 mM), DTT and EDTA (5 mM) but was inhibited by Hg^{2+}, Ag^{+}, SDS, Tween 20 and Triton X-100. The recombinant *Pichia* expressing *FSL* obtained using the constitutive promoter (*P. pastoris*/pGAPZαA-*FSL*) showed the highest specific activity of 18.8 U/mg after 3 days. Optimal lipase activity was observed at 35°C and pH 7.0 using *p*-nitrophenyl laurate as the substrate. Lipase activity was enhanced by Mn^{2+}, Ba^{2+}, Li^{+}, Ca^{2+}, Ni^{2+}, CHAPS (1 mM) and Triton X-100 (0.1% and 1% v/v) but was inhibited by Hg^{2+}, Ag^{+} and SDS. Recombinant lipases from *P. pastoris*/pPICZαA-*AML* and *P. pastoris*/pGAPZαA-*FSL* were stable in both water-miscible and water-immiscible organic solvents. The addition of 10% v/v of octanol and *p*-xylene increased activity of both lipases. For application in wastewater pretreatment, cultivation of *P. pastoris*/pGAPZαA-*FSL* in synthetic wastewater containing 1% w/v palm oil resulted in degradation of 87% of the oil within 72 h. K_M and V_{max} value of *P. pastoris*/pGAPZαA-*FSL* for palm oil degradation were 1% w/v and 38.4 µM TAG degraded/h/OD_{600}, respectively. From the results, *P. pastoris* expressing *FSL* by constitutive expression system could be used as an alternative microorganism for the wastewater pretreatment.

Field of Study: Biotechnology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Associate Professor Warawut Chulaluksananukul, Ph. D. and my co-advisor Professor Alain Marty, Ph. D. who made useful suggestiond and guidance throughout the process of this research and for spending time discussing on various points.

I would like to thank Assistant Professor Tosak Seelanan, Ph.D., Assistant Professor Chompunuch Glinwong, Ph.D., Assistant Professor Kittinan Komolpis, Ph.D., Chalermchai Ruangchainikom, Ph.D. and Rungtiwa Piamtongkam, Ph.D., the member of thesis committee, for their useful comments.

I would like to acknowledge the Institute for the Promotion of the Teaching Science and Technology, Thailand (IPST), the Development and Promotion of Science and Technology Talents Project (DPST) for their financial support during my thesis and extend my thankful to Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés (LISBP) de l'INSA de Toulouse, France for facilitate experiments in laboratory.

Special thanks are also extending to Dr. Sophie Duquesne, Dr. Florence Bordes and Dr. Pablo Alvarez for their help, kind assistance, suggestion and friendship throughout my research time at INSA-Toulouse, and all members of EAD1 for their kindness help, guidance and friendship during my stay in France.

Helpful advice from Dr. Sirawut Klinbunga and Miss Kanchana Sittikankaew, Aquatic Molecular Genetics and Biotechnology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) was greatly appreciated.

I would like to acknowledge the Department of Botany, Faculty of Science, Chulalongkorn University, for offering laboratory facilities in this research. And I am also thankful to all members of Biofuel by Biocatalyst Research Unit, Chulalongkorn University and friends for for their friendship, kind assistance and support.

Finally, I would like to express my wholeheartedly thanks and the deepest appreciation to my father, my mother, my brother and my husband for their unlimited love, support, strong encouragement, understanding, moral support and never leave me alone.

CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AML	*Aureobasidium melanogenum* lipase gene
BMGY	buffered complex glycerol medium
BMMY	buffered complex methanol medium
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CTAB	cetyltrimethylammonium bromide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
FFA	free fatty acid
FSL	*Fusarium solani* lipase gene
OD	optical density
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
p-NPB	*p*-nitrophenyl butyrate
p-NPC	*p*-nitrophenyl caprylate
p-NPD	*p*-nitrophenyl decanoate
p-NPL	*p*-nitrophenyl laurate
p-NPM	*p*-nitrophenyl myristate
p-NPP	*p*-nitrophenyl palmitate
RACE	rapid amplification of cDNA ends
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAG	triglyceride

CHAPTER I

INTRODUCTION

Most municipal and some industrial wastewaters contain lipids as the major component of the organic matter. These lipids contribute 30%–40% of the chemical oxygen demand in wastewater (Dueholm *et al.*, 2000). High concentration of these compounds causes major problem in biological wastewater treatment process. Pretreatment that hydrolyse these lipids may improve the biological degradation of lipid-containing wastewaters (Cammarota and Freire, 2006). Lipases can hydrolyse long-chain triglycerides (TAGs) to glycerol and free fatty acids (FFAs) at the lipid–water interface. There are researches about isolation and screening of lipase-producing microorganisms from various environments. Recently, Vitisant *et al.* (2013) isolated lipase-producing yeast, *Aureobasidium melanogenum* SRY14-3, from oil-contaminated foam board found in a shipyard area (Sichang Island, Chonburi Province, Thailand). The lipase from *A. melanogenum* has not previously been studied. The lipase-producing fungus *Fusarium solani* NAN103 was isolated from a soil sample in the deciduous dipterocarp forest in Nan province, Thailand (Malilas, 2010). This fungus can produce extracellular lipase; however, the yield of lipase obtained from this strain is quite low.

To improve the yield of lipase and construct the efficient microorganism for application in wastewater pretreatment, the methylotrophic yeast *Pichia pastoris* has become an interesting host for the heterologous expression of proteins. The

advantages of *P. pastoris* include a rapid growth rate in minimal medium, low levels of endogenous protein secretion, ease of genetic manipulation, the ability to efficiently secrete heterologous proteins and the ability to perform eukaryotic post-translational modifications, such as protein folding, glycosylation, disulfide bond formation and proteolytic processing (Ferrer *et al.*, 2009; Li *et al.*, 2007). For these reasons, *P. pastoris* is a very interesting host for lipase expression of *A. melanogenum* and *F. solani*.

Objectives

1. To clone lipase genes from *A. melanogenum* SRY14-3 and *F. solani* NAN103 for increasing lipase production via *P. pastoris* expression system and investigate the biochemical characteristics of the crude recombinant lipases

2. To apply the recombinant *P. pastoris* expressing lipase for lipid degradation in lipid-containing wastewater

Scope of study

The lipase genes of *A. melanogenum* SRY14-3 and *F. solani* NAN103 were cloned and expressed into *P. pastoris* systems (inducible promoter and constitutive promoter). The nucleotide sequence of *A. melanogenum* lipase has not been studied before; therefore, the full-length nucleotide sequence of gene-encoding lipase was identified. The recombinant lipases produced from *P. pastoris* were determined the biochemical characteristics. Finally, the recombitant *P. pastoris* producing the highest lipase activity would be selected for application in lipid-containing wastewater pretreatment. The synthetic wastewater was used in this experiment.

Expected results

The expression of *A. melanogenum* and *F. solani* lipase in *P. pastoris* could produces more lipase than wild-type. The recombinant *P. pastoris* could have the potential to be used as an alternative microorganism for the pretreatment of lipid-containing wastewater.

CHAPTER II

LITERATURE REVIEW

Lipase

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) hydrolyse tri-, di- and monoglycerides of long-chain fatty acids into glycerol and free fatty acids. However, there is no strict definition available for the term “long-chain”, but glycerol esters with an acyl chain length more than 10 carbon atoms can be regarded as lipase substrates (Andualema and Gessesse, 2012). Lipases are serine hydrolases that act at the lipid–water interface. The active site is formed by a catalytic triad consisting of Ser-Asp/Glu-His and a consensus sequence (Gly-x-Ser-x-Gly) is found around the active site of serine (Gupta *et al.*, 2004). The three-dimensional structures of lipases reveal the characteristic α/β-hydrolase fold (Nardini and Dijkstra, 1999).

Reactions catalyzed by lipases

The lipase-catalyzed reactions are shown in Figure II-1. Lipases can catalyse a wide range of reactions, including hydrolysis, esterification, interesterification, alcoholysis, acidolysis and aminolysis (Sharma and Kanwar, 2014; Singh and Mukhopadhyay, 2012). For the hydrolysis reaction, a triglyceride can be hydrolyzed to form glycerol and release free fatty acids. The reaction is reversible and the direction of the reaction depends on the water content in the reaction (Vakhlu, 2006). Esterification reactions between alcohols and free fatty acids are catalysed by lipases

in a low-water environment or even solvent free systems (Singh and Mukhopadhyay, 2012; Villeneuve *et al.*, 2000). Hydrolysis and esterification can occur simultaneously in a process known as interesterification. Depending on the substrates, lipases can catalyze acidolysis, alcoholysis, aminolysis and transesterification (Singh and Mukhopadhyay, 2012).

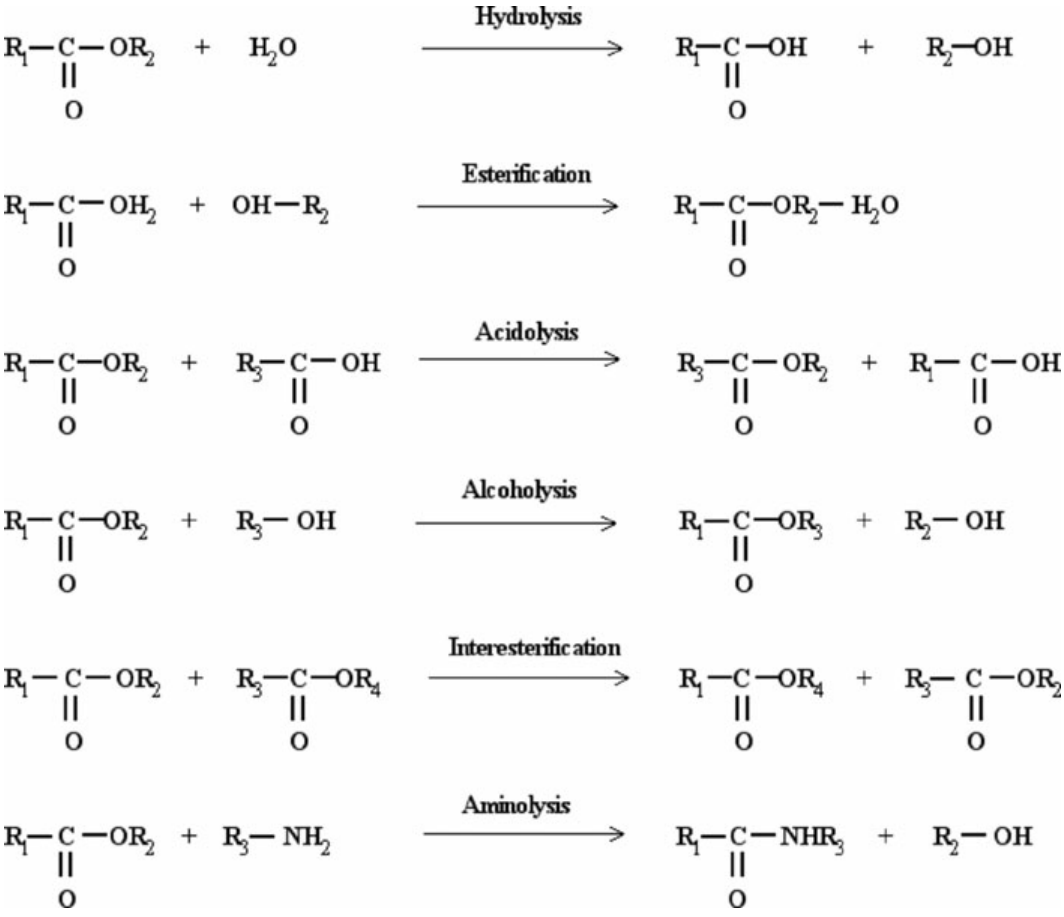

Figure II-1 The lipase-catalyzed reactions (Singh and Mukhopadhyay, 2012)

Lipase-producing microorganisms

Lipases are produced by animals, plants and microorganisms but only microbial lipases are found to be industrially important because they are diversified in their enzymatic properties and substrate specificity (Sharma and Kanwar, 2014). Some of the lipase-producing microorganisms are presented in Table II-1.

Table II-1 Some lipase-producing microorganisms (Sharma *et al.*, 2001)

Source	**Genus**	**Species**
Bacteria	*Bacillus*	*B. cereus*
		B. subtilis
		B. coagulans
	Staphylococcus	*S. aureus*
		S. warneri
	Pseudomonas	*P. aeruginosa*
		P. fragi
		P. cepacia
		P. fluorescens
	Aeromonas	*Ae. hydrophila*
Fungi	*Rhizopus*	*R. delemar*
		R. oryzae
		R. arrhizus
		R. chinensis
	Aspergillus	*A. flavus*
		A. niger
		A. fumigatus
		A. oryzae
	Penicillium	*Pe. cyclopium*
		Pe. citrinum
	Mucor	*M. miehei*
	Humicola	*H. lanuginosa*
	Rhizomucor	*R. miehei*
	Fusarium	*F. oxysporum*
		F. heterosporum

Table II-1 Some lipase-producing microorganisms (Sharma *et al.*, 2001) (continued)

Source	Genus	Species
Yeast	*Candida*	*C. rugosa*
		C. albicans
		C. tropicalis
		C. antarctica
		C. parapsilosis
		C. deformans
		C. curvata
		C. valida
	Geotrichum	*G. candidum*
	Yarrowia	*Y. lipolytica*
	Rhodotorula	*Rho. glutinis*
		Rho. pilimornae
	Pichia	*P. bispora*
		P. maxicana
		P. sivicola
		P. xylosa
		P. burtonii
	Saccharomyces	*S. lipolytica*
		S. crataegenesis
	Trichosporon	*T. asteroides*

There are researches about isolation and screening of novel lipase-producing microorganisms from various environments. Thongekkaew and Boonchird (2007) isolated Thermotolerant yeast *Candida thermophila* SRY-09 from natural sources in Thailand. Bussamara *et al.* (2010) have isolated yeasts and yeast-like strains from the phylloplane of *Hibiscus rosa-sinensis*. The highest lipase producer was *Pseudozyma hubeiensis* HB85A. The lipase-producing fungus *Fusarium solani* NAN103 was isolated from a soil sample in the deciduous dipterocarp forest in Nan province, Thailand (Malilas, 2010).

Recently, Lipase-producing yeast, *A. melanogenum* SRY14-3, from oil-contaminated foam board found in a shipyard area (Sichang Island, Chonburi Province, Thailand). The maximum hydrolysis activity at 4.85 ± 0.15 U/ml was obtained (Vitisant *et al.*, 2013). *A. pullulans* is well known as a black yeast because it produces dark pigment, which is a melanin-like compound (Chi *et al.*, 2009). *Aureobasidium* sp. comprises four species: *A. pullulans* (formerly known as *A. pullulans* var. *pullulans), A. melanogenum* (formerly known as *A. pullulans* var. *melanogenum*), *A. subglaciale* (formerly known as *A. pullulans* var. *subglaciale*) and *A. namibiae* (formerly known as *A. pullulans* var. *namibiae)* (Gostin *et al.*, 2014). *Aureobasidium* spp. produced various enzymes such as cellulase, amylase, proteinase, lipase, xylanase, mannanase and transferases (Chi *et al.*, 2009). *A. pullulans* HN2-3, isolated from a sea saltern of Qingdao, China, could secrete a large amount of extracellular lipase (Liu, Chi, *et al.*, 2008). Furthermore, the lipase gene was cloned from *A. pullulans* HN2-3 and expressed in *Escherichia coli* BL21 (DE3) by (Liu, Li, *et al.*, 2008). However, lipase of *A. melanogenum* has not been studied in cloning, characterization and expression.

Many fungal lipase genes have been cloned, such as *Fusarium heterosporum* (Nagao *et al.*, 1994), *Rhizopus niveus* (Kohno *et al.*, 1998), *Nectria haematococca* (anamorph *F. solani* f. sp. *pisi*) (Eddine *et al.*, 2001), *Fusarium graminearum* (Feng *et al.*, 2005), *Rhizopus oryzae* (Salah *et al.*, 2006), *Pleurotus sapidus* (Zelena *et al.*, 2009) and *Aspergillus niger* (Yang *et al.*, 2010).

More than 50% of reported lipase-producing yeasts, they produce lipases in the forms of various isozymes. These lipase isozymes are produced by various lipase encoding genes. Among many lipase-producing yeasts, *C. rugosa* is the most frequently used yeast as the source of lipase commercially. The genes encoding lipases from *C. albicans*, *C. antarctica*, *C. rugosa*, *C. deformans*, *C. parapsilosis*, *Geotrichum candidum*, *Trichosporon fermentans*, *Yarrowia lipolytica*, *Arxula adeninivorans* and *Kluyveromyces lactis* have already been cloned, characterized and expressed (Vakhlu, 2006).

***Pichia pastoris* expression system**

Recombinant DNA technology represents a very attractive feature that can be used to overcome the cost limitation of the industrial application of lipases. Lipase gene is cloned into a suitable expression system for production and purification in large quantities. Recombinant DNA technology can decrease 40% in the cost of raw material, water, steam and electricity compared with the native enzymes production (Houde *et al.*, 2004). *Escherichia coli* is one of the most widely used hosts for heterologous proteins production. However, the recombinant proteins expressed in *E. coli* became disadvantages due to the problems of protein folding, inclusion bodies and safety issue, leading to the investigation of other potential host systems (Kademi *et al.*, 2003).

The use of the methylotrophic yeast, *P. pastoris*, as an excellent host for the expression of recombinant proteins has become increasing popular in recent times. The advantages of *P. pastoris* include (Ferrer *et al.*, 2009; Li *et al.*, 2007):

1. rapid growth rate in minimal medium

2. low levels of endogenous protein secretion

3. ease of genetic manipulation

4. the ability to efficiently secrete heterologous proteins

5. the ability to perform eukaryotic post-translational modifications, such as protein folding, disulfide bond formation, glycosylation and proteolytic processing

General features of expression vectors

The insertion of a foreign coding sequence into the expression vector is usually carried out in *E. coli*. The expression vectors of *P. pastoris* have been designed as *E. coli/P. pastoris* shuttle vectors. The expression cassette contain an origin of replication for plasmid maintenance in bacteria and selectable markers for transformation of the vector in both organisms. Some secreted expression vectors, such as pPICZα and pGAPZα (Figure II-2), contain sequences encoding a secretion signal that are in frame with the foreign gene for secretion of foreign proteins. This secretion signal derives from *S. cerevisiae* α-mating factor (α-MF) (Li *et al.*, 2007).

The most commonly used signal sequence in *P. pastoris* secretion systems is the α-mating factor pre–pro leader sequence (α-MF) of *S. cerevisiae*. This sequence consists of a 19 amino acid signal peptide as pre-sequence, followed by a 60 amino acid pro-region. After translation, the signal sequence is removed by signal peptidase and pro-region cleavage site is recognized by the yeast kex2 protease, resulting in the release of the mature protein (Daly and Hearn, 2005).

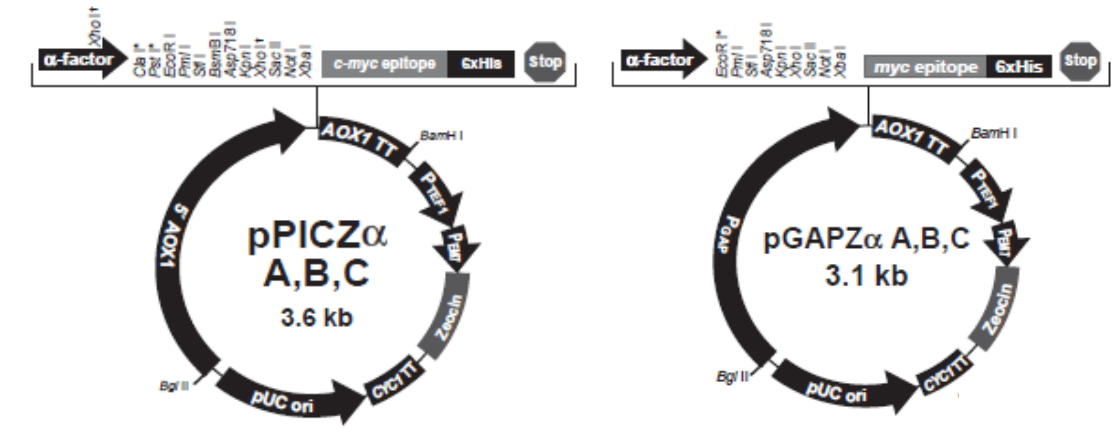

Figure II-2 Schematic map of the pPICZα and pGAPZα expression vector for *P. pastoris.*

Promoters used in *P. pastoris* systems

Pichia pastoris is an excellent host for the expression of heterologous proteins. Primarily, two promoters are used in *P. pastoris* expression system.

1. *AOX1* promoter

Alcohol oxidase (AOX), the first enzyme in the inducible methanol utilization pathway, represents up to 35% total cell protein in cells grown on methanol, indicating the power of this promoter. In the presence of glucose, ethanol or glycerol as a sole carbon source, its expression is undetectable (Potvin *et al.*, 2012). Most *P. pastoris* expression systems take advantage of the methanol-induced alcohol oxidase 1 (AOX1) system. Also, an expression vector p*AOX2* from the alcohol oxidase II has been reported but the expression level was lower than p*AOX1* (Zhang *et al.*, 2009).

The *AOX1* promoter (p*AOX1*), a strong inducible promoter, is used for the construction of recombinant proteins upon induction with methanol. The vectors containing p*AOX1* are pPICZ and pPICZα. Lipases from several microorganisms have been successfully expressed and secreted using the p*AOX1* system, including those from *R. oryzae* (Minning *et al.*, 1998), *C. parapsilosis* (Brunel *et al.*, 2004), *C. thermophila* (Thongekkaew and Boonchird, 2007), *Y. lipolytica* (Yu *et al.*, 2007), *R. chinensis* (Yu *et al.*, 2009), *C. rugosa* (Ferrer *et al.*, 2009) and *C. antarctica* (Liu *et al.*, 2012; Pfeffer *et al.*, 2006).

