PRODUCTION, CLONING AND CHARACTERIZATION OF THERMOSTABLE LIPASE FROM *Geobacillus thermodenitrificans* IBRL-nra

ANURADHA BALAN

UNIVERSITI SAINS MALAYSIA

2015

PRODUCTION, CLONING AND CHARACTERIZATION OF THERMOSTABLE LIPASE FROM *Geobacillus thermodenitrificans* IBRL-nra

by

ANURADHA BALAN

Thesis submitted in fulfilment of the requirements

for the Degree of

Doctor of Philosophy

April 2015

ACKNOWLEDGEMENT

First and foremost I would like extend my gratitude to my supervisor, Prof. Darah Ibrahim. Thank you for all the guidance, motivation and moral support given to me throughout my studies. I also appreciate the trust you had in me and giving me my own space and time.

Dr. Rashidah, you too were a wonderful person. Thank you for all the guidance, inspiration and motivations. Thank you for being a good friend and you always encouraged me to think outside the box.

Besides my supervisors, I would like to extend my deepest gratitude to Prof. Razip. Thank you for all the knowledge and ideas you have shared with me. It helped me to solve the problems I faced in my research in some ways.

To all my friends and colleagues at IBRL, Lab 406 and Lab 414, you all are amazing and helpful friends. Thanks for sharing all the skills and knowledge with me.

My appreciation also goes to all the staff of School of Biological Sciences especially staff of Microbiology and Biotechnology laboratories for all the technical supports. Special thanks to USM for the financial assistance awarded to me.

My deepest and heartfelt gratitude to my beloved parents, Mr. Balan and Madam Vilasany and my siblings for all the prayers, motivation and moral support. Thanks for having faith in me. My appreciation to my husband Mr. Ragubalan for all his contribution and endless support. Millions thanks for your understanding. Not forgetting my two little angels, Nelesh and Thanieshka, they always cheered me up when I was down. They are worth all the struggles I went through and the reason for me to bounce back. Above all, my appreciation and gratitude to God, for all His blessings and mercy upon me to complete my studies.

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS	iii
LIST OF TABLES	xvi
LIST OF FIGURES	xviii
LIST OF PLATES	xxii
LIST OF SYMBOLS AND ABBREVIATIONS	xxv
ABSTRAK	xxvii
ABSTRACT	xxix
CHAPTER 1 - INTRODUCTION	
1.0 Introduction	1
1.1 Rational and Research Objectives	3
CHAPTER 2 - LITERATURE REVIEW	
2.1 Lipases	5

2.1.1 Sources of Lipases 5

2.1.2 Structure of Lipases 7

2.1.3 Classification of Lipases 10

2.1.4 Lipase-catalyzed reactions 10

2.1.5 Catalytic mechanism of lipases 11

2.2 Applications of lipase 14

2.2.1 Lipase in detergent industry 16

2.2.2 Lipase in food and dairy processing industry 17

2.2.3 Lipase in fats and oils processing industry 18

2.2.4 Lipase in pulp and paper industry 19

2.2.5 Lipase in pharmaceuticals 19

2.2.6 Lipase as biosensors 20

2.2.7 Lipase in biodiesel production 21

2.3 Production of lipases via submerged fermentation 22

2.3.1 Production of lipases in shake flasks system 22

2.3.2 Production of lipases in bioreactor system 23

2.4 Recombinant DNA technology and overexpression of recombinant protein 26

in *E. coli*

2.4.1 Cloning and overexpression of thermostable lipase genes 27

2.5 Purification and characterization of thermostable lipases 28

2.6 Protein crystallization 34

2.6.1 Crystallization of thermostable lipase 38

2.6.2 X-ray crystallography of thermostable lipase 40

(Three-dimensional structures of thermostable lipases)

2.7 Extremophiles 42

2.7.1 Thermophiles 44

2.7.1.1 Molecular adaption of thermophiles 45

2.7.1.2 Thermophiles as the thermostable enzyme sources 45

2.7.1.3 *Geobacillus thermodenitrificans* 46

2.8 Thermostable Lipases 48

CHAPTER 3 - GENERAL METHODOLOGY

3.1 Microorganisms and culture maintenance 50

3.2 Preparation of medium 50

3.2.1 Nutrient agar 50

3.2.2 Nutrient broth 50

3.2.3 Luria Bertani (LB) broth 51

3.2.4 Luria Bertani agar + ampicillin 51

3.2.5 Luria Bertani agar (LA) + ampicillin + X-gal + IPTG 51

3.3 Preparations of lipase screening medium 51

3.3.1 Preparations of nutrient agar plates supplemented with tributyrin, olive oil and Rhodamine B-olive oil agar plants 51

3.3.2 Preparations of Luria Bertani (LB) agar plates supplemented with tributyrin, olive oil and Rhodamine B-olive oil agar plants 52

3.4 Determination of lipase activity 52

3.5 Determination of protein content 53

3.6 Determination of the growth of the microorganism 54

3.7 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 55

3.7.1 Coomassie blue staining 57

3.7.2 Silver staining 57

CHAPTER 4 - PRODUCTION OF THERMOSTABLE LIPASE BY *Geobacillus thermodenitrificans* IBRL-nra

4.1 INTRODUCTION 59

4.2 MATERIALS AND METHODS

4.2.1 Growth temperature of *G. thermodenitrificans* IBRL-nra 61

4.2.2 Screening of thermostable lipase activity by *G. thermodenitrificans* IBRL-nra on agar plates 61

4.2.3 Production of thermostable lipase by *G. thermodenitrificans* IBRL-nra in a shake flask system 62

4.2.4 Production of thermostable lipase by *G. thermodenitrificans* IBRL-nra in a stirred tank bioreactor 62

4.2.5 Enhancement of fermentation parameters in stirred tank bioreactors for thermostable lipase production by *G. thermodenitrificans* IBRL-nra 63

4.3 RESULTS AND DISCUSSION

4.3.1 Growth temperature of *G. thermodenitrificans* IBRL-nra 64

4.3.2 Screening of thermostable lipase activity by *G. thermodenitrificans* IBRL-nra on agar plates 64

4.3.3 Production of thermostable lipase by *G. thermodenitrificans* IBRL-nra in a shake flask system 68

4.3.4 Intial time-course for the production of themostable lipase by *G. thermodenitrificans* IBRL-nra in a stirred tank bioreactor 70

4.