Pfeffer *et al.* (2006) cloned lipase A gene from *C. antarctica* (*CalA*) into the pPICZαA vector and transformed into *P. pastoris* X-33. Lipase concentrations of 0.88 and 0.55 g/l were obtained using the fed-batch and semi-continuous processes, respectively. Yu *et al.* (2007) reported that the extracellular lipase gene from *Y. lipolytica* (*YlLip2*) was cloned into the pPICZαA and integrated into the genome of *P. pastoris* X-33. The lipase was successfully expressed and secreted under the control of AOX1 promoter. The lipase activity of 12,500 U/ml (0.63 g lipase/l) was obtained in fed-batch cultivation.

2. *GAP* promoter

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in glycolysis. Its promoter (p*GAP*), a strong constitutive promoter, has also been used for the constitutive expression of heterologous proteins. It has been used to express many heterologous proteins in cells using glucose or glycerol as a carbon source. The advantage of using this promoter is that methanol is not required for induction, and it

is not necessary to switch cultures from a different carbon source to methanol. This promoter is more suitable for large-scale production of heterologous recombinant proteins (Zhang *et al.*, 2009). Lipase genes have been expressed under the control of the p*GAP*, including those from *C. rugose* (Chang *et al.*, 2006; Chang *et al.*, 2005; Zhao, Wang, *et al.*, 2008) and *Y. lipolytica* (Wang *et al.*, 2012).

Chang *et al.* (2005) converted the 19 non-universal CTG-serine codons in *LIP1* into universal TCT-serine codons by multiple site-directed mutagenesis and successfully expressed a highly active recombinant *C. rugosa LIP1* in the *Pichia* expression system using pGAPZαC vector. The lipolytic activity of the recombinant LIP1 was 253.3 U/ml which was 2-fold higher than that achieved by a methanol induction system of Brocca *et al.* (1998), which synthetic *LIP1* gene was expressed using pPICZαB vector in *P. pastoris*. The lipase activity of 150 U/ml was obtained.

Selectable Markers

The *Sh ble* gene from *Streptoalloteichus hindustanus* is used in resistance to the bleomycin-related drug zeocin.

Integration of expression vectors into the *P. pastoris* genome

The expression vectors are usually integrated into the *P. pastoris* genome (Figure II-3). The recombinant *P. pastoris* that contain multiple integrated copies of an expression cassette produce larger amounts of recombinant protein than do single-copy strains.

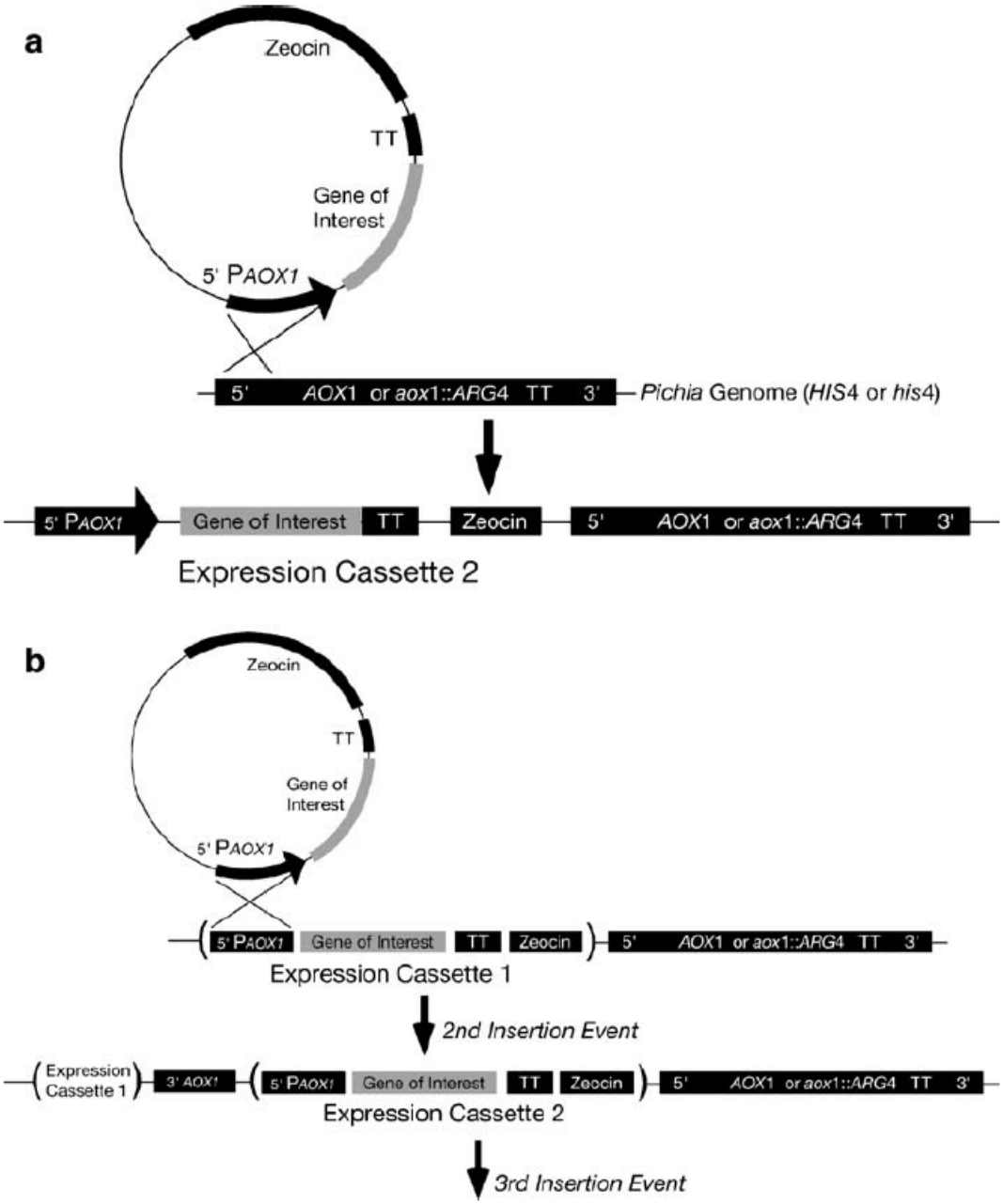

Figure II-3 Map of integration of a heterologous gene into *P. pastoris* genome. (a) Single copy integration; (b) Multiple copy integration (Li *et al.*, 2007)

The application of lipase in wastewater pretreatment

Wastewater from restaurants and some industrial plants are mostly rich in biodegradable organic molecules and nutrients and usually contain high levels of fats and proteins that have a low biodegradability. They cause high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) in water. Therefore the application of a pretreatment to hydrolyze and dissolve lipids may improve the biological degradation of lipid-rich wastewater, accelerating the process and improving time efficiency. It is necessary to reduce the concentration of fat and oils or to eliminate these materials, in order to enable the biological treatment to proceed without any inhibition of the organic matter in wastewater (Cammarota and Freire, 2006). Effective breakdown of solids and the clearing and prevention of fat blockage or filming in waste systems are important in many industrial operations, such as the degradation of organic debris by a commercial mixture of lipase, protease, cellulase, amylase, inorganic nutrients, etc. and sewage treatment by cleaning of holding tanks, grease traps and septic tanks (Gandhi, 1997).

Conventional pretreatment systems for oil and grease removal

1. Physical pretreatment

The main technique for separating lipids from wastewater is the grease-trap method (gravity separation of floatable fat/oil). Alternatively, oil/water separators (tilted plate separators; TPS) and dissolved air flotation (DAF) units have been employed to reduce lipid content in wastewater (Willey, 2001).

2. Chemical pretreatment

Chemical treatments may be used to reduce total fatty acid matter. Ferric chloride, aluminum sulfate, or lime, may be used to break the fat emulsion and coagulate fat particles, which can be readily separated by flotation or sedimentation. However, the cost of such reagents is high, the removal efficiency of dissolved and/or emulsified oil and grease is low and extremely problematic sludge is produced (Cammarota and Freire, 2006; Willey, 2001).

3. Biological treatment

Lipases are applied in activated sludge and other aerobic waste processes where thin layers of fats constantly removed from the surface of aerated tanks to permit oxygen transport (Singh and Mukhopadhyay, 2012). Some reports have described the use of lipases during wastewater pretreatment. Lipase from *P. restrictum* was used to treat dairy wastewater with initial content of oils and grease of 1,200 mg/l and then submitted to an anaerobic biological treatment. The COD removal rate was 80% (Leal *et al.*, 2002).

Jeganathan *et al.* (2007) evaluated the hydrolysis of wastewater with high oil and grease concentration from a pet food industry using immobilized *C. rugosa lipase* (CRL) as a pretreatment process for anaerobic treatment through batch and continuous-flow experiments.

The application of a commercial lipase (Lipolase 100T, Novozymes) and a lipase obtained by solid state fermentation (SSF) of *P. restrictum* was studied in the

wastewater pretreatment of anaerobic biodegradation from swine and bovine meat industry. When the wastewater containing 1200 mg/l of oil and grease was pretreated with the lipase obtained by SSF, COD removal showed an increase of 22% (Rigo *et al.*, 2008).

There are researches that have described the use of microorganisms for the lipid-containing wastewater pretreatment. Biodegradation of waste cooking oil by *Y. lipolytica* CECT 1240 have been investigated. The result showed that COD was diminished up to nearly 90% (Domínguez et al., 2010). The immobilized cell of *Y. lipolytica* W29 in calcium alginate degraded 2000 mg/l oil and 2000 mg/l COD within 50 h (Lan *et al.*, 2009).

A bacterial consortium comprising *P. aeruginosa* LP602, *Bacillus* sp. B304 and *Acinetobacter calcoaceticus* LP009 was used in treatment of lipid-rich wastewater from kitchen. The lipid content was reduced from 20,000 mg/l to less than 20 mg/l within 12 days under aerobic conditions (Mongkolthanaruk and Dharmsthiti, 2002). Lipase-producing bacteria like *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *Serratia marsescens*, *P. aeruginosa* and *Staphylococcus aureus* have also been used as a bacterial consortium during the pretreatment of lipid-rich wastewater from palm oil mill, dairy, slaughter house, soap industry and domestic wastewater. The lipid content was reduced from 25,000 mg/l to 80 mg/l within 12 days of incubation (Prasad and Manjunath, 2011).

Molecular technique used in the study

Rapid amplification of cDNA ends (RACE)

RACE is a technique for generating the full-length cDNA in reverse transcription reactions using SMART™ (Switching Mechanism At 5' end of RNA Transcript) technology (Zhu *et al.*, 2001). This is made possible by the joint action of the oligonucleotide and reverse transcriptase. As shown in Figure II-4, first-strand cDNA synthesis is primed using a modified oligo (dT) primer. After reverse transcriptase reaches the end of the mRNA template, several dC residues are added to the 3' end of the first-strand cDNA. The Oligonucleotide anneals to the tail of the cDNA and serves as an extended template for reverse transcriptase (Clontech, 2003).

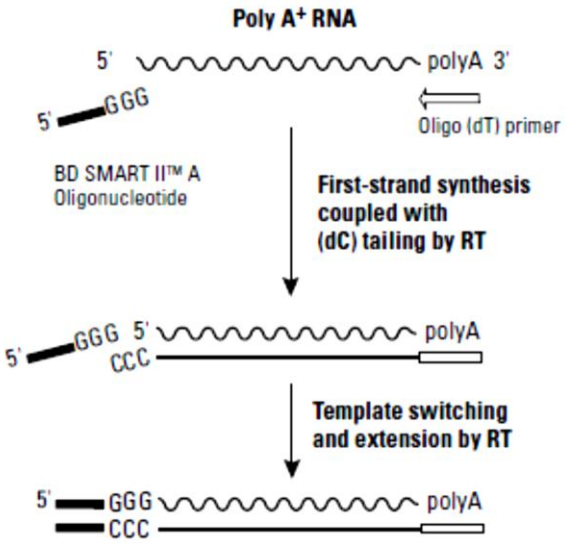

Figure II-4 Mechanism of SMART™ cDNA synthesis (Clontech, 2003)

The gene specific primers (GSP) are designed from partial sequence of the interested gene for 5'-RACE and 3'-RACE PCR to produce overlapping RACE products (Figure II-5). The overview of 5'-RACE and 3'-RACE PCR amplification is shown as Figure II-6 and Figure II-7, respectively.

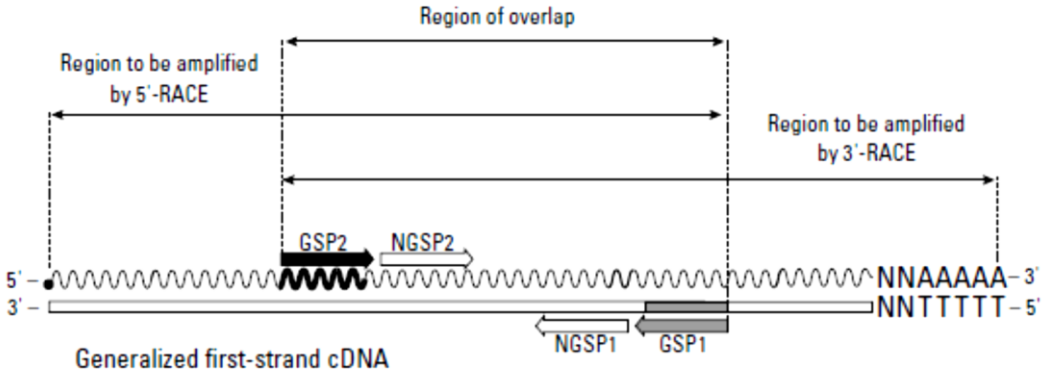

Figure II-5 The diagram of relationship of gene-specific primers to the cDNA template (Clontech, 2003)

The RACE technique was used to determine the full-length sequence of the genes such as endo-1,4-β-xylanase from *A. pullulans* ATCC 20524 (Tanaka *et al.*, 2006), lipase gene from *A. pullulans* HN2-3 (Liu, Li, *et al.*, 2008) and β-glucosidase gene from *Periconia* sp. (Harnpicharnchai *et al.*, 2009).

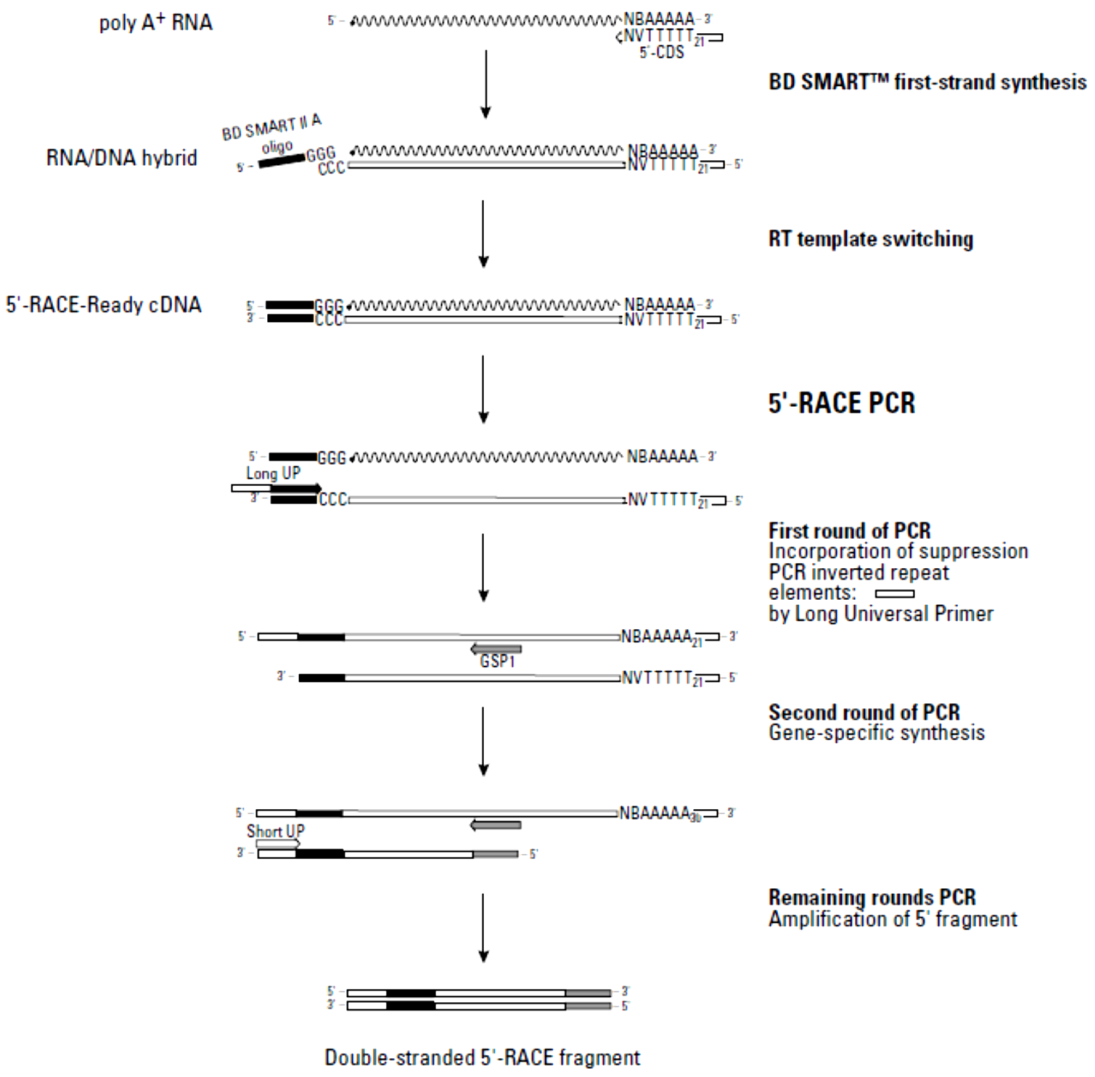

Figure II-6 The overview of 5 '-RACE PCR amplification (Clontech, 2003)

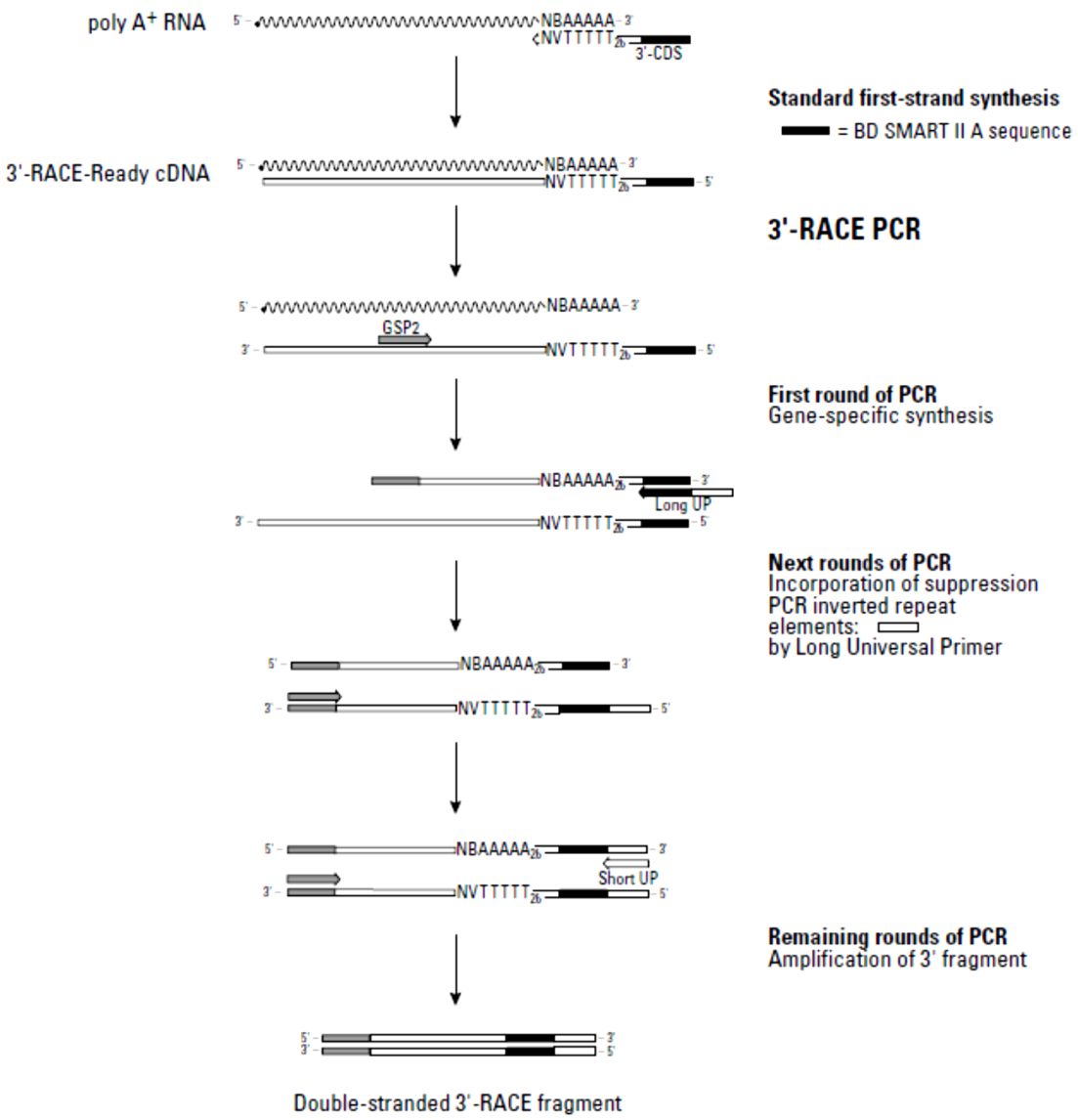

Figure II-7 The overview of 3'-RACE PCR amplification (Clontech, 2003)

CHAPTER III

MATERIAL AND METHODS

Part I Cloning and expression of *Aureobasidium melanogenum* lipase in *Pichia pastoris*

1. Strains, culture conditions and plasmid

The lipolytic yeast strain *Aureobasidium melanogenum* SRY14-3, isolated from an oil-contaminated soil sample, collected at Srichang Island, Chonburi Province, Thailand (Vitisant *et al.*, 2013), was obtained from Biofuels by Biocatalysts Research Unit, Chulalongkorn University. This strain was used as a source of the lipase gene. *E. coli* DH5α (Gibco, USA) and *P. pastoris* (Invitrogen, USA) were used as host cells for plasmid propagation and expression, respectively, of the target gene. The pGEM-T easy vector system was used for the cloning of the PCR product. pPICZαA and pGAPZαA vectors (Invitrogen, USA) were used for lipase expression. *E. coli* was grown at 37°C in Luria–Bertani broth (LB) supplemented with ampicillin (100 μg/ml) or zeocin (25 μg/ml), whereas *P. pastoris* was grown in YPD (10 g yeast extract/l, 20 g peptone/l and 20 g glucose/l).

2. Total RNA isolation and RT-PCR

A. melanogenum was cultured in 50 ml of lipase-inducing production medium (11.7 g yeast extract/l, 14 g sucrose/l, 10 g KH_2PO_4, 1 g $MgSO_4.7H_2O$/l and 1.44% v/v cottonseed oil, pH 5.2) at 30°C , 200 rpm for 2 days (Vitisant *et al.*, 2013) and

cells were harvested by centrifugation. Total RNA was extracted using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was generated from the total mRNA using an Improm-II™ reverse transcription system (Promega, USA). The cDNA obtained was used as a template for partial lipase cDNA amplification by PCR. The primers (Table III-1) were designed from conserved regions of the lipase gene using the nucleotide sequence of *A. pullulans* HN2-3 (Liu, Li, *et al.*, 2008) obtained from GenBank (accession EU082005.1). The PCR reaction contained 1× DyNAzyme buffer, 2 mM of Mg^{2+}, 200 μM of each dNTP, 0.3 μM of each primer (F1 and R1), 500 ng of template cDNA and 1 U of DyNAzyme II DNA polymerase (Thermo Scientific, USA) in a total volume of 50 μl. The PCR conditions were as follows: one cycle of 3 min at 94°C, 30 cycles of 30 s at 94°C, 45 s at 50°C and 1 min at 72°C and finally one cycle of 7 min at 72°C. The PCR products from the first reaction were used as a template for nested PCR using primers NF2 and NR2. The PCR products from the nested PCR, representing a partial sequence of the putative lipase gene, were cloned into a pGEM®-T Easy Vector System (Promega, USA) and sequenced (Macrogen, South Korea). The partial sequence obtained was then used to design another set of internal primers (Table III-1) against the putative lipase gene for rapid amplification of cDNA ends (RACE).

3. Rapid amplification of cDNA ends (RACE)

To obtain the full-length sequence of the lipase gene from cDNA, the 5′ and 3′ ends of the lipase gene were generated by RACE using a BD SMART™ RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's

instructions. The PCR products were cloned into a cloning vector and sequenced. The resulting 5′ and 3′ halves of the lipase gene were assembled with overlapping contigs into full-length constructs using BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The full-length nucleotide sequence was compared with previously deposited sequences in GenBank using BlastX (http://www.ncbi.nlm.nih.gov/blast/). The amino acid sequence of the cDNA fragments was deduced using the programs of DNAMAN 8.0. Putative signal sequence was identified with the SignalP prediction program (http://www.cbs.dtu.dk/Services/SignalP/).

4. Cloning of genomic DNA sequence encoding lipase

The gene specific primers were designed according to the full-length sequence of lipase cDNA. Genomic DNA was extracted with an E.Z.N.A.® Yeast DNA Kit (Omega Bio-Tek, USA) and used as a template for amplification of the lipase gene. The PCR reaction was performed in a total volume of 50 µl. The PCR reaction contained 1× Optimized DyNAzyme EXT Buffer, 200 µM of each dNTP, 0.5 µM of each primer (DNALip-F and DNALip-R, Table III-1), 10 ng of template DNA and 1 U of DyNAzyme EXT DNA polymerase (Thermo Scientific, USA) in a total volume of 50 µl. The reaction was performed at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min, and then finally at 72°C for 7 min. The PCR products were cloned into a cloning vector and sequenced.

The nucleotide sequence of cDNA encoding lipase from *A. melanogenum* has been submitted to the GenBank database under accession number KT067768.

5. Construction of yeast expression plasmid and *P. pastoris* transformation

The cDNA from *A. melanogenum* lipase (*AML*) lacking its signal sequence was amplified using PCR with gene-specific primers containing recognition sites for the restriction enzymes *Eco*RI and *Not*I. The PCR reaction mixtures contained 1×Phusion GC buffer, 200 µM of each dNTP, 0.5 µM of each primer (Lip-F and Lip-R, Table III-1), 10 ng of template DNA and 1 U of Phusion DNA polymerase (New England Biolabs, USA) in a total volume of 50 µl. The reaction was performed at 98°C for 30 s followed by 35 cycles of 98°C for 10 s, 58°C for 30 s and 72°C for 1 min, and then finally at 72 °C for 10 min. The amplified fragment was digested with the restriction enzymes and ligated into the same cleavage sites of the expression vectors pPICZαA (*AOX1* promoter) and pGAPZαA (*GAP* promoter) as shown in Figure III-1. The ligation product was transformed into *E. coli* DH5α. Recombinant plasmids were extracted from transformants and sequenced for confirmation of the correct insertion of the lipase gene. The plasmids were linearised using *Pme*I and *BspH*I for pPICZαA and pGAPZαA, respectively, and transformed into *P. pastoris* X-33 strain by electroporation. Recombinants were selected on YPDS-zeocin agar (10 g yeast extract/l, 20 g peptone/l, 20 g glucose/l and 1 M D-sorbitol) containing zeocin at several concentrations (100, 500, 1000 and 2000 µg/ml).

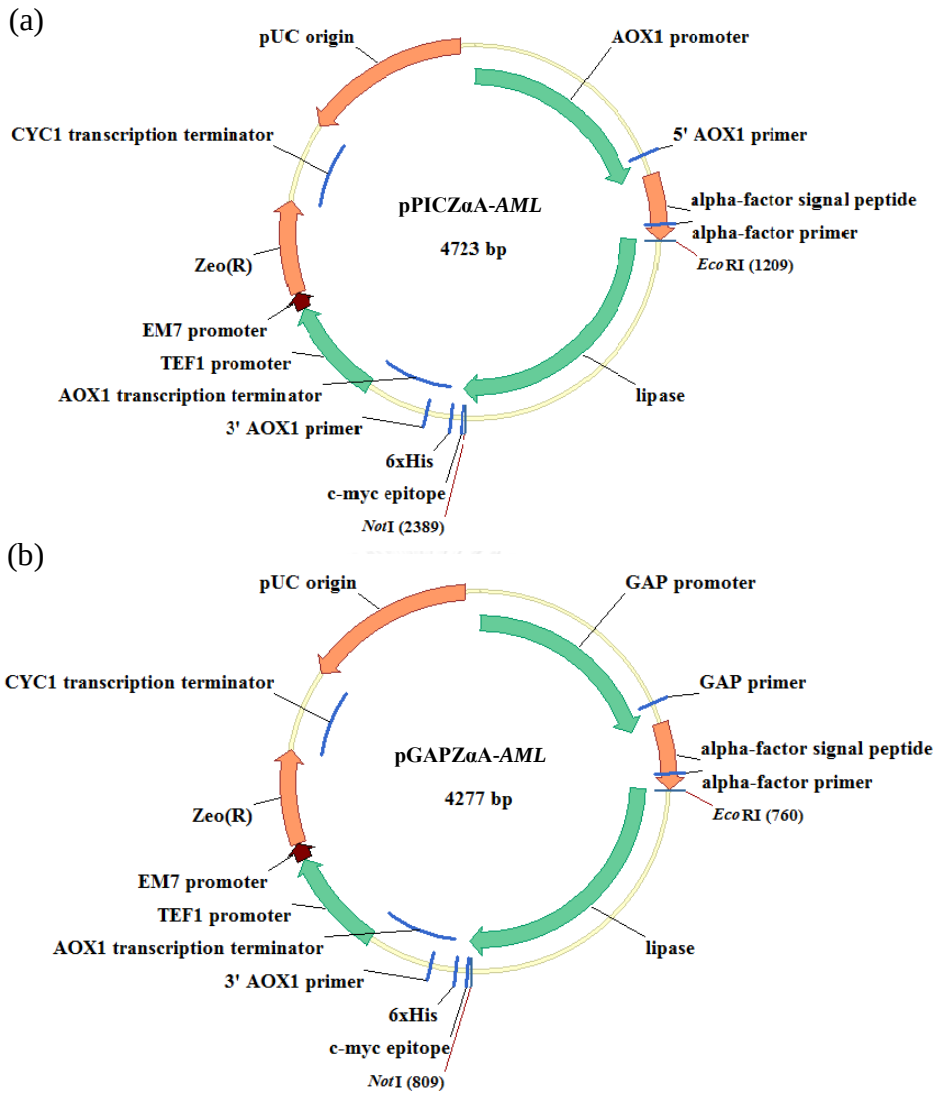

Figure III-1 Schematic diagram of the expression vector for *A. melanogenum* lipase gene insertion. (a) pPICZαA vector and (b) pGAPZαA vector. Both vectors contain α-factor secretion signal for efficient secretion of most proteins from *Pichia* and zeocin resistance gene for selection.

Table III-1 Primer used in PCR reactions

Amplification reaction	Primer name	Sequence (5'- 3')
cDNA partial sequence	F1	CTTATTGTGTAGGTCTCACCG
	R1	TGCTACCACTTACAGAATGGG
	NF2	GCGTTCCGTGGCACCTACT
	NR2	CACCTTTGGAGAGCCCCG
RACE	3'GSP	CCAGAAACTCCGCGATGCAACAACT
	5'GSP	TGGCCGACAAGTGTCAAGGCGTAATTC
	3'NGSP	TGCCGGACTTGGAAGCTGCCGTTGC
	5'NGSP	GTCCGGCAAGATGGCAGAGGAAGCTA
	Universal Primer A Mix (UPM)	Long: CTAATACGACTCACTATAGGGCAAG CAGTGGTATCAACGCAGAGT Short: CTAATACGACTCACTATAGGGC
	Nested Universal Primer A (NUP)	AAGCAGTGGTATCAACGCAGAGT
Genomic DNA sequence	DNALip-F	ATGACATGGATCATCACAATAA
	DNALip-R	TCAGGTCGACTCCTGTCC
Expression	Lip-F	CGGAATTCCTTCCAGCTCAACAAGCTC
	Lip-R	ATAGTTTAGCGGCCGCGGTCGACTCCTGT CCATTA

*Eco*RI recognition site within Lip-F primer and *Not*I recognition site within Lip-R primer are underlined.

6. Lipase expression in *P. pastoris*

P. pastoris containing *AML* gene were precultured in 5 ml of YPD at 30°C and 250 rpm for 24 h. For the *AOX1* promoter expression system, *P. pastoris*/pPICZαA-*AML* was cultured in 25 ml of buffered complex glycerol medium (BMGY; 10 g yeast extract/l, 20 g peptone/l, 100 mM potassium phosphate buffer, pH 6.0, 13.4 g YNB/l, 4×10^{-5} g biotin/l and 1% v/v glycerol) at 30°C, 250 rpm for 18 h. The cell pellets

were harvested, resuspended in 50 ml of buffered complex methanol medium (BMMY; BMGY containing 0.5% v/v methanol instead of glycerol) and incubated at 30°C, 250 rpm for 7 days. To induce expression, methanol was added daily to final concentrations of 0.5%, 1%, 2% and 3% v/v. For the *GAP* promoter expression system, *P. pastoris*/pGAPZαA-*AML* was cultured in 50 ml of YPD at 30°C, 250 rpm for 3 days. The supernatants were harvested every day by centrifugation at 10,000 g for 5 min.

7. Lipase activity and protein assay

Lipase activity was measured using *p*-nitrophenyl laurate (*p*NPL) solubilised in absolute ethanol as a substrate. The assay was modified from method by Leelaruji *et al.* (2013). The reaction contained 250 µl of lipase, 710 µl of 50 mM phosphate buffer, pH 7.0 and 40 µl of 25 mM *p*-NPL. The hydrolytic reaction was incubated at 35°C for 10 min. The mixture was then centrifuged at 14,000×*g* for 5 min. The supernatant was measured at OD 405 nm with a Eon microplate spectrophotometer (BioTek Instruments, USA). One unit of lipase activity was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol per minute under the assay conditions used. The total protein content was determined by Bradford's method and bovine serum albumin was used as the standard.

8. SDS-PAGE analysis

The produced proteins were analysed by SDS-PAGE. Proteins were mixed with 2× sample buffer (Biorad, USA), denatured for 10 min at 95°C and loaded at 30 µl/lane on a NuPAGE 10% Bis–Tris pre-cast gel (Biorad, USA). Electrophoresis was

performed in Tris/glycine/SDS (TGS) buffer for 30 min at 200 V. After electrophoresis, the gel was subjected to Coomassie blue staining and destained with 10% v/v ethanol. Precision Plus protein standard (Biorad, USA) was used as a molecular mass standard. Molecular masses were estimated from the migration distance and logarithm of molecular weights.

9. Enzyme characterization

To prepare crude recombinant lipase for characterisation, the culture broth was centrifuged at 10,000 *g* for 15 min at 4°C. The collected supernatant was concentrated approximately 10 times by ultrafiltration through a Macrosep Advance Centrifugal Device with a 10 kDa cut-off membrane (PALL, USA).

9.1 Effects of pH on lipase activity and stability

The optimal pH of lipase activity was determined in several buffers with different pH (pH 6.0–10.0) under lipase assay conditions, *p*-NPL as the substrate and a reaction time of 10 min at 37°C. The following buffer systems (50 mM each) were used: citric acid–sodium citrate buffer (pH 6.0), Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0–8.0) and glycine–NaOH buffer (pH 9.0–10.0).The pH stability of lipase was determined by pre-incubating lipase in several pH buffers for 24 h at 4°C and then analysing the residual activity under lipase assay conditions with pH 7.0 as a reference.

9.2 Effects of temperature on lipase activity and stability

The effects of temperature on lipase activity were studied between 20°C–100°C using the *p*-NPL assay in phosphate buffer (pH 7.0). The thermostability of lipase was determined by preincubating the lipase at 20°C–60°C for 30 min in phosphate buffer (pH 7.0). The residual activity was measured using *p*-NPL as the substrate at 37°C for 10 min. Lipase incubated at 4°C was used as a reference.

9.3 Substrate specificity of lipase

The substrate specificity of lipase towards various *p*-nitrophenyl esters was examined using the substrates *p*-nitrophenyl butyrate (*p*-NPB), *p*-nitrophenyl caprylate (*p*-NPC), *p*-nitrophenyl decanoate (*p*-NPD), *p*-nitrophenyl laurate (*p*-NPL), *p*-nitrophenyl myristate (*p*-NPM) and *p*-nitrophenyl palmitate (*p*-NPP) with carbon chain lengths as C4–C16, respectively, at a final concentration of 1 mM. The lipase activity was measured in phosphate buffer (pH 7.0) at 37°C for 10 min.

9.4 Effects of metal ions on lipase activity

The effects of metal ions on lipase activity were determined by measuring the residual activity after preincubating lipase in phosphate buffer (pH 7.0) with different metal ions at a final concentration of 1 or 5 mM at 4°C for 1 h. The metal salts used were LiCl, $BaCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $CuSO_4$, $HgCl_2$, $AgNO_3$, $ZnSO_4$, $CoCl_2$, $NiCl_2$, $FeSO_4$ and $FeCl_3$. The residual activity was measured using *p*-NPL as substrate at 37°C for 10 min. A sample without the addition of metal ions was used as the control.

9.5 Effects of detergents and inhibitors and on lipase activity

The effects of detergents (SDS, CTAB, EDTA, CHAPS, Triton X-100 and Tween-20) and inhibitors (DTT and PMSF) on lipase activity were determined after preincubating lipase with various detergents in phosphate buffer (pH 7.0) at 4°C for 1 h. Detergents and inhibitors were present at a final concentration of 1 or 5 mM, except that Triton X-100 and Tween-20 were used at 0.1% and 1% v/v. The residual activity was measured using *p*-NPL as substrate at 37°C for 10 min. A sample incubated without addition of detergent or inhibitor was taken as the control.

9.6 Effects of organic solvents on lipase activity

The effects of various organic solvents at 10% v/v (50 µl of organic solvents plus 450 µl of crude enzyme (30 U/ml)) on lipase activity were investigated using various water-immiscible organic solvents (chloroform, hexane, 1-octanol and *p*-xylene) and water-soluble organic solvents (methanol, ethanol, propanol, isopropanol, butanol, acetone, acetonitrile and DMSO). The mixture was incubated at 37 °C, 200 rpm for 30 min and then incubated further at 4 °C for 24 h. The residual activity was measured using pNPL as substrate at 37 °C for 10 min. The sample without addition organic solvents was taken as control.

Statistical analysis

All experiments were performed in triplicate. Data are expressed as mean ± SD. The data were statistically tested using analysis of variance (ANOVA) and Duncan's new multiple range test and considered significant when $P < 0.05$.

Part II Cloning and expression of *Fusarium solani* lipase in *Pichia pastoris*

1. Strains, plasmids and culture conditions

The mature lipase gene from *Fusarium solani* NAN103 (*FSL*) (Genbank accession number KT003282, Appendix A, Figure A-1) inserted in the plasmid vector pPICZαA was obtained from the Biofuels by Biocatalysts Research Unit, Chulalongkorn University. *Escherichia coli* DH5α (Gibco, USA) and *P. pastoris* X-33 (Invitrogen, USA) were used as the host cells for plasmid propagation and expression of the target gene, respectively. pPICZαA and pGAPZαA vectors (Invitrogen, USA) were used for the expression of the lipase gene in *P. pastoris*. *E. coli* DH5α was grown in Luria–Bertani broth at 37°C. *P. pastoris* was grown in yeast extract peptone dextrose (YPD) medium (10 g/l of yeast extract, 20 g/l of peptone and 20 g/l of glucose) at 30°C.

2. Cloning of lipase gene from *F. solani*

The *FSL* gene lacking its signal sequence was amplified by PCR with gene-specific primers containing recognition sites for the restriction enzymes *Kpn*I and *Not*I. The forward primer was 5′-CAGGTACCGGCCCAGTGCCCTCTGTTGATGAAA-3′, and the reverse primer was 5′-TCGCGGCCGCAGTCATCTGCTTAACAAATTCCTGATCCAG-3′ (the restriction enzyme recognition sites are underlined). The PCR reaction mixtures contained 1× Phusion high-fidelity (HF) buffer, 200 µMof each dNTP, 0.5 µM of each primer, 10 ng of template DNA and 1 U of Phusion DNA polymerase (New England Biolabs Inc., USA) in a total volume of 50 µl. The reaction

was performed at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 62°C for 30 s and 72°C for 30 s and then finally at 72°C for 10 min. The amplified fragment was digested with the appropriate restriction enzymes and ligated into similarly digested expression vectors pPICZαA (p*AOX1*) and pGAPZαA (p*GAP*) as shown in Figure III-2.

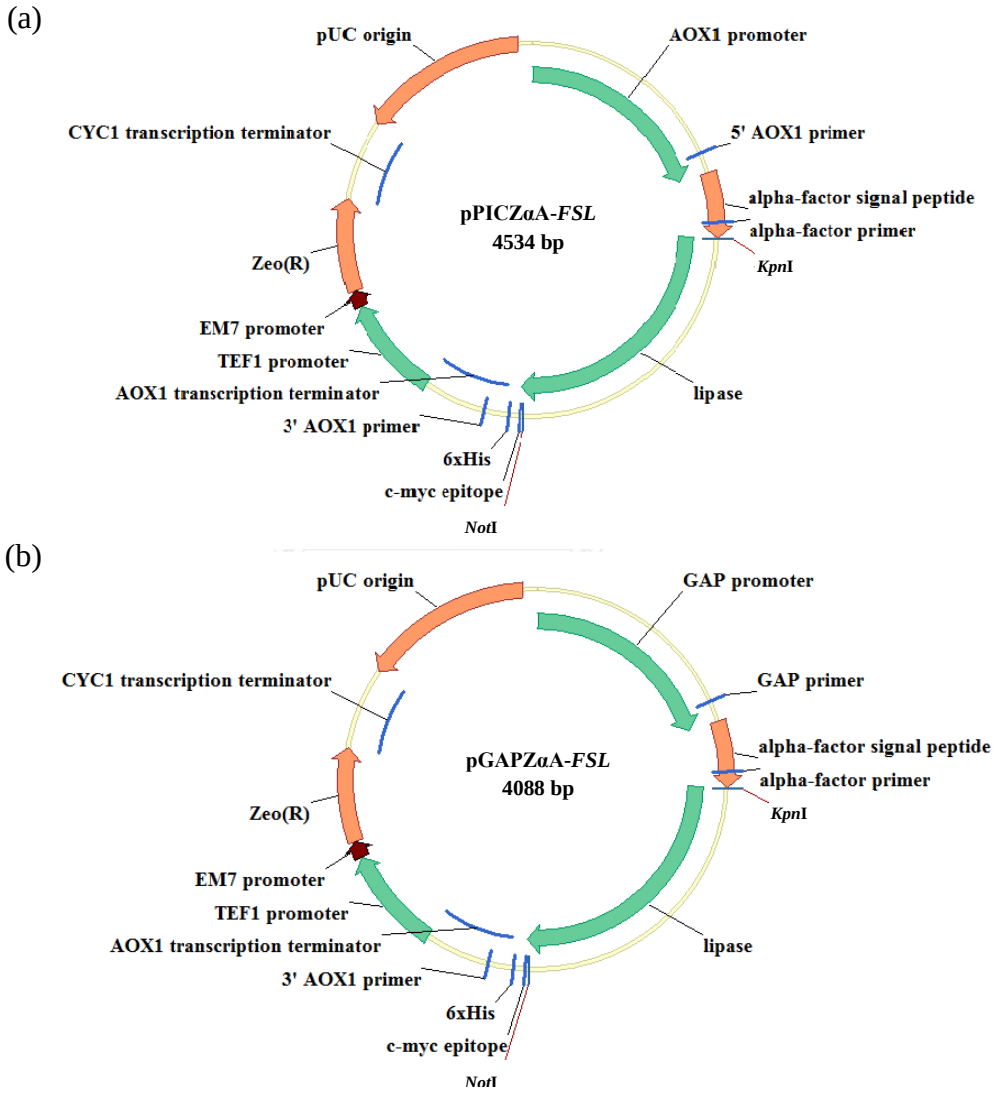

Figure III-2 Schematic diagram of the expression vector for *F.solani* lipase gene insertion. (a) pPICZαA vector and (b) pGAPZαA vector. Both vectors contain α-factor secretion signal for efficient secretion of most proteins from *Pichia* and zeocin resistance gene for selection.

The ligation products were used to transform *E. coli* DH5α. To verify the nucleotide sequence of the lipase genes, plasmid DNAs extracted from selected clones were sequenced by Pacific Science (Bangkok, Thailand) using the α-factor (5′-TACTATTGCCAGCATTGCTGC-3′) and 3′ AOX (5′-GCAAATGGCATTCTGACA TCC-3′) sequencing primers.

3. *P. pastoris* transformation

The recombinant plasmids were linearized using *Pme*I and *Avr*II for pPICZαA and pGAPZαA, respectively, and then used to transform competent *P. pastoris* X-33 using electroporation performed with the Gene Pulser Xcell (Bio-Rad, USA). Transformants were selected on YPDS-zeocin agar (10 g/l of yeast extract, 20 g/l of peptone, 20 g/l of glucose and 1 M of D-sorbitol) containing zeocin at 100, 500, 1,000 and 2,000 μg/ml. The plates were incubated at 30°C for 3–4 days.

4. Lipase expression in *P. pastoris*

P. pastoris containing the *FSL* gene were precultured in 5 ml of YPD shaken at 250 rpm for 24 h at 30°C. For the p*AOX1* expression system, *P. pastoris*/pPICZαA-*FSL* was cultured in 25 ml of buffered glycerol-complex medium (BMGY; 10 g/l of yeast extract, 20 g/l of peptone, 13.4 g/l of YNB, 4×10^{-4} g/l of biotin, 10 g/l of glycerol and 0.1 M of potassium phosphate buffer, pH 6.0) shaken at 250 rpm for 18 h at 30°C. The cell pellets were harvested and resuspended in 50 ml of buffered methanol-complex medium (BMMY; BMGY medium containing 5 ml/l of methanol instead of glycerol). Different methanol concentrations (0.5, 1, 2 and 3% v/v) were

added to the BMMY medium every 24 h for 7 days to induce expression (Invitrogen, 2010a). For the p*GAP* expression system, *P. pastoris*/pGAPZαA-*FSL* was cultured in 50 ml of YPD medium shaken at 250 rpm for 3 days at 30°C (Invitrogen, 2010b). The supernatants were harvested every day by centrifugation at 10,000 g for 5 min.

5. Lipase activity and protein assay

Lipase activity in the culture supernatant was determined by the hydrolysis of *p*-nitrophenyl laurate (*p*-NPL) into lauric acid and *p*-nitrophenol. The assay was modified from the method of Leelaruji *et al.* (2013). Absolute ethanol was used to solubilize *p*-NPL. Reactions contained 250 µl of lipase, 710 µl of 50 mM phosphate buffer, pH 7.0, and 40 µl of 25 mM *p*-NPL. This hydrolytic reaction was incubated at 37°C for 10 min and then centrifuged at 14,000 *g* for 5 min. The absorbance of the supernatant was measured at 405 nm using an Eon microplate spectrophotometer (BioTek Instruments, Inc., USA). One unit of lipase activity was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol per min under the assay conditions. The total protein content was determined using Bradford's method with bovine serum albumin used as the standard (Bradford, 1976).

6. SDS-PAGE analysis

The recombinant proteins were analysed using SDS-PAGE (Laemmli, 1970) with a 12% separating gel and a 4% stacking gel. Protein-containing samples were mixed with a 3× sample buffer (New England Biolabs Inc., USA) and denatured for 10 min at 95°C before being loaded (30 µl/lane) on the gel. The migration was

performed in Tris/glycine/SDS buffer at 30 mA for 120 min. After migration of the proteins, the gel was stained with Blue Lightning Stain solution (Vivantis, USA). Precision Plus Protein Standards (Bio-Rad, USA) were used as molecular mass standards. Molecular masses were estimated from the migration distance and the logarithm of their molecular weights.

7. Enzyme characterization

To prepare crude recombinant lipase for characterisation, the culture broth was centrifuged at 10,000 *g* for 15 min at 4°C. The collected supernatant was concentrated approximately 10 times by ultrafiltration through a Macrosep Advance Centrifugal Device with a 10 kDa cut-off membrane (PALL, USA).

7.1 Effects of pH on lipase activity and stability

The optimal pH of lipase activity was determined under lipase assay conditions using buffers of different pH, *p*-NPL as the substrate and a reaction time of 10 min at 37°C. The following buffer systems (50 mM each) were used: citric acid–sodium citrate buffer (pH 5.0–6.0), Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0–8.0) and glycine–NaOH buffer (pH 9.0–10.0). The pH stability of lipase was determined by incubating lipase in buffers of different pH for 24 h at 4°C and then analysing the residual activity under lipase assay conditions with pH 7.0 as a reference.

7.2 Effects of temperature on lipase activity and stability

The effects of temperature on lipase activity were studied between 20°C and 100°C using the *p*-NPL assay in phosphate buffer (pH 7.0). The thermostability of

lipase was determined by preincubating the lipase at 20°C–80°C for 30 min in phosphate buffer (pH 7.0). The residual activity was measured using *p*-NPL as the substrate at 37°C for 10 min. Lipase incubated at 4°C was used as a reference.

7.3 Substrate specificity of lipase

The substrate specificity of lipase towards various *p*-nitrophenyl esters was examined using the substrates *p*-NPB, *p*-NPC, *p*-NPD, *p*-NPL, *p*-NPM and *p*-NPP with carbon chain lengths as C4–C16, respectively, at a final concentration of 1 mM. The lipase activity was measured in phosphate buffer (pH 7.0) at 35°C for 10 min.

7.4 Effects of metal ions on lipase activity

The effects of metal ions on lipase activity were determined by measuring the residual activity after preincubating lipase in phosphate buffer (pH 7.0) with different metal ions at a final concentration of 1 or 5 mM at 4°C for 1 h. The metal salts used were LiCl, $BaCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $CuSO_4$, $HgCl_2$, $AgNO_3$, $ZnSO_4$, $CoCl_2$, $NiCl_2$, $FeSO_4$ and $FeCl_3$. The residual activity was measured using *p*-NPL as substrate at 35°C for 10 min. A sample without the addition of metal ions was used as the control.

7.5 Effects of detergents and inhibitors and on lipase activity

The effects of detergents (SDS, CTAB, EDTA, CHAPS, Triton X-100 and Tween-20) and inhibitors (DTT and PMSF) on lipase activity were determined after preincubating lipase with various detergents in phosphate buffer (pH 7.0) at 4°C for 1 h. Detergents and inhibitors were present at a final concentration of 1 or 5 mM, except

that Triton X-100 and Tween-20 were used at 0.1% and 1% v/v. The residual activity was measured using *p*-NPL as substrate at 35°C for 10 min. A sample incubated without addition of detergent or inhibitor was taken as the control.

7.6 Effects of organic solvents on lipase activity

The effects of various organic solvents at 10% v/v (50 µl of organic solvents plus 450 µl of crude enzyme (30 U/ml)) on lipase activity were investigated using various water-immiscible organic solvents (chloroform, hexane, 1-octanol and *p*-xylene) and water-soluble organic solvents (methanol, ethanol, propanol, isopropanol, butanol, acetone, acetonitrile and DMSO). The mixture was incubated at 35 °C, 200 rpm for 30 min and then incubated further at 4 °C for 24 h. The residual activity was measured using pNPL as substrate at 35 °C for 10 min. The sample without addition organic solvents was taken as control.

Statistical analysis

All experiments were performed in triplicate. Data are expressed as mean ± SD. The data were statistically tested using analysis of variance (ANOVA) and Duncan's new multiple range test and considered significant when $P < 0.05$.

Part III The application of recombinant *Pichia pastoris* in lipid-containing wastewater pretreatment

P. pastoris/pGAPZαA-*FSL* was precultured in 10 ml YPD at 30°C, 250 rpm for 24 h. Subsequently, the inoculum (4% v/v) was added to 25 ml of synthetic wastewater medium in a 250-ml flask containing (in g/l) palm oil, 10; peptone, 0.6; beef extract, 0.4; urea, 0.1; Na_2HPO_4, 0.1; NaCl, 0.03; $CaCl_2$, 0.014; KCl, 0.014 and $MgSO_4$, 0.01 (the composition of the synthetic wastewater medium was adapted from Matsumiya *et al.* (2007). The flask was incubated at 30°C for 72 h, with shaking at 150 rpm. Effect of initial oil concentrations on TAG content was investigated using different oil concentrations (0.5, 0.75, 1 and 1.5% w/v). Samples of the culture were taken and treated with 5 ml of chloroform to extract the lipids. The chloroform layer was used for the determination of TAG and FFA content by HPLC. The HPLC analysis was performed using a Shimadzu LC-20A (Shimadzu, Japan) equipped with an Apollo Silica column (25 cm × 4.6 mm; Alltech, USA) connected to an evaporative light scattering detector equipped with nitrogen gas. The mobile phase was composed of a mixture of solution A (hexane/isopropanol/ethyl acetate/formic acid, 85:10:10:0.1 by volume) and solution B (hexane/formic acid, 100:0.2 v/v). A flow rate of 1.5 ml/min and 40°C column temperature were used.

CHAPTER IV

RESULTS AND DISCUSSION

Part I Cloning and expression of *Aureobasidium melanogenum* lipase in *Pichia pastoris*

Cloning of lipase gene from *A. melanogenum*

The full-length sequence of cDNA encoding lipase from *A. melanogenum* was obtained using RACE-PCR. It had an open reading frame of 1,254 bp, whereas the genomic DNA encoding lipase gene comprised 1,311 bp with one intron (57 bp). It encodes 417 amino acid residues with a putative signal peptide of 26 amino acids as determined by SignalP (Figure IV-1). The deduced amino acid sequence of *A. melanogenum* lipase had an estimated molecular weight of 46.2 kDa.

A BLAST search of *A. melanogenum* lipase in the protein sequence database showed identity to lipases of other *Aureobasidium* sp. and fungi, with 99%, 88%, 87%, 53%, 31% and 25% amino acid identity pertaining to lipases from *A. melanogenum* CBS 110374 (accession no. KEQ63038.1), *A. namibiae* CBS 147.97 (accession no. KEQ71427.1), *A. pullulans* (accession no. ABV03820.1), *Aspergillus fumigatus* Af293 (accession no. XP_750543.1), *Y. lipolytica* (AFH77825.1) and *N. haematococca* (*F. solani*) (CAC19602.1), respectively (Fig IV-2). This result indicated that lipase from *A. melanogenum* was highly similar to *Aureobasidium* sp.

The protein sequence contained a lipase consensus sequence (Gly-X-Ser-X-Gly) that is highly conserved among all known lipases (Liu, Li, *et al.*, 2008).

```
1     ATGACATGGATCATCACAATAAACATGCACTTTCTTCTGCTATCTCTCCTGAGTGCGCTA
1      M  T  W  I  I  T  I  N  M  H  F  L  L  L  S  L  L  S  A  L
61    TCAGTCATCACGAGCGCGCTTCCAGCTCAACAAGCTCCTCTGCTTCGCCATGCTGAAGAC
21     S  V  I  T  S  A  L  P  A  Q  Q  A  P  L  L  R  H  A  E  D
121   AATCGCACAGTGTCTGCCGAGTTATTCTCTGACCTCGAGGAGCTCGCGCGCATTGTCGAT
41     N  R  T  V  S  A  E  L  F  S  D  L  E  E  L  A  R  I  V  D
181   ATCTCTTATTGCGTAGGTCTCACTGGCACAGGCATATCAAGGCCTTTCAAATGTCTCAGT
61     I  S  Y  C  V  G  L  T  G  T  G  I  S  R  P  F  K  C  L  S
241   CGCTGCTCCGAATTTCCAGATTTCGAACTTGTCAAGgtaagagagtgtgtgtttgtattg
81     R  C  S  E  F  P  D  F  E  L  V  K
301   accatatttgaccatgtgccgacatgttactagACATGGAACACTGGCCAGTTGATGTCT
93                                      T  W  N  T  G  Q  L  M  S
361   GACTCCTGTGGCTACATCGCCTTGTCTCACTCACAATCAAATCCTCGCATCATCGTCGCG
102    D  S  C  G  Y  I  A  L  S  H  S  Q  S  N  P  R  I  I  V  A
421   TTTCGTGGCACCTACTCTATCGCCAACACTGTTGTCGATCTTTCGACGGTCCCTCAAGAA
122    F  R  G  T  Y  S  I  A  N  T  V  V  D  L  S  T  V  P  Q  E
481   TATATTCCCTATCCTGGTGACCCGGATTCAGATGCCTCAAAAGCTGGCCAAAAGAAACCA
142    Y  I  P  Y  P  G  D  P  D  S  D  A  S  K  A  G  Q  K  K  P
541   GAAACTCCGCGATGCAACAACTGCACTGTGCATACGGGCTTCTACAAATCTTGGAAAGTA
162    E  T  P  R  C  N  N  C  T  V  H  T  G  F  Y  K  S  W  K  V
601   GCTTCCTCTGCCATCTTGCCGGACTTGGAAGCTGCCGTTGCAGCGTACCCGAATTACGCC
182    A  S  S  A  I  L  P  D  L  E  A  A  V  A  A  Y  P  N  Y  A
661   TTGACACTTGTCGGCCATTCCCTCGGAGGAGCTGTTGCTGCACTCGCAGGACTGGAACTT
202    L  T  L  V [G  H  S  L  G] G  A  V  A  A  L  A  G  L  E  L
721   GACTCGCGAGGCTGGAACCCGACTGTCACTACTTTTGGCGAGCCTAGGCTTGGAAACGCT
222    D  S  R  G  W  N  P  T  V  T  T  F  G  E  P  R  L  G  N  A
781   GCCCTGAACAAATATCTTGACCAGCAATTCAACCTGGTCGGCTCTTCCAGCGAAGCTTGG
242    A  L  N  K  Y  L  D  Q  Q  F  N  L  V  G  S  S  S  E  A  W
841   GCAAACACTTTCGATGAGCGACAATTGCGTTATCGCCGTGTCACTCACATAGATGATCCA
262    A  N  T  F  D  E  R  Q  L  R  Y  R  R  V  T  H  I  D  D  P
901   GTACCTCTGTTGCCACTCACAGAATGGGGTTATCGTATGCACGCTGGCGAGATCTACATC
282    V  P  L  L  P  L  T  E  W  G  Y  R  M  H  A  G  E  I  Y  I
961   TCTAAGTCTGCCTTGACTCCGGATGTGCAAGATCTGCAGCACTGTGTTGGTGATGAGGAC
302    S  K  S  A  L  T  P  D  V  Q  D  L  Q  H  C  V  G  D  E  D
1021  CATCAATGCATTGCCGGACAAGATGGCAGTCTAGAGTCGACTGTCGCTACCCACGATGAC
322    H  Q  C  I  A  G  Q  D  G  S  L  E  S  T  V  A  T  H  D  D
1081  CTGAGAGCGCAGGTAAAACGCTCAGTCGATGACCTTGCAGAGGAGCGTGAGTTGGAGAAG
342    L  R  A  Q  V  K  R  S  V  D  D  L  A  E  E  R  E  L  E  K
1141  CGAGCCATCGGTTCTTGGGTCGTCCCATCTCGCTACAAGTTGTGGCAGTTGTTCTTCTCC
362    R  A  I  G  S  W  V  V  P  S  R  Y  K  L  W  Q  L  F  F  S
1201  CATCGCGACTACTTCTGGCGTCTCGGTTTGTGTGTTCCTGGTGGAGACCCTTGGGACTGG
382    H  R  D  Y  F  W  R  L  G  L  C  V  P  G  G  D  P  W  D  W
1261  AACCGCAAGCCGTACGCACCACTCGATGGTAATGGACAGGAGTCGACCTGA
402    N  R  K  P  Y  A  P  L  D  G  N  G  Q  E  S  T  *
```

Figure IV-1 Nucleotide sequence of the lipase gene of *A. melanogenum* and its deduced amino acid sequence. The upper lines show the nucleotide sequence with an intron (in small letter). The lower lines show the deduced amino acid with signal peptide (underlined). The two N-linked glycosylation sites are shaded. The conserved motifs (Gly-X-Ser-X-Gly) are boxed. The stop codon is indicated with an asterisk.

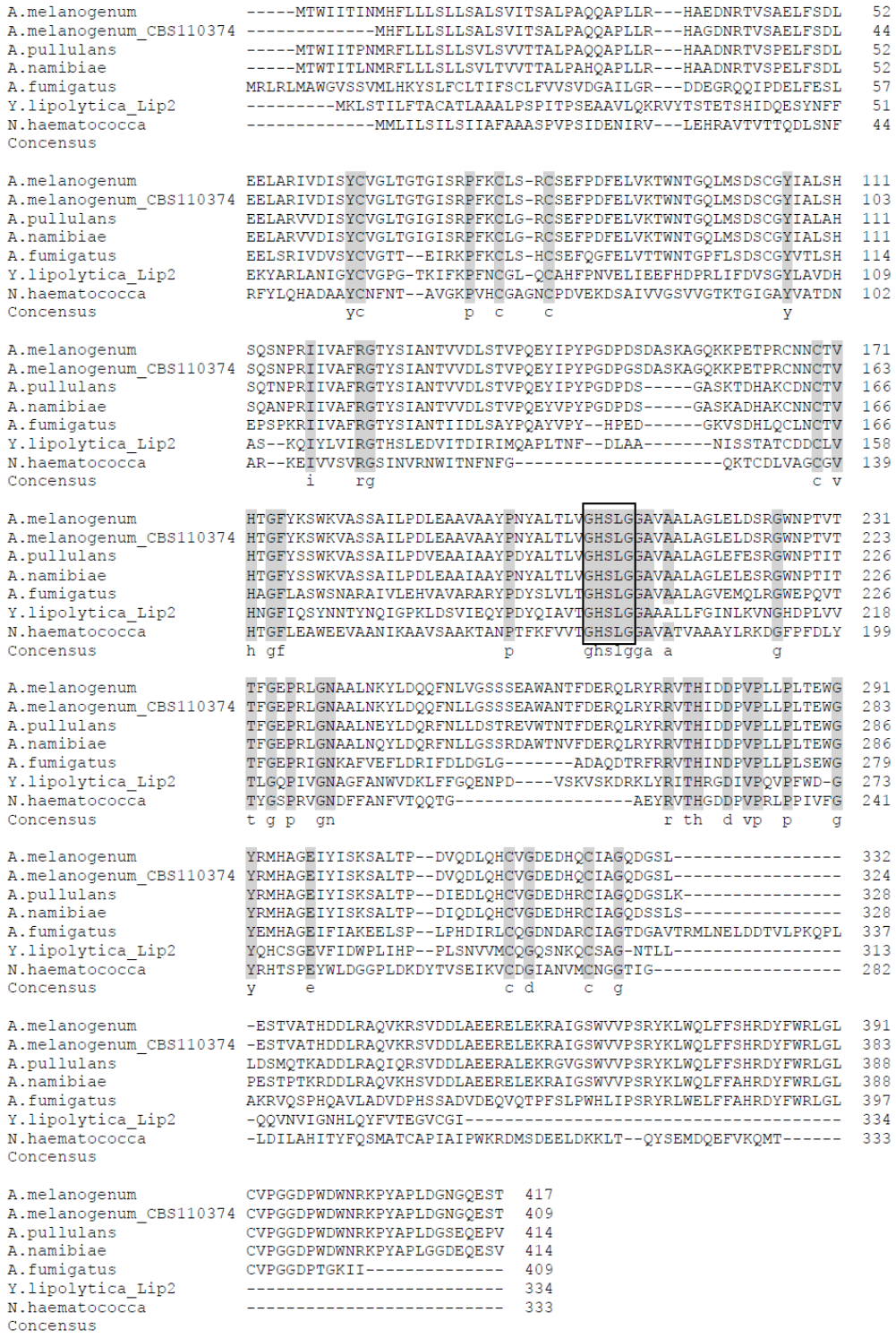

Figure IV-2 Multiple alignment of the deduced amino acid sequence of lipase from *A. melanogenum* with other lipases from *A. melanogenum* CBS 110374 (KEQ63038.1), *A. pullulans* (ABV03820.1), *A. namibiae* CBS 147.97 (KEQ71427.1), *A. fumigatus* Af293 (XP_750543.1), *Y. lipolytica* (AFH77825.1) and *Nectria haematococca* (CAC19602.1) obtained from GenBank. Multiple sequence alignment of proteins was carried out using the DNAMAN 8.0. Identical residues are shaded. The conserved motifs (Gly-X-Ser-X-Gly) are boxed.

Expression of *A. melanogenum* lipase in *P. pastoris*

In this study, *AML* gene lacking signal peptide sequence was amplified and transformed into *P. pastoris*. The sequence encoding the α-factor secretion signal from *Saccharomyces cerevisiae* was inserted into the pPICZαA and pGAPZαA vectors used to secrete proteins. Secretion allowed easier purification of the lipase from the extracellular medium (Lin-Cereghino *et al.*, 2013). Therefore, this expression system may be used to produce extracellular lipase.

The *AML* gene was successfully overexpressed in *P. pastoris* system. *P. pastoris*/pPICZαA-*AML* induced by 2% v/v methanol had the highest specific lipase activity of 9.9 ± 0.9 U/mg after 6 days (Figure IV-2a), whereas *P. pastoris*/pGAPZαA-*AML* had a specific activity of 6.8 ± 0.2 U/mg (Figure IV-3b). The inducible expression of *AML* was higher than that obtained from constitutive expression by 1.5-fold; therefore, *P. pastoris*/pPICZαA-*AML* and the enzyme it produced were selected for further characterisation. The specific lipase activity of recombinant *P. pastoris* was higher than that of *A. pullulans* HN2-3 lipase expressed in *E. coli* (0.96 U/mg) (Liu, Li, *et al.*, 2008). The specific activity of lipase from *A. melanogenum* wild type was 1.1 ± 0.3 U/mg. The inducible and constitutive expressions of *A. melanogenum* lipase in *P. pastoris* were approximately 9-fold and 6.2-fold, respectively, higher than those of the wild type (Table IV-1). *P. pastoris*/pPICZαA-*AML* may have a higher gene copy number than the other transformant given that it could grow on YPDS containing high antibiotic concentrations (1000 μg/ml Zeocin), whereas *P. pastoris*/pGAPZαA-*AML* could grow on YPDS agar plates containing 500 μg/ml. The level of antibiotic resistance reflects the number of recombinant genes integrated into the *P. pastoris* genome (Nordén *et*

al., 2011). Similarly, the activity of glucan 1,3-beta-glucosidase A under the control of an inducible expression system was higher than under a constitutive expression system (Boonvitthya *et al.*, 2012).

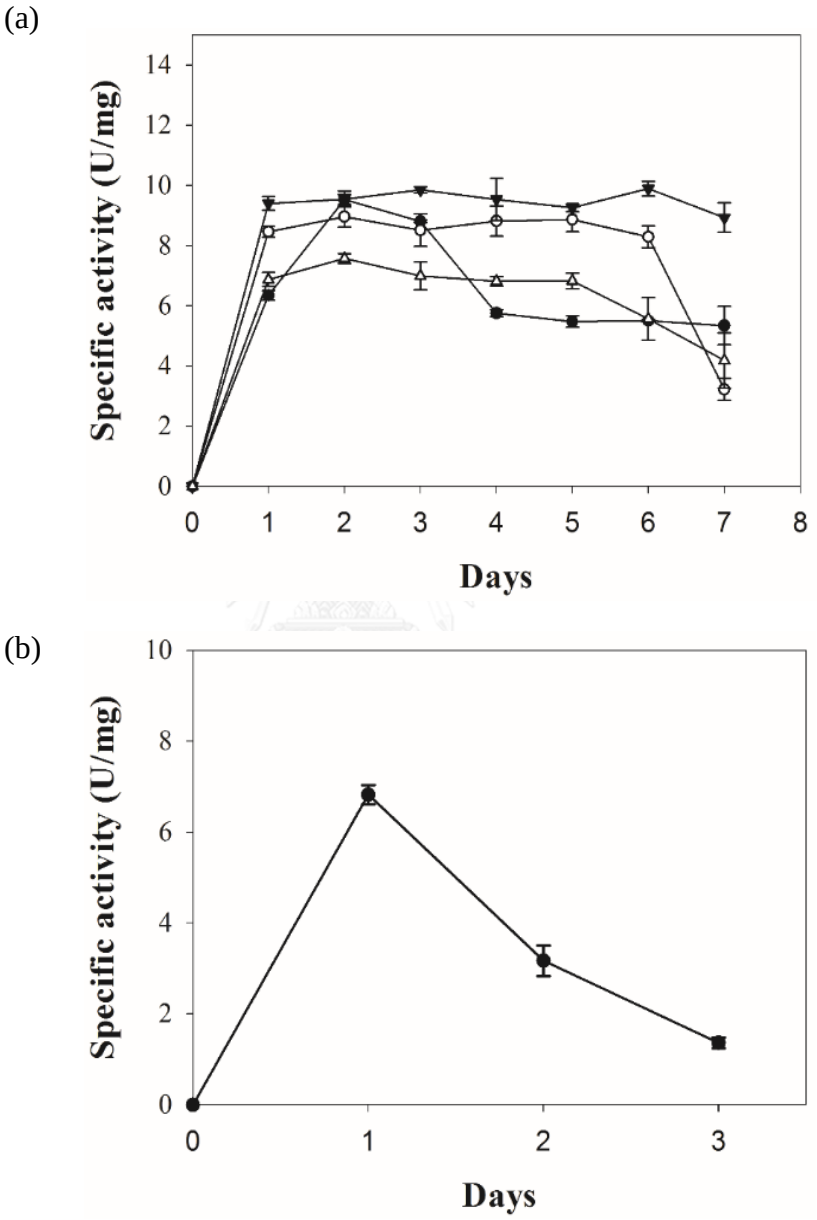

Figure IV-3 Specific activity of (a) *P. pastoris*/pPICZαA-*AML* cultured in 50 ml of BMMY in a 500-ml baffled flask at 30°C and 250 rpm. Initially, 0.5% v/v methanol was added. Thereafter, methanol at 0.5% v/v (●), 1% v/v (○), 2% v/v (▼) and 3% v/v (△) was added every day. (b) *P. pastoris*/pGAPZαA-*AML* was cultured in 50 ml of YPD in a 500-ml baffled flask at 30°C, 250 rpm. Data are means of three independent experiments and error bars indicate standard deviation.

Table IV-1 Lipase activity of recombinant *P. pastoris* and wild type *A. melanogenum*

Source of lipase	Cultivation time (days)	Lipase activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)
P. pastoris/pPICZαA-*AML*	6	3.8 ± 0.2	0.4 ± 0.1	9.9 ± 0.9
P. pastoris/pGAPZαA-*AML*	1	0.5 ± 0.1	0.08 ± 0.01	6.8 ± 0.2
P. pastoris X-33	3	0.03 ± 0.0	0.10 ± 0.0	0.30 ± 0.0
A. melanogenum SRY14-3	4	1.6 ± 0.3	1.5 ± 0.0	1.1 ± 0.3

One unit of lipase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min under the assay conditions.

There were two potential N-linked glycosylation sites of the protein [-N-R-T-(41) and -N-C-T-(168)] in the sequence of *A. melanogenum* lipase predicted by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). However, SDS-PAGE showed that the supernatant from both *P. pastoris* recombinants showed one specific band with a molecular mass of approximately 43 kDa (Figure IV-4), a size similar to that (43.4 kDa) estimated from the deduced amino acid sequence of *A. melanogenum* lipase lacking its signal sequence. This result suggests the absence of glycosylation by *P. pastoris*. When endoglycosidase H was used for protein deglycosylation, the molecular weight of lipase did not decrease, indicating that the recombinant lipase was not glycosylated by *Pichia* expression system (Figure IV-5); however, the recombinant lipase could express lipase activity.

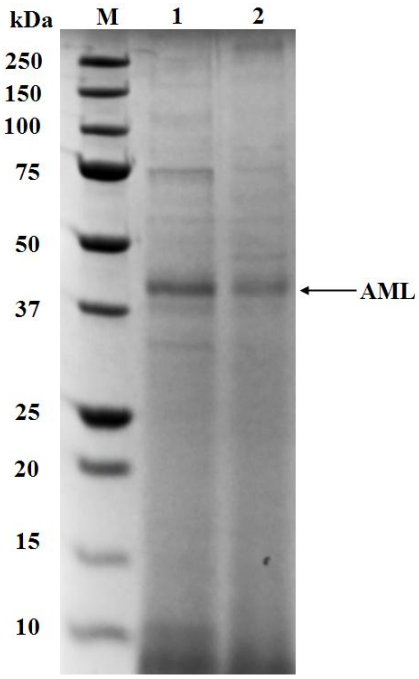

Figure IV-4 SDS-PAGE analysis of the recombinant lipase produced from *P. pastoris*/pPICZαA-*AML* and *P. pastoris*/pGAPZαA-*AML*. Lane M: protein marker; lane 1: supernatant of *P. pastoris*/pPICZαA-*AML*; lane 2: supernatant of *P. pastoris*/pGAPZαA-*AML*. The main band in the gel corresponds to *A. melanogenum* lipase (arrow).

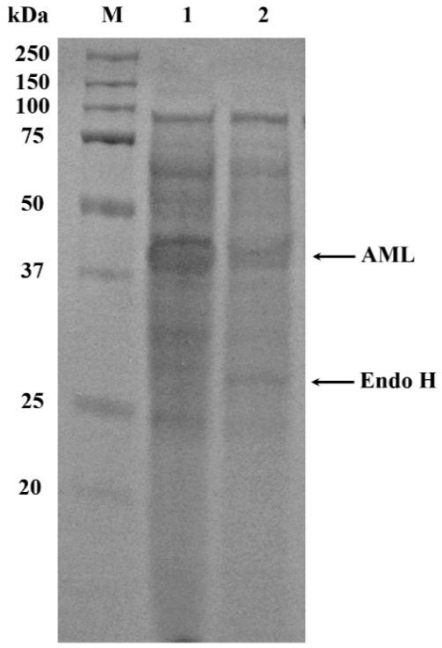

Figure IV-5 SDS-PAGE analysis of recombinant lipase produced by of *P. pastoris*/pPICZαA-*AML* before (lane 1) and after (lane 2) deglycosylation with Endo H. The position of Endo H (29 kDa) and recombinant lipase (43 kDa) are indicated on the right. Lane M contains molecular weight markers.

Effects of pH and temperature on recombinant lipase activity and stability

The crude lipase from *P. pastoris*/pPICZαA-*AML* showed optimum lipase activity at pH 7.0. The enzyme was stable only at pH 7.0 with 81% residual activity after incubation for 24 h (Figure IV-6a). The enzyme was stable only at pH 7.0. Unlike *A. pullulans* HN2-3, the lipase showed optimum activity at alkaline pH (Liu, Chi, *et al.*, 2008; Liu, Li, *et al.*, 2008). The difference may be associated with the sea saltern environment where *A. pullulans* HN2-3 was isolated. The optimum temperature of the recombinant lipase from *P. pastoris* was 35°C–37°C. The lipase activity decreased rapidly above 40°C. The recombinant lipase was stable at 20°C–40°C and maintained residual activity at 64%–77% after incubation for 30 min in

phosphate buffer (pH 7.0). Marked loss of lipase activity was observed above 40°C, and no activity was detected after incubation for 30 min at 60°C, as shown in Figure IV-6b. The optimum temperature of the recombinant lipase from *P. pastoris* was 35°C–37°C. Similarly, Liu, Li, *et al.* (2008) reported that crude lipase of *A. pullulans* HN2-3 expressed in *E. coli* showed highest activity at 35°C and was inactivated at 60°C.

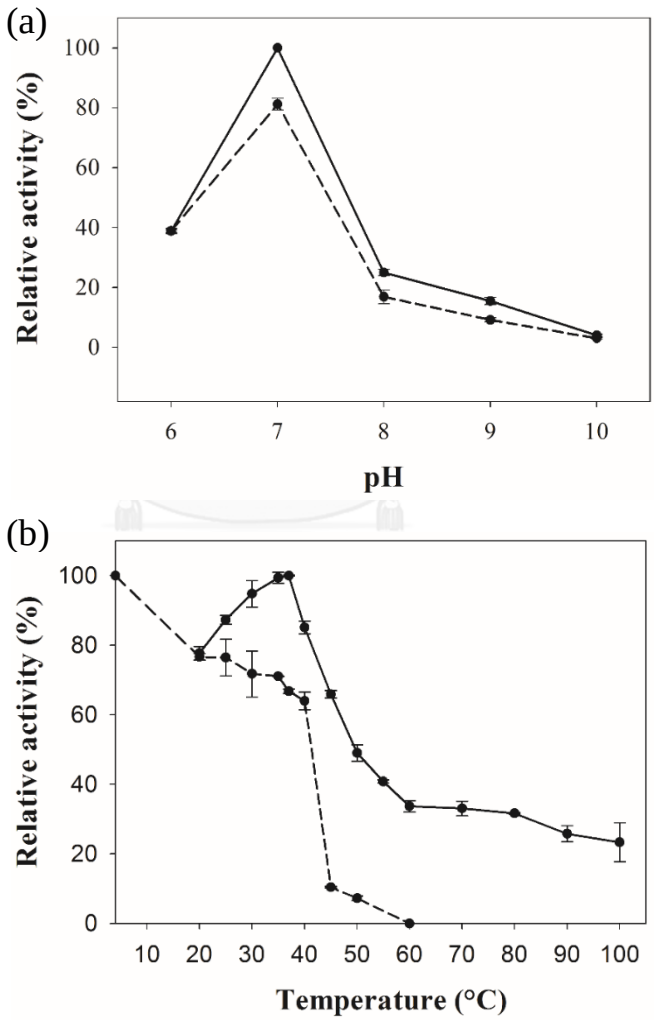

Figure IV-6 Effect of pH (a) and temperature (b) on lipase activity (solid line) and stability (dashed line) of the recombinant lipase produced from *P. pastoris*/pPICZαA-*AML*. Data are means of three independent experiments and error bars indicate standard deviation.

Substrate specificity of lipase

The substrate specificity of the crude lipase towards various *p*-nitrophenyl esters was investigated (Figure IV-7). The recombinant lipase of *P. pastoris*/pPICZαA-*AML* had optimum activity for substrates with medium-chain length from 8 to 12. The highest lipase activity with *p*-NPC (C8) and *p*-NPL (C12) was obtained which was not significantly different. The activity declined below 40% with *p*-NPP (C16), *p*-NPM (C14) and *p*-NPB (C4).

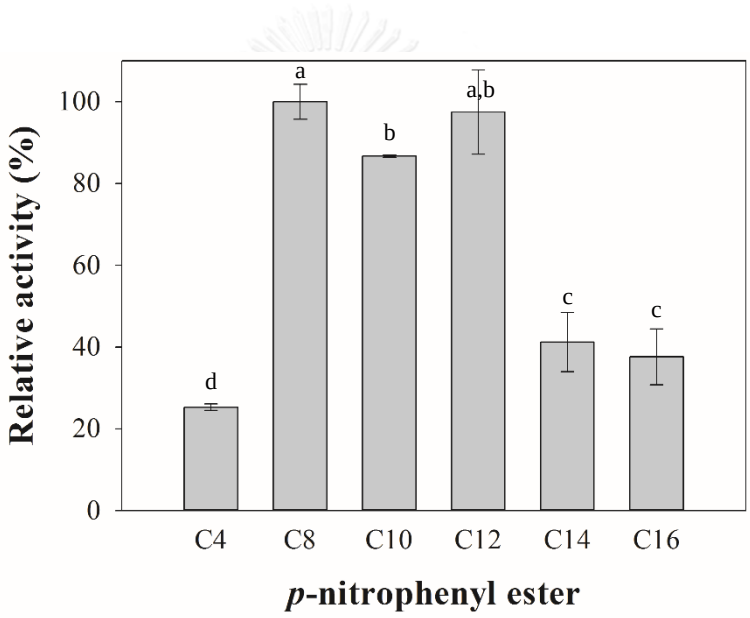

Figure IV-7 Substrate specificity of recombinant lipase produced from *P. pastoris*/pPICZαA-*AML* towards *p*-nitrophenyl esters. Data are means of three independent experiments and error bars indicate standard deviation. The same letters above bars indicate that the lipase activity was not significantly different ($P > 0.05$).

Effects of metal ions on lipase activity

Various metal ions had different effects on lipase activity, as shown in Table IV-2. Mg^{2+}, Mn^{2+}, Li^{+}, Ca^{2+} and Ni^{2+} (1 mM) stimulated lipase activity by 16–28% which was not significantly different, whereas Ag^{+} and Hg^{+} strongly inhibited lipase activity. The lipase activity was greatly inhibited by all metal ions at high concentrations (5 mM). Unlike the result of Liu, Chi, *et al.* (2008), Zn^{2+} and Fe^{2+} (1.0 mM) showed an inhibitory effect in decreasing activity of the purified lipase of *A. pullulans* HN2.3 which was different from this study. The presence of Zn^{2+} and Fe^{2+} (1 mM) did not significantly affect the *P. pastoris*/pPICZαA-*AML* lipase activity. Metal ions enhance enzyme activity by maintenance the structural stability of the protein by binding to negative charge of amino acid residues (Çolak *et al.*, 2005). Generally, the metal ions form complexes with ionized fatty acids, changing their solubility and behavior at interfaces. The fatty acids released could be affected by metal ions. However, the effects of metal ions depend on particular lipase (Hasan *et al.*, 2009). Each metal ion might have affinity to different amino acid residues of lipase resulting in conformational change which could stimulate or inhibit the activity of lipase.

Table IV-2 Effects of various metal ions on the lipase activity of recombinant lipase produced from *P. pastoris*/pPICZαA-*AML*

Metal ions	Relative activity (%)	
	1 mM	5 mM
Control	100.0 ± 0.0	100.0 ± 0.0
$HgCl_2$	17.7 ± 1.0	14.7 ± 0.6
$AgNO_3$	8.1 ± 1.2	7.5 ± 1.3
$ZnSO_4$	103.5 ± 5.7	34.2 ± 1.2
$FeSO_4$	99.3 ± 7.2	41.1 ± 3.3
$CoCl_2$	98.9 ± 6.1	27.1 ± 0.4
$CuSO_4$	50.2 ± 2.9	10.6 ± 1.2
$FeCl_3$	67.9 ± 0.1	31.7 ± 0.1
$MgSO_4$	125.1 ± 6.4	27.0 ± 8.4
$MnSO_4$	128.0 ± 1.1	49.3 ± 0.8
$BaCl_2$	69.8 ± 4.5	28.8 ± 2.9
LiCl	116.3 ± 6.7	63.1 ± 0.1
$CaCl_2$	125.1 ± 4.9	27.9 ± 0.3
$NiCl_2$	115.9 ± 5.5	27.2 ± 2.1

Relative lipase activity was measured by *p*-NPL assay at 37°C for 10 min. Data are means of three independent experiments and error bars indicate standard deviation.

Effects of detergents and inhibitors on lipase activity

The lipase activity enhanced in the presence of CHAPS at 1 mM resulting in relative increases of 60% activity. At a high concentration of SDS, CHAPS, Tween-20 and Triton X-100 completely inactivated activity, whereas DTT and EDTA enhanced lipase activity with an increase of 7 and 16%, respectively (Table IV-3). This result suggests that the structure of lipase from *P. pastoris*/pPICZαA-*AML* was unfolded by anionic detergents (SDS) and non-ionic detergents (Tween 20, and Triton

X-100) whereas the lipase structure was not affected by low concentration of zwitterionic detergent (CHAPS). The recombinant lipase can be potentially applied in the industries using CHAPS at low concentration as surfactant. Lipases belong to the class of serine hydrolases, and in most cases their activity are irreversibly inhibited by PMSF because it binds directly to the active site at serine residue (Peng *et al.*, 2014). In this study, PMSF had an inhibitory effect on the recombinant lipase from *P. pastoris*/pPICZαA-*AML*. DTT is a strong reducing agent. It can cleavage disulfide bond of protein resulting in change of the protein conformation which can enhance or inhibit the activity of lipase. Similar inhibitory effects were reported by Chang *et al.* (2006), the lipase activity of recombinant LIP3 of *C. rugosa* in the *P. pastoris* system was completely inactivated by SDS (0.1 and 1% w/v) and CHAPS (1% w/v).

The major interactions between lipases and detergents are hydrophobic. However, the charged groups of anionic and cationic detergents play an important role to this interaction. Related effects of the detergents can be observed on many different lipases, but there are no general rules. Because many factors affect the lipase-substrate-detergent interactions including micelle formation, concentrations of free and micellar substrate, their availability to enzyme, the degree and mode of the enzyme activation by the hydrophobic interactions, enzyme denaturation or inactivation by detergents, and the structure of the enzyme itself defining the location of the enzyme in the water-oil interphase. However, some common behavior has been demonstrated such as the maximal stimulation by zwitterionic detergents (Helistö and Korpela, 1998).

Table IV-3 Effects of various detergents and inhibitors on the lipase activity of recombinant lipase produced from *P. pastoris*/pPICZαA-*AML*

Detergents and inhibitors	Low concentration	Relative activity (%)	High concentration	Relative activity (%)
Control		100.0 ± 0.8		100.0 ± 0.8
Inhibitors				
DTT	1 mM	87.0 ± 2.9	5 mM	107.4 ± 2.1
PMSF	1 mM	49.2 ± 7.0	5 mM	27.0 ± 4.6
Detergents				
SDS	1 mM	14.2 ± 0.7	5 mM	0.0 ± 0.0
CTAB	1 mM	23.3 ± 3.1	5 mM	76.4 ± 5.7
EDTA	1 mM	76.0 ± 6.7	5 mM	116.3 ± 2.3
CHAPS	1 mM	159.2 ± 6.2	5 mM	0.0 ± 0.0
Tween 20	0.1% v/v	0.0 ± 0.0	1% v/v	0.0 ± 0.0
Triton X-100	0.1% v/v	0.0 ± 0.0	1% v/v	0.0 ± 0.0

Relative lipase activity was measured by *p*-NPL assay at 37°C for 10 min. Data are means of three independent experiments and error bars indicate standard deviation.

Effects of organic solvents on lipase activity

The stability in organic solvents is an important characteristic of lipase. It can determine whether lipase can be used to catalyze synthetic reactions and to predict which solvent would be better to perform the reaction (Lima *et al.*, 2004). Log *P* (the logarithm of the partition coefficient of a given solvent between 1-octanol and water) is commonly used to describe and predict the impact of solvent on enzymatic activity (Liu *et al.*, 2012).

From Table IV-4, *P. pastoris*/pPICZαA-*AML* lipase was stable in both water-miscible ($-2.5<\log P<1$) and water-immiscible organic solvents ($1<\log P<4$). Lipase activity increased after incubation for 24 h in 10% v/v of acetone, DMSO, *p*-xylene and octanol with a residual activity of 121.6%, 118.3%, 117.2% and 103.9%, respectively. The addition of propanol and butanol strongly inhibited the lipase activity. Like previous studies, lipase from *Serratia marcescens* ECU1010 displayed high stability in many water-miscible and water-immiscible organic solvents (Zhao, Xu, *et al.*, 2008). However, lipase activity of some microorganisms decreased with the decreasing of the log *P* of organic solvents. Lipase from *Penicillium aurantiogriseum* (Lima *et al.*, 2004), *Staphylococcus saprophyticus* M36 (Fang *et al.*, 2006) and *C. antarctica* ZJB09193 (Liu *et al.*, 2012) showed a good stability only in water-immiscible organic solvents.

The use of organic solvents in reaction could increase solubility of non-polar substrates and products, which markedly speeds up overall reaction rates. Stability and activity of lipase in organic solvents depend not only on the properties and concentration of the organic solvents, but also on the structure of the enzymes (Torres and Castro, 2004). Lipase is activated by organic solvents, which keep the lid of lipase in the open conformation, facilitating the access of the substrate to the active site of lipase (Su *et al.*, 2015). The differences between lipase activities in different solvents may be due to direct interactions between the solvent molecules and enzyme molecules. Some solvents can induce the opening of the lid of lipase. Some solvents can act as a competitive inhibitor in the reaction catalyzed (Adlercreutz, 2013).

According to this results, propanol and butanol seem to be the competitive inhibitors for lipase from *P. pastoris*/pPICZαA-*AML*.

Table IV 4 Effects of various organic solvents on lipase activity of recombinant lipase produced from *P. pastoris*/pPICZαA-*AML*

Organic solvents	Log *P*[*]	Relative activity (%)[**]
Control	-	100 ± 0.6
DMSO	-1.35	118.3 ± 8.6
Methanol	-0.77	94.7 ± 0.3
Acetonitrile	-0.34	90.6 ± 6.5
Ethanol	-0.31	84.2 ± 4.6
Acetone	-0.24	121.6 ± 4.4
Isopropanol	0.05	62.6 ± 4.6
Propanol	0.25	9.6 ± 0.4
Butanol	0.88	4.0 ± 0.4
Chloroform	2.0	86.8 ± 1.1
Octanol	2.9	103.9 ± 0.9
p-Xylene	3.1	117.2 ± 4.3
Hexane	3.9	78.9 ± 4.2

[*] Log *P* value is the logarithm of partition coefficient of an organic solvent between 1-octanol and water phases. In this study, the organic solvents were used at concentration of 10% v/v.

[**] Relative lipase activity was measured by *p*NPL assay at 37 °C for 10 min. Data are means of three independent experiments and error bars indicate standard deviation.

Part II Cloning and expression of *Fusarium solani* lipase in *Pichia pastoris*

The lipase gene of *F. solani* NAN103 has an open reading frame of 999 bp. It encodes 333 amino acid residues of a protein with a putative signal peptide of 16 amino acids (Appendix A, Figure A-1). *FSL* gene lacking signal peptide sequence was amplified and transformed into *P. pastoris*. To compare inducible and constitutive expression in *P. pastoris*, the vectors pPICZαA and pGAPZαA were used for cloning, respectively. The results showed that *P. pastoris*/pPICZαA-*FSL* induced by 2% v/v methanol had the highest specific lipase activity of 16.5 ± 1.2 U/mg after 5 days (Figure IV-8a), while *P. pastoris*/pGAPZαA-*FSL* produced the highest specific lipase activity, 18.8 U/mg, after 3 days (Figure IV-8b). The constitutive expression of *FSL* was higher than that obtained from inducible expression by 1.2-fold. This is possible that *P. pastoris*/pGAPZαA-*FSL* may have a higher gene copy number than the other transformant given that it could grow on YPDS containing high antibiotic concentrations (1000 μg/ml Zeocin), whereas *P. pastoris*/pPICZαA-*FSL* could grow on YPDS agar plates containing 100 μg/ml.

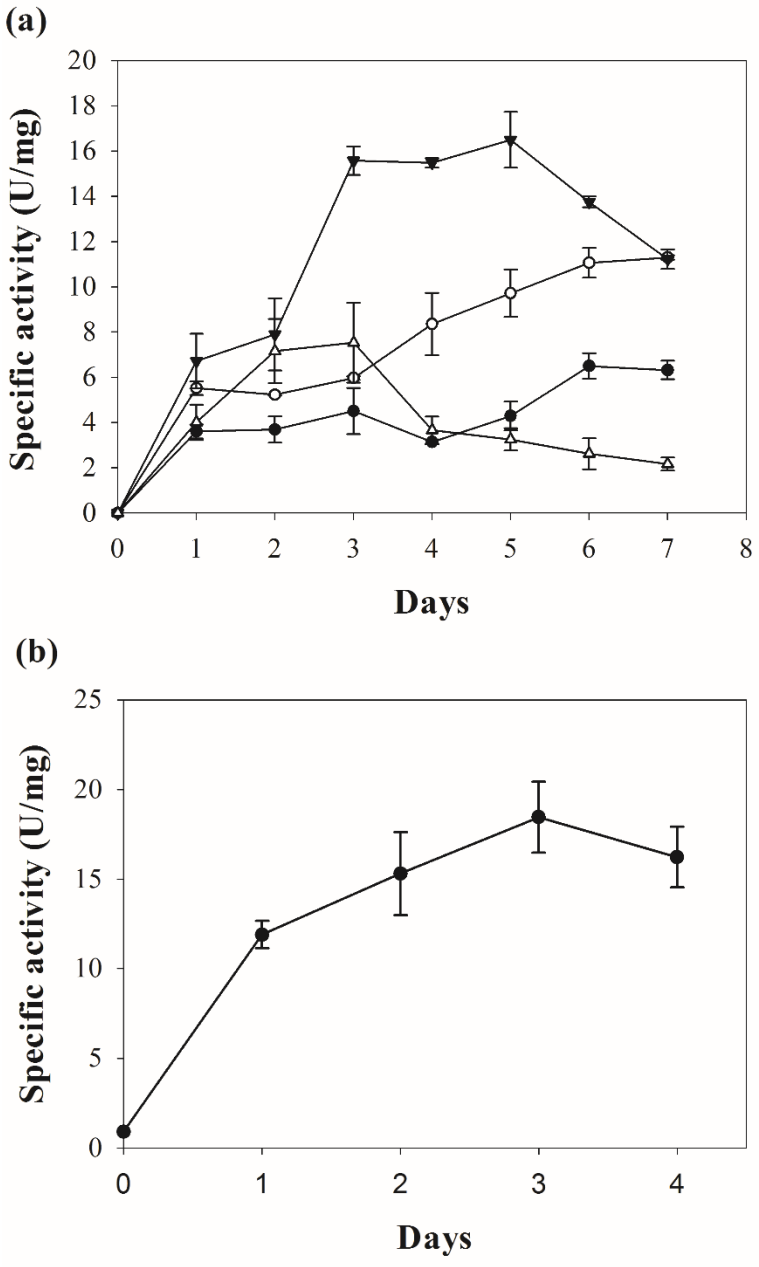

Figure IV-8 Specific activity of (a) *P. pastoris*/pPICZαA-*FSL* cultured in 50 ml of BMMY in a 500-ml baffled flask at 30°C and 250 rpm. Initially, 0.5% v/v methanol was added. Thereafter, methanol at 0.5% v/v (●), 1% v/v (○), 2% v/v (▼) and 3% v/v (△) was added every day. (b) *P. pastoris*/pGAPZαA-*FSL* was cultured in 50 ml of YPD in a 500-ml baffled flask at 30°C, 250 rpm. Data are means of three independent experiments and error bars indicate standard deviation.

The expression of recombinant *P. pastoris* was higher than that observed in wild-type *F. solani* NAN103, by approximately 190-fold for inducible expression and 216-fold for constitutive expression (Table IV-5). The *FSL* gene has been produced via other heterologous expression systems, including the *S. cerevisiae* expression system. *N. haematococca* (anamorph *F. solani* f. sp. *pisi*) was cloned into three different strains of *S. cerevisiae*. They produced lipase activities of 1.2–1.5 U/ml when *p*-NPP was used in the lipase assay (Eddine *et al.*, 2001). The activity of the recombinant lipase produced by *P. pastoris*/pGAPZαA-*FSL* seems to be close to that of the recombinant lipase produced from *S. cerevisiae*. Although both p*AOX1* and p*GAP* are strong promoters that can drive the expression of a target gene, resulting in the production of large amounts of target protein, constitutive expression systems are more suitable for a large-scale production because the use of methanol causes environmental pollution (Zhang *et al.*, 2009). The yield of *F. solani* lipase from the constitutive expression system was higher than that from the induced expression system, and it required a shorter incubation time. Therefore, *P. pastoris*/pGAPZαA-*FSL* and the enzyme it produced were selected for further characterisation and application studies.

Table IV-5 Lipase activity of recombinant *P. pastoris* and wild type *F. solani*

Source of lipase	Cultivation time (days)	Lipase activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)
P. pastoris/pPICZαA-*FSL*	5	2.8 ± 0.2	0.17 ± 0.0	16.5 ± 1.2
P. pastoris/pGAPZαA-*FSL*	3	3.0 ± 0.5	0.16 ± 0.0	18.8 ± 2.0
P. pastoris X-33*	3	0.03 ± 0.0	0.10 ± 0.0	0.30 ± 0.0
F. solani NAN103**	3	3.2 ± 0.1	36.3 ± 0.7	0.087 ± 0.0

One unit of lipase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min under the assay conditions.

**P. pastoris* X-33 without the *FSL* gene was cultured in YPD as a control.

******Malilas (2010) reported the lipase activity of *F. solani* NAN103 cultured in lipase production medium containing (in g/l) soybean flour, 75; glucose, 20; KH_2PO_4, 1; $NaNO_3$, 1; $MgSO_4$, 0.5 and 10 ml/l of palm oil with pH 7.0 at 30°C, 200 rpm for 3 days using *p*-NPP as a substrate for lipase assay.

SDS-PAGE analysis showed that the recombinant lipase produced in the supernatant of *P. pastoris*/pGAPZαA-*FSL* exhibited one specific band with a molecular mass of approximately 30 kDa (Figure IV-9). This is similar to that from a previous study; Jallouli *et al.* (2012) reported that the purified lipase from *F. solani* isolated from the forest wood in the north of Tunisia had a molecular mass of 30 kDa.

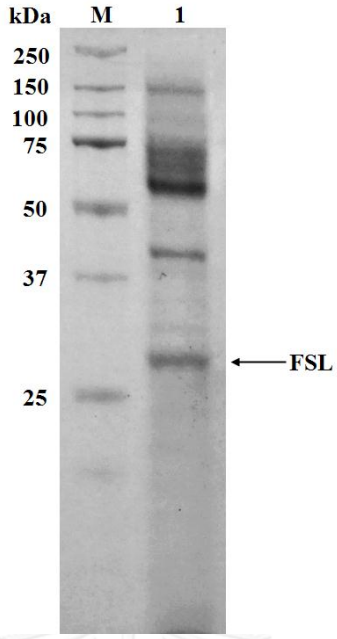

Figure IV-9 SDS-PAGE (12% w/v acrylamide) analysis of the recombinant lipase produced from *P. pastoris*/pGAPZαA-*FSL*. Lane M: molecular mass marker proteins; Lane 1: concentrated crude lipase.

Effects of pH and temperature on lipase activity and stability

As seen in Figure IV-10a, the crude lipase from *P. pastoris*/pGAPZαA-*FSL* exhibited optimal lipase activity at pH 7.0. The enzyme was stable within a pH range of 6.0–7.0 and retained at least 80% activity after 24 h of incubation. At pH 8.0–9.0, the enzyme showed approximately 50% activity. In contrast, Jallouli *et al.* (2012) reported that purified lipase from *F. solani* showed optimal lipase activity at pH 8.5–9 and was most stable at pH 9.0. The optimum temperature of the enzyme was 35°C. The lipase activity decreased rapidly above 40°C. In fact, most fungal lipases are not stable above 40°C (Lima *et al.* 2004). Above 40°C, the recombinant lipase was less active, which was similar to the result obtained with purified lipase from *F. solani* (Jallouli *et al.*, 2012). With respect to the enzyme's thermostability, the residual lipase

activities remaining after incubation at 20°C and 25°C for 30 min in phosphate buffer (pH 7.0) were 96% and 81%, respectively. Significant losses of lipase activity were observed when the enzyme was incubated for 30 min at temperatures above 25°C, as shown in Figure IV-10b.

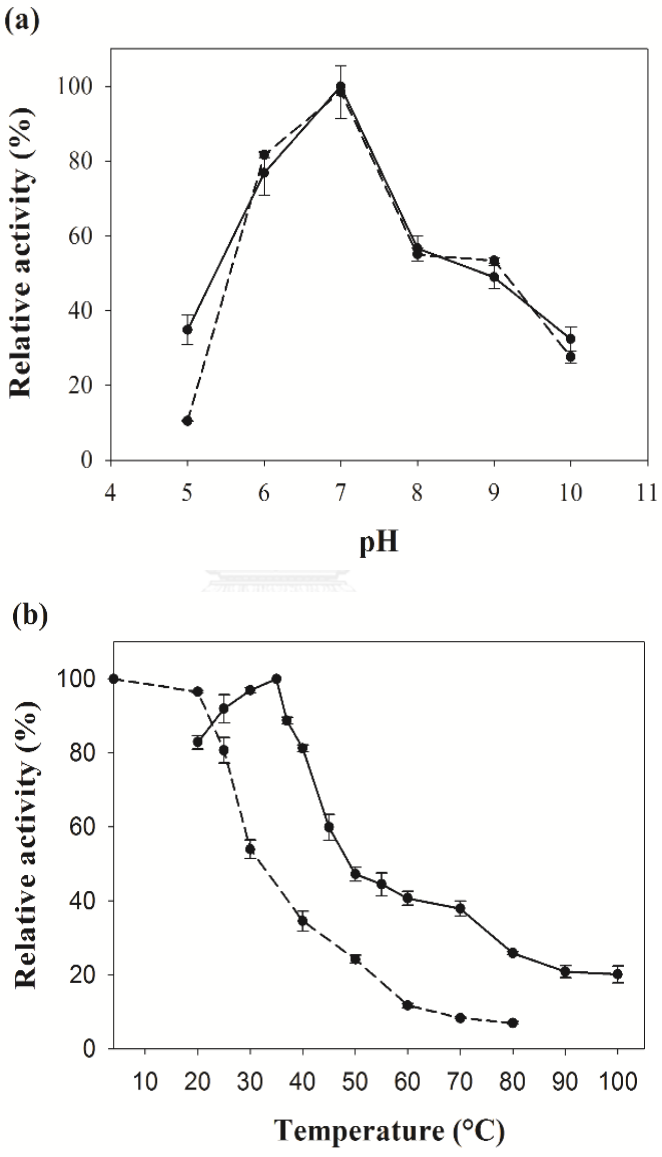

Figure IV-10 Effect of pH (a) and temperature (b) on lipase activity (solid line) and stability (dashed line) of the recombinant lipase produced from *P. pastoris*/pGAPZαA-*FSL*. Data are means of three independent experiments and error bars indicate standard deviation.

Substrate specificity of lipase

The substrate specificity of the crude lipase towards various *p*-nitrophenyl esters was investigated (Figure IV-11). The relative activity was above 70% with carbon chain lengths from C4 to C14 and displayed the highest lipase activity with *p*-NPL (C12) but declined to 50% with *p*-NPP (C16). The recombinant lipase produced from *P. pastoris*/pGAPZαA-*FSL* had wide substrate specificity. Lipases are different from esterases in terms of their substrate preferences. Preferred lipase substrates have acyl chain lengths of more than 10 carbon atoms (Peng *et al.*, 2014). The recombinant lipase from *P. pastoris*/pGAPZαA-*FSL* could hydrolyse *p*-nitrophenyl esters containing more than 10 carbon atoms; therefore, it could be concluded that this recombinant lipase could be classified as a true lipase.

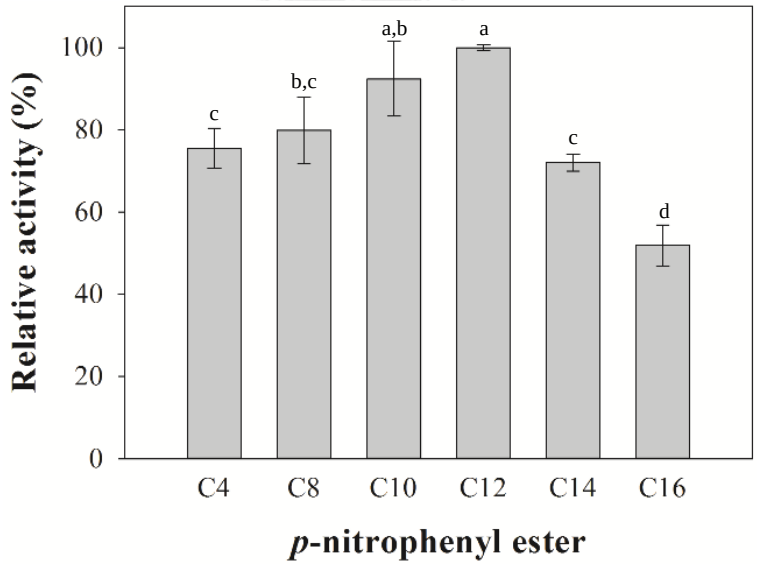

Figure IV-11 Substrate specificity of recombinant lipase produced from *P. pastoris*/pGAPZαA-*FSL* towards *p*-nitrophenyl esters. Data are means of three independent experiments and error bars indicate standard deviation. The same letters above bars indicate that the lipase activity was not significantly different ($P > 0.05$).

Effects of metal ions on lipase activity

Various metal ions had different effects on lipase activity, as shown in Table IV-6. Mn^{2+}, Ba^{2+}, Li^{+}, Ca^{2+} and Ni^{2+} (1 mM) stimulated lipase activity, whereas Ag^{+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+} and Fe^{3+} ions decreased lipase activity. The addition of Ni^{2+} result increased the relative activity by 90%. The presence of Hg^{2+} and Ag^{+} strongly inhibited lipase activity same as the result of *P. pastoris*/pPICZαA-*AML* lipase. The lipase activity was greatly inhibited by high concentrations (5 mM) of most of the metal ions, except that Li^{+} and Ni^{2+} were found to enhance lipase activity. Each metal ion might have affinity to different amino acid residues of lipase resulting in change of the lipase conformation which could stimulate or inhibit the activity of lipase. The activity of recombinant lipase decreased in the presence of Hg^{2+}, as was observed with the lipases from *Mucor sp.* (Abbas *et al.*, 2002), *Penicillium aurantiogriseum* (Lima *et al.*, 2004) and *A. pullulans* (Liu, Chi, *et al.*, 2008). Hg^{2+} may affect enzyme conformation because the thiol group in sulfur-containing amino acid residues may have a high affinity to Hg^{2+} (Liu, Chi, *et al.*, 2008). The increased lipase activity in the presence of Ca^{2+} (1 mM) is somewhat similar to that of purified *F. solani* lipase, which requires Ca^{2+} to stimulate its lipase activity (Jallouli *et al.*, 2012). Many lipases possess a calcium-binding motif around the catalytic site. Therefore, calcium ions might remove the fatty acids as insoluble salts and stabilize the enzyme (Fickers *et al.*, 2011). In addition, Li^{+} and Ni^{2+} at 1 mM resulted in enhancing lipase activity, similar to a lipase from *C. antarctica* ZJB09193 (Liu *et al.*, 2012).

Table IV-6 Effects of various metal ions on the lipase activity of recombinant lipase produced from *P. pastoris*/pGAPZαA-*FSL*

Metal ions	Relative activity (%)	
	1 mM	5 mM
Control	100.0 ± 0.0	100.0 ± 0.0
$HgCl_2$	0.0 ± 0.0	0.0 ± 0.0
$AgNO_3$	19.9 ± 2.2	4.0 ± 1.1
$ZnSO_4$	46.9 ± 6.5	41.7 ± 2.6
$FeSO_4$	54.4 ± 5.6	29.9 ± 1.8
$CoCl_2$	65.6 ± 1.1	57.0 ± 5.7
$CuSO_4$	73.3 ± 0.3	25.6 ± 3.5
$FeCl_3$	84.9 ± 3.8	54.2 ± 0.8
$MgSO_4$	109.9 ± 5.8	79.7 ± 3.0
$MnSO_4$	126.5 ± 0.8	72.1 ± 3.4
$BaCl_2$	142.3 ± 8.6	62.0 ± 1.7
LiCl	158.6 ± 3.3	116.4 ± 3.6
$CaCl_2$	181.3 ± 5.6	102.4 ± 4.5
$NiCl_2$	190.6 ± 1.7	183.7 ± 6.0

Relative lipase activity was measured by *p*-NPL assay at 35°C for 10 min. Data are means of three independent experiments and error bars indicate standard deviation.

Effects of detergents and inhibitors on lipase activity

Lipase activity was strongly inhibited by SDS. A low concentration of CTAB showed greater inhibitory effect than a high concentration. The addition of CHAPS (1 mM) and Triton X-100 (0.1% v/v) activated lipase activity, resulting in relative increases of 87% and 31% activity, respectively. At a high concentration of DTT, Tween 20 and Triton X-100 enhanced lipase activity, whereas inhibitors such as PMSF, CHAPS and EDTA reduced activity by 42%, 40% and 11%, respectively (Table IV-7). The recombinant lipase from *P. pastoris*/pGAPZαA-*FSL* appears to be

the most active in the buffer containing 1 mM of CHAPS or 1% v/v of Triton X-100. Therefore, the recombinant lipase could be potentially applied using CHAPS and Triton X-100 as surfactants. This result suggests that the structure of lipase from *P. pastoris*/pGAPZαA-*FSL* was not affected by non-ionic detergents (Tween 20, and Triton X-100) which was different from lipase obtained from *P. pastoris*/pPICZαA-*AML*. It might be due to the difference of lipase structure which had an effect on the different interaction with detergents. Similar effects were reported by Prazeres *et al.* (2006) in the case of the lipase from *F. oxysporum*. This enzyme was reported to be stimulated by Triton X-100 and Triton X-114 and strongly inhibited by SDS.

Table IV-7 Effects of various detergents and inhibitors on the lipase activity of recombinant lipase produced from *P. pastoris*/pGAPZαA-*FSL*

Detergents and inhibitors	Low concentration	Relative activity (%)	High concentration	Relative activity (%)
Control		100.0 ± 0.0		100.0 ± 0.0
Inhibitions				
DTT	1 mM	45.3 ± 3.9	5 mM	123.3 ± 12.8
PMSF	1 mM	82.7 ± 4.2	5 mM	57.9 ± 2.6
Detergents				
SDS	1 mM	25.0 ± 3.6	5 mM	16.2 ± 1.6
CTAB	1 mM	26.4 ± 2.6	5 mM	76.8 ± 2.6
EDTA	1 mM	93.6 ± 0.9	5 mM	88.7 ± 6.0
CHAPS	1 mM	186.5 ± 4.6	5 mM	59.1 ± 2.5
Tween 20	0.1% v/v	88.5 ± 2.3	1% v/v	152.4 ± 7.0
Triton X-100	0.1% v/v	131.3 ± 12.5	1% v/v	167.9 ± 11.5

Relative lipase activity was measured by *p*-NPL assay at 35°C for 10 min. Data are means of three independent experiments and error bars indicate standard deviation.

Effects of organic solvents on lipase activity

As shown in Table IV-8, *P. pastoris*/pGAPZαA-*FSL* lipase was stable in both water-miscible and water-immiscible organic solvents like *P. pastoris*/pPICZαA-*AML*. Lipase activity increased after incubation for 24 h in 10% v/v of octanol, *p*-xylene, hexane and isopropanol with a residual activity of 129.3%, 119.5%, 115.5% and 108.7%, respectively. Lipase activity was not strongly inhibited by any organic solvent except that butanol decreased lipase activity by 50%. From the results, butanol seems to be the competitive inhibitor for lipase from *P. pastoris*/pGAPZαA-*FSL*. Unlike the result of crude lipase from *F. solani* FS1, lipase activity increased by incubation in *n*-hexane and toluene but decreased by incubation with water-miscible organic solvents (Maia *et al.*, 2001).

Table IV-8 Effects of various organic solvents on lipase activity of recombinant lipase produced from *P. pastoris*/pGAPZαA-*FSL*

Organic solvents	Log *P*[*]	Relative activity (%)[**]
Control	-	100 ± 0.0
DMSO	-1.35	78.2 ± 2.0
Methanol	-0.77	95.7 ± 9.6
Acetonitrile	-0.34	98.7 ± 9.0
Ethanol	-0.31	85.3 ± 5.1
Acetone	-0.24	84.3 ± 5.9
Isopropanol	0.05	108.7 ± 4.8
Propanol	0.25	96.0 ± 4.2
Butanol	0.88	50.9 ± 2.9
Chloroform	2.0	94.1 ± 2.8
Octanol	2.9	129.3 ± 5.7
p-Xylene	3.1	119.5 ± 1.3
Hexane	3.9	115.5 ± 6.0

[*] Log *P* value is the logarithm of partition coefficient of an organic solvent between 1-octanol and water phases. In this study, the organic solvents were used at concentration of 10% v/v.

[**] Relative lipase activity was measured by *p*NPL assay at 35 °C for 10 min. Data are means of three independent experiments and error bars indicate standard deviation.

Part III The application of recombinant *Pichia pastoris* in lipid-containing wastewater pretreatment

From the previous result, *P. pastoris*/pGAPZαA-*FSL* showed the specific lipase activity of 18.8 U/mg which is approximately 1.9-fold higher than the specific lipase activity produced *P. pastoris*/pPICZαA-*AML* (9.9 U/mg). For this reason, the ability of *P. pastoris*/pGAPZαA-*FSL* to degrade palm oil in synthetic wastewater was tested. There was almost no difference between using 10^6 and 10^7 colony-forming units (CFU)/ml of yeast cells in the reduction of TAG content (Figure IV-12a). The TAG content decreased rapidly within 24 h of yeast cell addition. Subsequently, the TAG content decreased gradually when 10^7 CFU/ml of yeast cells were added, whereas the TAG content was rather constant after 24 h when 10^6 CFU/ml were added. The higher cell concentration resulted in a greater decrease of FFA content. When 10^7 CFU/ml of *P. pastoris*/pGAPZαA-*FSL* was added to synthetic wastewater medium, the palm oil decreased from 10 to 1.3 g/l within 72 h, which was equal to 87% degradation (Figure IV-12b).

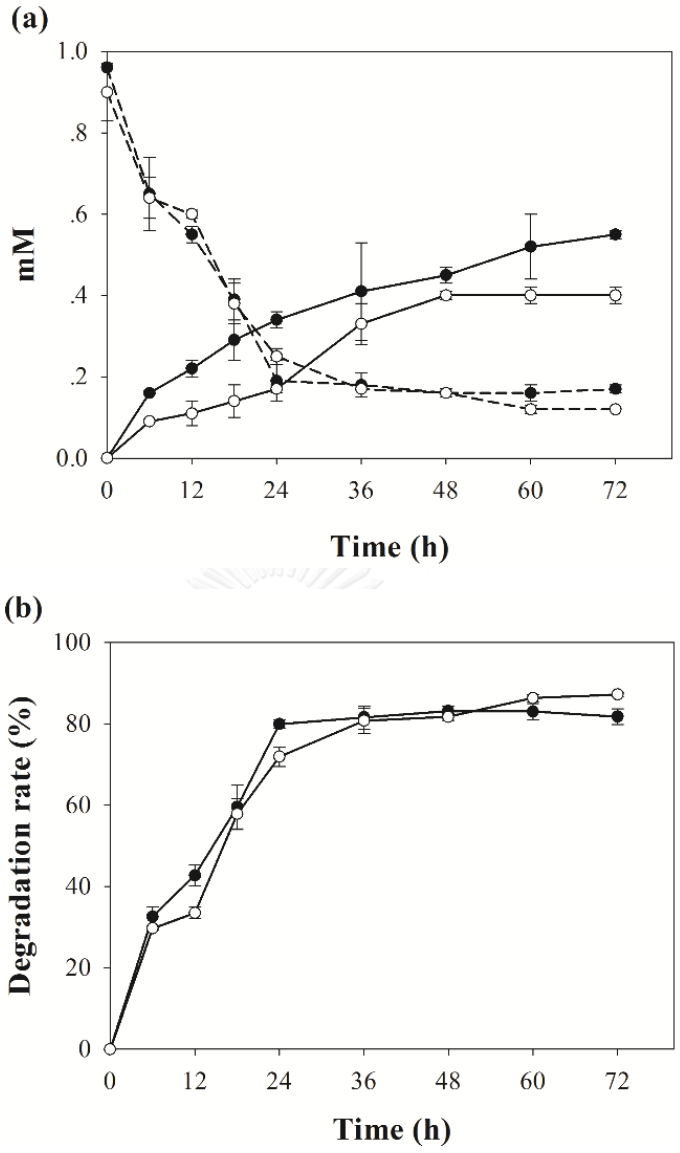

Figure IV-12 Analysis of oil degradation by *P. pastoris*/pGAPZαA-*FSL* cultured in synthetic wastewater containing 10 g/l of palm oil shaken at 150 rpm for 3 days at 30°C. (a) TAG (dashed line) and FFA (solid line) content (mM) and (b) amount degraded (%). The different concentrations of yeast cells were 10^6 CFU/ml (close circle) and 10^7 CFU/ml (open circle), which equal to OD_{600} of 0.15 and 0.9, respectively. Data are means of three independent experiments and error bars indicate standard deviation.

The use of lipase alone in lipid hydrolysis cannot remove FFA. Therefore, lipase-producing microorganisms should be used for lipid degradation because they can consume the FFA via β-oxidation. *P. pastoris*/pGAPZαA-*FSL* showed the ability to degrade lipids and reduce the concentration of FFA. Similarly, Matsumiya *et al.* (2007) reported that *Burkholderia* sp. DW2-1 grew well in synthetic wastewater medium and efficiently degraded lipids at high oil concentrations (10 g/l), reducing the lipid content by more than 90% after 48 h of cultivation. The recombinant *Pichia* grew in the synthetic wastewater medium containing palm oil and produces lipase to hydrolyse lipids to FFAs and glycerol. Although the synthetic wastewater medium has no sole carbon source such as glucose, it is possible that *P. pastoris*/pGAPZαA-*FSL* can use glycerol as a carbon source for the growth and expression of recombinant lipase under the control of p*GAP*. Glucose, glycerol, oleic acid and methanol have been used as the carbon source for the p*GAP* expression system in *P. pastoris* (Zhang *et al.*, 2009).

The ability of *P. pastoris*/pGAPZαA-*FSL* in degradation of different initial oil concentrations shown as Figure IV-13. After 24 h, the TAG content remained at 0.2 mM which there was no significant difference between the TAG content when 0.5, 0.75 and 1% w/v of initial oil concentrations added in synthetic wastewater. While the TAG content slightly decreased when synthetic wastewater contained 1.5% w/v of oil concentrations. This may be because the high concentration of palm oil at the beginning affects the growth of yeast. Within 24 h, *P. pastoris*/pGAPZαA-*FSL* cannot produce lipase enough to hydrolyse substrate resulting in high TAG content. Approximately 88% of degradation occurred within 48 h when synthetic wastewater

contained 0.5% and 0.75% w/v whereas 82% and 70% of degradation occurred when synthetic wastewater contained 1% and 1.5% w/v, respectively.

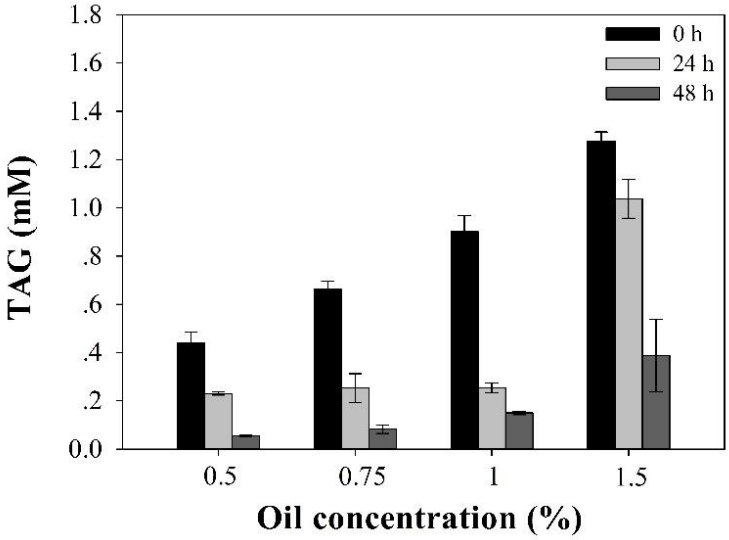

Figure IV-13 Effect of initial oil concentrations (% w/v) on TAG content using 10^7 CFU/ml of *P. pastoris*/pGAPZαA-*FSL* cultured in synthetic wastewater at 30 °C, 150 rpm for 48 h. Data are means of three independent experiments and error bars indicate standard deviation.

The kinetics of oil degradation rate of *P. pastoris*/pGAPZαA-*FSL* can be expressed by the Michaelis-Menten curve (Appendix A, Figure A-2) and equation as shown below:

$$V = \frac{V_{max}\,[S]}{K_M + [S]} \qquad (1) \qquad \text{(Berg } et\ al.\text{, 2002b)}$$

[S] is oil concentration (% w/v), V is the initial rate of degradation (TAG degraded/h/OD_{600}), V_{max} is the maximum degradation rate (TAG degraded/h/OD_{600}), t

is the degradation time (h), and K_M (Michaelis constant) is the oil concentration at which the degradation rate is half of maximal value.

Michaelis-Menten equation is transformed into one that gives a straight-line plot. Taking the reciprocal of both sides of equation (1) gives

$$\frac{1}{V} = \left(\frac{K_M}{V_{max}}\right)\left(\frac{1}{[S]}\right) + \left(\frac{1}{V_{max}}\right) \qquad (2)$$

A Lineweaver-Burk plot is generated by plotting $1/V_0$ versus 1/[S]. The slope is the K_M/V_{max}, the intercept on the Y-axis is $1/V_{max}$, and the intercept on the X-axis is $-1/K_M$ (Berg *et al.*, 2002a); therefore, K_M and V_{max} value of *P. pastoris*/pGAPZαA-*FSL* for palm oil degradation were 1% w/v and 38.4 µM/h/OD_{600} (Figure IV-14). While K_M and V_{max} value of *C. rugosa* were 1% w/v and 24.2 µM/h/OD_{600} (Tanapong, 2015). This result indicated that K_M of *P. pastoris*/pGAPZαA-*FSL* and *C. rugosa* were equal; therefore both had similar affinity for the substrate. However, *P. pastoris*/pGAPZαA-*FSL* showed a faster oil degradation rate than *C. rugose* by 1.6-fold. The recombinant *P. pastoris* seems to be an efficient microorganism for lipid degradation in lipid-containing wastewater pretreatment. It might be possible to apply this recombinant for lipid degradation in aerobic treatment system.

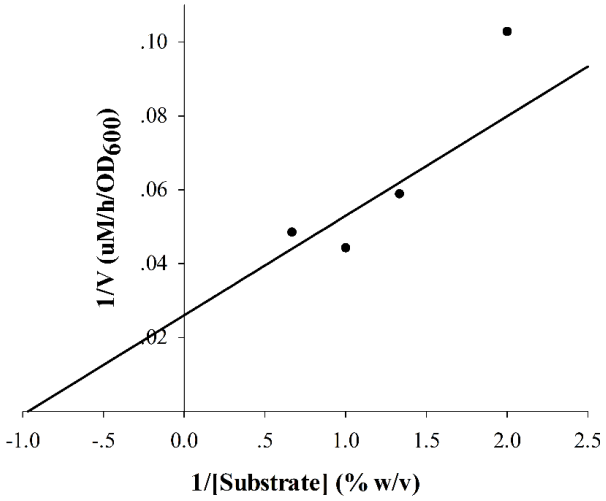

Figure IV-14 Lineweaver-Burk plot for palm oil degradation by 10^7 CFU/ml (OD_{600} = 0.9) of *P. pastoris*/pGAPZαA-*FSL*.

CHAPTER V

CONCLUSION AND FUTURE PERSPECTIVES

The full-length sequence of cDNA encoding *A. melanogenum* lipase was successfully cloned and expressed in *P. pastoris*. Recombinant lipase in an inducible expression system (*P. pastoris*/pPICZαA-*AML*) showed the highest specific lipase activity of 9.9 U/mg after 6 days of 2% v/v methanol induction. The lipase activity from inducible expression was greater than that obtained from constitutive expression and wild type *A. melanogenum* by 1.5-fold and 9-fold, respectively. The optimal lipase activity was observed at 35–37°C and pH 7.0 using *p*-nitrophenyl laurate as the substrate. Lipase activity was enhanced by Mg^{2+}, Mn^{2+}, Li^{+}, Ca^{2+} and Ni^{2+} (1 mM), DTT and EDTA (5 mM) but was inhibited by Hg^{2+}, Ag^{+}, SDS, Tween 20 and Triton X-100.

For *F. solani* lipase, the recombinant *Pichia* obtained under the control of constitutive promoter (*P. pastoris*/pGAPZαA-*FSL*) showed the highest activity of 18.8 U/mg after 3 days. The lipase activity from constitutive expression was greater than that obtained from inducible expression and wild type *F. solani* by 1.2-fold and 216-fold, respectively. The optimal lipase activity was observed at 35°C and pH 7.0 using *p*-nitrophenyl laurate as the substrate. Lipase activity was enhanced by Mn^{2+}, Ba^{2+}, Li^{+}, Ca^{2+}, Ni^{2+}, CHAPS (1 mM) and Triton X-100 (0.1% and 1% v/v) but was inhibited by Hg^{2+}, Ag^{+} and SDS. Recombinant lipases from *P. pastoris*/pPICZαA-

AML and *P. pastoris*/pGAPZαA-*FSL* were stable in both water-miscible and water-immiscible organic solvents. The addition of 10% v/v of octanol and *p*-xylene increased activity of both lipases.

P. pastoris/pGAPZαA-*FSL* showed the specific lipase activity higher than *P. pastoris*/pPICZαA-*FSL* by 1.9-fold; therefore, it was selected for application. The cultivation of *P. pastoris*/pGAPZαA-*FSL* in synthetic wastewater containing 1% w/v palm oil resulted in degradation of 87% of the oil within 72 h. K_M and V_{max} value for palm oil degradation were 1% w/v and 38.4 µM TAG degraded/h/OD_{600}, respectively. *P. pastoris*/pGAPZαA-*FSL* is expected to be useful for application in lipid-containing wastewater pretreatment. It may be possible to develop this organism into an aerobic wastewater treatment system. *P. pastoris*/pGAPZαA-*FSL* can be used as a whole-cell biocatalyst in the system without lipase separation step from yeast cell before using. It is better than the use of lipase alone in the pretreatment. The recombinant *P. pastoris* can grow and produce lipase simultaneously in wastewater. For the future research, the optimum cultivation conditions and low-cost medium should be studied to reduce the cost of lipid degradation in wastewater. In addition, lipid-containing wastewater from restaurants should be applied in the experiment to analyse other parameters. The co-cultivation of recombinant *P. pastoris* expressing lipase with other microorganisms having ability to produce other enzymes like protease amylase and cellulase is an interesting aspect for application in wastewater pretreatment in the future.

REFERENCES

Abbas, H., Hiol, A., Deyris, V., and Comeau, L. 2002. Isolation and characterization of an extracellular lipase from *Mucor* sp. strain isolated from palm fruit. *Enzyme and Microbial Technology.* 31(7): 968-975.

Adlercreutz, P. 2013. Immobilisation and application of lipases in organic media. *Chemical Society Reviews.* 42(15): 6406-6436.

Andualema, B. and Gessesse, A. 2012. Microbial lipases and their industrial applications: review. *Biotechnology.* 11(3): 100.

Berg, J. M., Tymoczko, J. L., and Stryer, L. 2002a. *Appendix: Vmax and KM Can Be Determined by Double-Reciprocal Plots*. [online]. Avaliable from: http://www.ncbi.nlm.nih.gov/books/NBK22557/. [29 May 2015]

Berg, J. M., Tymoczko, J. L., and Stryer, L. 2002b. *The Michaelis-Menten model accounts for the kinetic properties of many enzymes*. [online]. Avaliable from: http://www.ncbi.nlm.nih.gov/books/NBK22430/. [29 May 2015]

Boonvitthya, N., Tanapong, P., Kanngan, P., Burapatana, V., and Chulalaksananukul, W. 2012. Cloning and expression of the *Aspergillus oryzae* glucan 1, 3-beta-glucosidase A (*exgA*) in *Pichia pastoris*. *Biotechnology Letters.* 34(10): 1937-1943.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* 72(1): 248-254.

Brocca, S., Schmidt-Dannert, C., Lotti, M., Alberghina, L., and Schmid, R. D. 1998. Design, total synthesis, and functional overexpression of the *Candida rugosa*

lip1 gene coding for a major industrial lipase. *Protein Science: a publication of the Protein Society*. 7(6): 1415.

Brunel, L., Neugnot, V., Landucci, L., Boze, H., Moulin, G., Bigey, F., *et al.* 2004. High-level expression of *Candida parapsilosis* lipase/acyltransferase in *Pichia pastoris*. *Journal of Biotechnology*. 111(1): 41-50.

Bussamara, R., Fuentefria, A. M., de Oliveira, E. S., Broetto, L., Simcikova, M., Valente, P., *et al.* 2010. Isolation of a lipase-secreting yeast for enzyme production in a pilot-plant scale batch fermentation. *Bioresource Technology*. 101(1): 268-275.

Cammarota, M. and Freire, D. 2006. A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content. *Bioresource Technology*. 97(17): 2195-2210.

Chang, S.-W., Lee, G.-C., and Shaw, J.-F. 2006. Efficient production of active recombinant *Candida rugosa* LIP3 lipase in *Pichia pastoris* and biochemical characterization of the purified enzyme. *Journal of Agricultural and Food Chemistry*. 54(16): 5831-5838.

Chang, S., Shieh, C., Lee, G., and Shaw, J. 2005. Multiple mutagenesis of the *Candida rugosa* LIP1 gene and optimum production of recombinant LIP1 expressed in *Pichia pastoris*. *Applied Microbiology and Biotechnology*. 67(2): 215-224.

Chi, Z., Wang, F., Chi, Z., Yue, L., Liu, G., and Zhang, T. 2009. Bioproducts from *Aureobasidium pullulans*, a biotechnologically important yeast. *Applied Microbiology and Biotechnology*. 82(5): 793-804.

Clontech. 2003. *BD SMART™ RACE cDNA Amplification Kit User Manual*. [online]. Avaliable from: www.bdbiosciences.com. [25 October 2011]

Çolak, A., Şişik, D., Saglam, N., Güner, S., Çanakçi, S., and Beldüz, A. O. 2005. Characterization of a thermoalkalophilic esterase from a novel thermophilic bacterium, *Anoxybacillus gonensis* G2. *Bioresource Technology.* 96(5): 625-631.

Daly, R. and Hearn, M. T. 2005. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. *Journal of Molecular Recognition.* 18(2): 119-138.

Domínguez, A., Deive, F. J., Angeles Sanromán, M., and Longo, M. A. 2010. Biodegradation and utilization of waste cooking oil by *Yarrowia lipolytica* CECT 1240. *European Journal of Lipid Science and Technology.* 112(11): 1200-1208.

Dueholm, T., Andreasen, K., and Nielsen, P. 2000. Transformation of lipids in activated sludge. *Water Science and Technology.* 43(1): 165-172.

Eddine, A. N., Hannemann, F., and Schafer, W. 2001. Cloning and expression analysis of *NhL1*, a gene encoding an extracellular lipase from the fungal pea pathogen *Nectria haematococca* MP VI (*Fusarium solani* sp. pisi) that is expressed in planta. *Molecular Genetics and Genomics.* 265: 215-224.

Fang, Y., Lu, Z., Lv, F., Bie, X., Liu, S., Ding, Z., *et al.* 2006. A newly isolated organic solvent tolerant *Staphylococcus saprophyticus* M36 produced organic solvent-stable lipase. *Current Microbiology.* 53(6): 510-515.

Feng, J., Liu, G., Selvaraj, G., Hughes, G. R., and Wei, Y. 2005. A secreted lipase encoded by *LIP1* is necessary for efficient use of saturated triglyceride lipids in *Fusarium graminearum*. *Microbiology.* 151(12): 3911-3921.

Ferrer, P., Alarcón, M., Ramón, R., Benaiges, M. D., and Valero, F. 2009. Recombinant *Candida rugosa* LIP2 expression in *Pichia pastoris* under the control of the *AOX1* promoter. *Biochemical Engineering Journal.* 46(3): 271-277.

Fickers, P., Marty, A., and Nicaud, J. M. 2011. The lipases from *Yarrowia lipolytica*: genetics, production, regulation, biochemical characterization and biotechnological applications. *Biotechnology advances.* 29(6): 632-644.

Gandhi, N. N. 1997. Applications of lipase. *Journal of the American Oil Chemists' Society.* 74(6): 621-634.

Gostin, C., Ohm, R. A., Kogej, T., Sonjak, S., Turk, M., Zajc, J., *et al.* 2014. Genome sequencing of four *Aureobasidium pullulans* varieties: biotechnological potential, stress tolerance, and description of new species. *BMC Genomics.* 15(1): 549.

Gupta, R., Gupta, N., and Rathi, P. 2004. Bacterial lipases: an overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology.* 64(6): 763-781.

Harnpicharnchai, P., Champreda, V., Sornlake, W., and Eurwilaichitr, L. 2009. A thermotolerant β-glucosidase isolated from an endophytic fungi, *Periconia* sp., with a possible use for biomass conversion to sugars. *Protein Expression and Purification.* 67(2): 61-69.

Hasan, F., Shah, A. A., and Hameed, A. 2009. Methods for detection and characterization of lipases: a comprehensive review. *Biotechnology advances.* 27(6): 782-798.

Helistö, P. and Korpela, T. 1998. Effects of detergents on activity of microbial lipases as measured by the nitrophenyl alkanoate esters method. *Enzyme and Microbial Technology*. 23(1): 113-117.

Houde, A., Kademi, A., and Leblanc, D. 2004. Lipases and their industrial applications. *Applied Biochemistry and Biotechnology*. 118(1-3): 155-170.

Invitrogen. 2010a. *EasySelect™ Pichia expression kit for expression of recombinant proteins using pPICZ and pPICZα in Pichia pastoris*. [online]. Avaliable from: http://www.lifetechnologies.com. [29 May 2015]

Invitrogen. 2010b. *pGAPZ A, B, and C pGAPZα A, B, and C: Pichia expression vectors for constitutive expression and purification of recombinant proteins*. [online]. Avaliable from: http://www.lifetechnologies.com. [29 May 2015]

Jallouli, R., Khrouf, F., Fendri, A., Mechichi, T., Gargouri, Y., and Bezzine, S. 2012. Purification and Biochemical Characterization of a Novel Alkaline (Phospho) lipase from a Newly Isolated *Fusarium solani* Strain. *Applied Biochemistry and Biotechnology*. 168(8): 2330-2343.

Jeganathan, J., Nakhla, G., and Bassi, A. 2007. Hydrolytic pretreatment of oily wastewater by immobilized lipase. *Journal of Hazardous Materials.* 145(1): 127-135.

Kademi, A., Lee, B., and Houde, A. 2003. Production of heterologous microbial lipases by yeasts. *Indian Journal of Biotechnology*. 2(3): 346-355.

Kohno, M., Enatsu, M., and Kugimiya, W. 1998. Cloning of genomic DNA of *Rhizopus niveus* lipase and expression in the yeast *Saccharomyces cerevisiae*. *Bioscience, Biotechnology, and Biochemistry.* 62(12): 2425-2427.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227(5259): 680-685.

Lan, W., Gang, G., and Jinbao, W. 2009. Biodegradation of oil wastewater by free and immobilized *Yarrowia lipolytica* W29. *Journal of Environmental Sciences.* 21(2): 237-242.

Leal, M., Cammarota, M., Freire, D., and Anna Jr, S. 2002. Hydrolytic enzymes as coadjuvants in the anaerobic treatment of dairy wastewaters. *Brazilian Journal of Chemical Engineering.* 19(2): 175-180.

Leelaruji, W., Piamtongkam, R., Chulalaksananukul, S., and Chulalaksananukul, W. 2013. Biodiesel production from *Jatropha curcas* oil catalyzed by whole cells of *Aureobasidium pullulans* var. melanogenum SRY 14-3. *African Journal of Biotechnology.* 12(27): 4380-4386.

Li, P., Anumanthan, A., Gao, X.-G., Ilangovan, K., Suzara, V. V., Düzgüneş, N., *et al.* 2007. Expression of recombinant proteins in *Pichia pastoris*. *Applied Biochemistry and Biotechnology.* 142(2): 105-124.

Lima, V., Krieger, N., Mitchell, D., and Fontana, J. 2004. Activity and stability of a crude lipase from *Penicillium aurantiogriseum* in aqueous media and organic solvents. *Biochemical Engineering Journal.* 18(1): 65-71.

Lin-Cereghino, G. P., Stark, C. M., Kim, D., Chang, J., Shaheen, N., Poerwanto, H., *et al.* 2013. The effect of α-mating factor secretion signal mutations on recombinant protein expression in *Pichia pastoris*. *Gene.* 519(2): 311-317.

Liu, Z.-Q., Zheng, X.-B., Zhang, S.-P., and Zheng, Y.-G. 2012. Cloning, expression and characterization of a lipase gene from the *Candida antarctica* ZJB09193 and its application in biosynthesis of vitamin A esters. *Microbiological Research.* 167(8): 452-460.

Liu, Z., Chi, Z., Wang, L., and Li, J. 2008. Production, purification and characterization of an extracellular lipase from *Aureobasidium pullulans* HN2-3 with potential application for the hydrolysis of edible oils. *Biochemical Engineering Journal.* 40(3): 445-451.

Liu, Z., Li, X., Chi, Z., Wang, L., Li, J., and Wang, X. 2008. Cloning, characterization and expression of the extracellular lipase gene from *Aureobasidium pullulans* HN2-3 isolated from sea saltern. *Antonie Van Leeuwenhoek.* 94(2): 245-255.

Maia, M., Heasley, A., De Morais, M. C., Melo, E., Morais, M., Ledingham, W., *et al.* 2001. Effect of culture conditions on lipase production by *Fusarium solani* in batch fermentation. *Bioresource Technology.* 76(1): 23-27.

Malilas, W. 2010. *Screening and induced mutation of lipolytic fungi to enhance hydrolytic activity*. [online]. Avaliable from: http://tar.thailis.or.th/handle/123456789/250. [1 June 2015]

Matsumiya, Y., Wakita, D., Kimura, A., Sanpa, S., and Kubo, M. 2007. Isolation and characterization of a lipid-degrading bacterium and its application to lipid-containing wastewater treatment. *Journal of Bioscience and Bioengineering.* 103(4): 325-330.

Minning, S., Schmidt-Dannert, C., and Schmid, R. D. 1998. Functional expression of *Rhizopus oryzae* lipase in *Pichia pastoris*: high-level production and some properties. *Journal of Biotechnology.* 66(2): 147-156.

Mongkolthanaruk, W. and Dharmsthiti, S. 2002. Biodegradation of lipid-rich wastewater by a mixed bacterial consortium. *International Biodeterioration & Biodegradation.* 50(2): 101-105.

Nagao, T., Shimada, Y., Sugihara, A., and Tominaga, Y. 1994. Cloning and nucleotide sequence of cDNA encoding a lipase from *Fusarium heterosporum*. *Journal of Biochemistry.* 116(3): 536-540.

Nardini, M. and Dijkstra, B. W. 1999. α/β hydrolase fold enzymes: the family keeps growing. *Current opinion in structural biology.* 9(6): 732-737.

Nordén, K., Agemark, M., Danielson, J. Å., Alexandersson, E., Kjellbom, P., and Johanson, U. 2011. Increasing gene dosage greatly enhances recombinant expression of aquaporins in *Pichia pastoris*. *BMC Biotechnology.* 11(1): 47.

Peng, Q., Wang, X., Shang, M., Huang, J., Guan, G., Li, Y., *et al.* 2014. Isolation of a novel alkaline-stable lipase from a metagenomic library and its specific application for milkfat flavor production. *Microbial Cell Factories.* 13(1).

Pfeffer, J., Richter, S., Nieveler, J., Hansen, C.-E., Rhlid, R. B., Schmid, R. D., *et al.* 2006. High yield expression of Lipase A from *Candida antarctica* in the methylotrophic yeast *Pichia pastoris* and its purification and characterisation. *Applied Microbiology and Biotechnology.* 72(5): 931-938.

Potvin, G., Ahmad, A., and Zhang, Z. 2012. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. *Biochemical Engineering Journal.* 64: 91-105.

Prasad, M. P. and Manjunath, K. 2011. Comparative study on biodegradation of lipid-rich wastewater using lipase producing bacterial species. *Indian Journal of Biotechnology.* 10: 121-124.

Prazeres, J. N. d., Cruz, J. A. B., and Pastore, G. M. 2006. Characterization of alkaline lipase from *Fusarium oxysporum* and the effect of different surfactants and detergents on the enzyme activity. *Brazilian Journal of Microbiology.* 37(4): 505-509.

Rigo, E., Rigoni, R. E., Lodea, P., Oliveira, D. d., Freire, D. M., and Luccio, M. D. 2008. Application of different lipases as pretreatment in anaerobic treatment of wastewater. *Environmental Engineering Science.* 25(9): 1243-1248.

Salah, R. B., Mosbah, H., Fendri, A., Gargouri, A., Gargouri, Y., and Mejdoub, H. 2006. Biochemical and molecular characterization of a lipase produced by *Rhizopus oryzae*. *FEMS Microbiology Letters.* 260(2): 241-248.

Sharma, R., Chisti, Y., and Banerjee, U. C. 2001. Production, purification, characterization, and applications of lipases. *Biotechnology Advances.* 19(8): 627-662.

Sharma, S. and Kanwar, S. S. 2014. Organic solvent tolerant lipases and applications. *The Scientific World Journal.* 2014.

Singh, A. K. and Mukhopadhyay, M. 2012. Overview of fungal lipase: a review. *Applied Biochemistry and Biotechnology.* 166(2): 486-520.

Su, J., Zhang, F., Sun, W., Karuppiah, V., Zhang, G., Li, Z., *et al.* 2015. A new alkaline lipase obtained from the metagenome of marine sponge *Ircinia* sp. *World Journal of Microbiology and Biotechnology*: 1-10.

Tanaka, H., Muguruma, M., and Ohta, K. 2006. Purification and properties of a family-10 xylanase from *Aureobasidium pullulans* ATCC 20524 and characterization of the encoding gene. *Applied Microbiology and Biotechnology.* 70(2): 202-211.

Tanapong, P. 2015. *Production and characterization of lipase from Candida rugosa and application in wastewater treatment*. (Unpublished raw data).

Thongekkaew, J. and Boonchird, C. 2007. Molecular cloning and functional expression of a novel extracellular lipase from the thermotolerant yeast *Candida thermophila*. *Fems Yeast Research.* 7(2): 232-243.

Torres, S. and Castro, G. R. 2004. Non-aqueous biocatalysis in homogeneous solvent systems. *Food Technology and Biotechnology.* 42(4): 271-277.

Vakhlu, J. 2006. Yeast lipases: enzyme purification, biochemical properties and gene cloning. *Electronic Journal of Biotechnology.* 9(1): 0-0.

Villeneuve, P., Muderhwa, J. M., Graille, J., and Haas, M. J. 2000. Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. *Journal of Molecular Catalysis B: Enzymatic.* 9(4): 113-148.

Vitisant, T., Leelaruji, W., Chulalaksananukul, S., Wattayakorn, G., and Chulalaksananukul, W. 2013. A high-activity lipolytic yeast isolated from Sichang Island, Thailand. *Maejo International Journal of Science and Technology.* 7: 96-105.

Wang, X., Sun, Y., Ke, F., Zhao, H., Liu, T., Xu, L., *et al.* 2012. Constitutive expression of *Yarrowia lipolytica* lipase LIP2 in *Pichia pastoris* using GAP as promoter. *Applied Biochemistry and Biotechnology.* 166(5): 1355-1367.

Willey, R. 2001. Fats, oils, and greases: the minimization and treatment of wastewaters generated from oil refining and margarine production. *Ecotoxicology and Environmental Safety.* 50(2): 127-133.

Yang, J., Sun, J., and Yan, Y. 2010. *lip*2, a novel lipase gene cloned from *Aspergillus niger* exhibits enzymatic characteristics distinct from its previously identified family member. *Biotechnology Letters.* 32(7): 951-956.

Yu, M., Lange, S., Richter, S., Tan, T., and Schmid, R. D. 2007. High-level expression of extracellular lipase Lip2 from *Yarrowia lipolytica* in *Pichia pastoris* and its purification and characterization. *Protein Expression and Purification.* 53(2): 255-263.

Yu, X.-W., Wang, L.-L., and Xu, Y. 2009. *Rhizopus chinensis* lipase: gene cloning, expression in *Pichia pastoris* and properties. *Journal of Molecular Catalysis B: Enzymatic.* 57(1): 304-311.

Zelena, K., Krügener, S., Lunkenbein, S., Zorn, H., and Berger, R. G. 2009. Functional expression of the lipase gene Lip2 of *Pleurotus sapidus* in *Escherichia coli*. *Biotechnology Letters.* 31(3): 395-401.

Zhang, A.-L., Luo, J.-X., Zhang, T.-Y., Pan, Y.-W., Tan, Y.-H., Fu, C.-Y., *et al.* 2009. Recent advances on the GAP promoter derived expression system of *Pichia pastoris*. *Molecular Biology Reports.* 36(6): 1611-1619.

Zhao, L.-L., Xu, J.-H., Zhao, J., Pan, J., and Wang, Z.-L. 2008. Biochemical properties and potential applications of an organic solvent-tolerant lipase isolated from *Serratia marcescens* ECU1010. *Process Biochemistry.* 43(6): 626-633.

Zhao, W., Wang, J., Deng, R., and Wang, X. 2008. Scale-up fermentation of recombinant *Candida rugosa* lipase expressed in *Pichia pastoris* using the GAP promoter. *Journal of Industrial Microbiology and Biotechnology.* 35(3): 189-195.

Zhu, Y., Machleder, E., Chenchik, A., Li, R., and Siebert, P. 2001. Reverse transcriptase template switching: A SMART™ approach for full-length cDNA library construction. *Biotechniques.* 30(4): 892-897.

APPENDIX

APPENDIX A

The nucleotide sequence of the cDNA lipase of *F. solani* NAN103 (Genbank accession number KT003282) shows as Figure A-1.

```
1      ATGATGCTCATCCTATCTATTCTTTCTATAATTGCCTTTACGGCAGCTGGCCCAGTGCCC
1       M  M  L  I  L  S  I  L  S  I  I  A  F  T  A  A  G  P  V  P
61     TCTGTTGATGAAAATACTCGGGTACTTGAGCATCGAGCTGTGACAGTCACGACGCAGGAT
21      S  V  D  E  N  T  R  V  L  E  H  R  A  V  T  V  T  T  Q  D
121    CTGTCAAACTTCAGGTTCTATCTCCAGCATGCTGATGCTGCGTATTGCAATTTCAATACA
41      L  S  N  F  R  F  Y  L  Q  H  A  D  A  A  Y  C  N  F  N  T
181    GCAGTTGGCAAACCAGTCTACTGCAGTGCCGGGAATTGCCCTGACATTGAAAAGGACGCT
61      A  V  G  K  P  V  Y  C  S  A  G  N  C  P  D  I  E  K  D  A
241    GCTATCGTTGTCAAATCGGTAATTGGTACAAAAACGGGCATCGGTGCCTATGTGGCAACT
81      A  I  V  V  K  S  V  I  G  T  K  T  G  I  G  A  Y  V  A  T
301    GACAACGCTCGTAAGGAGATCGTTGTCTCTGTACGTGGCAGCATCAACGTGCGAAACTGG
101     D  N  A  R  K  E  I  V  V  S  V  R  G  S  I  N  V  R  N  W
361    ATCACAAACTTCGACTTTGGTCAAAAGGCCTGCGACCTTGTTGCTGGCTGTGGCGTTCAC
121     I  T  N  F  D  F  G  Q  K  A  C  D  L  V  A  G  C  G  V  H
421    ACCGGCTTCTTGGATGCCTGGGAGGAGGTTGCAGCCAATATTAAAGCTGCTGTCACCGCA
141     T  G  F  L  D  A  W  E  E  V  A  A  N  I  K  A  A  V  T  A
481    GCGAAGGCTGCAAACCCGACTTTCAAGTTCGTCGCTACCGGACACTCCCTCGGTGGTGCC
161     A  K  A  A  N  P  T  F  K  F  V  A  T  G  H  S  L  G  G  A
541    GTTGCTACTATTGCGGCTGCGTACCTGCGCAAAGATGGCTTTCCTTTTGACCTCTATACC
181     V  A  T  I  A  A  A  Y  L  R  K  D  G  F  P  F  D  L  Y  T
601    TATGGCTCTCCAAGAGTAGGAAACGACTTCTTCGCCAACTTCGTCACACAACAGACGGGC
201     Y  G  S  P  R  V  G  N  D  F  F  A  N  F  V  T  Q  Q  T  G
661    GCTGAATATCGCGTCACACATGGTGATGACCCCGTCCCACGTCTTCCTCCTATCATCTTT
221     A  E  Y  R  V  T  H  G  D  D  P  V  P  R  L  P  P  I  I  F
721    GGATACCGCCACACTAGCCCAGAATACTGGCTTGACGGTGGCCCACTTGATAAGGACTAC
241     G  Y  R  H  T  S  P  E  Y  W  L  D  G  G  P  L  D  K  D  Y
781    ACCGTGACCGAAATCAAGGTTTGTGAGGGCATGCCGAACGTTATGTGCAATGGTGGCACG
261     T  V  T  E  I  K  V  C  E  G  M  P  N  V  M  C  N  G  G  T
841    GTAGGTCTGGACATTCTTGCGCACATCACCTATTTCCAGAGCATGGCCACTGGTGCACCA
281     V  G  L  D  I  L  A  H  I  T  Y  F  Q  S  M  A  T  G  A  P
901    ATCGCGATCCCATGGAAGCCGCACATGTCAGATGAGGAGCTGGAAAAGAAGTTGACTCGG
301     I  A  I  P  W  K  P  H  M  S  D  E  E  L  E  K  K  L  T  R
961    TATAGCGAGCTGGATCAGGAATTTGTTAAGCAGATGACTTAG
321     Y  S  E  L  D  Q  E  F  V  K  Q  M  T  *
```

Figure A-1 Nucleotide sequence of the cDNA lipase of *F. solani* NAN103 and its deduced amino acid sequence. The lower lines show the deduced amino acid with signal peptide (underlined). The conserved motifs (Gly-X-Ser-X-Gly) are boxed. The stop codon is indicated with an asterisk.

Michaelis–Menten kinetic of *P. pastoris*/pGAPZαA-*FSL* shows as Figure A-2

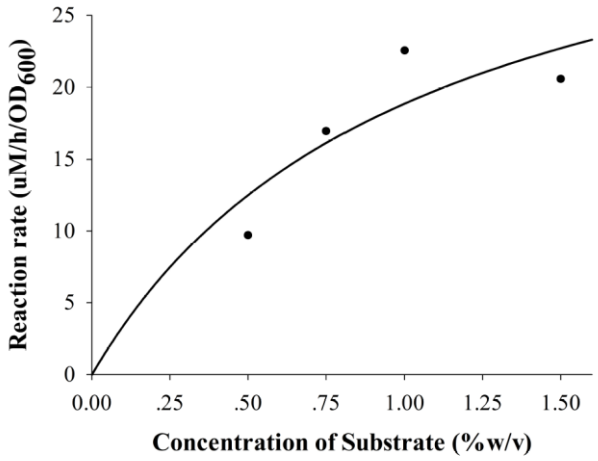

Figure A-2 Michaelis–Menten curve for the oil degradation by *P. pastoris*/pGAPZαA-*FSL*. Rate of degradation has been measured as µM of TAG degraded/h/OD_{600} and substrates are palm oil at different concentrations (% w/v).

VITA

Miss Jinaporn Wongwatanapaiboon was born on August 8th, 1984 in Bangkok, Thailand. She is a government scholarship student in the Development and Promotion of Science and Technology Talents Project (DPST). She got a Bacherlor's Degree of Science in Biology (First Class Honors) from Faculty of Science, Kasetsart University in 2005. Later in 2006, she furthered her Master's Degree of Science from Program in Biotechnology, Faculty of Science, Chulalongkorn University, at which she finished in May of 2008 with "very good" merit from her defense examination. Then, she continues studied in Program in Biotechnology, the same place, with financial support from DPST to do a research for one year under co-supervisor of Professor Alain Marty at Laboratory of Biological system engineering and Bioprocess (LISBP), Institut National des Sciences Appliquées (INSA), Toulouse, France.

Outcome from this study

2 publications:

1. Wongwatanapaiboon, J., Malilas, W., Lekhapan, P., Chanhom, T., Ruangchainikom, C., Thummadetsak, G., Marty, A., and Chulalaksananukul, W. 2015. Overexpression of Fusarium solani lipase in Pichia pastoris and its application in lipid-containing wastewater pretreatment. (submitted in Journal of Applied Microbiology)

2. Wongwatanapaiboon, J., Klinbunga, S., Ruangchainikom, C., Thummadetsak, G., Marty, A., and Chulalaksananukul, W. 2015. Cloning and expression of Aureobasidium melanogenum lipase in Pichia pastoris. (submitted in Biotechnology Letters)

3 patents (Thailand):

1. Patent no. 1401001488 (20 March 2014)

2. Patent no. 1401001489 (20 March 2014)

3. summited in Chulalongkorn University Intellectual Property Institute (29 June 2015)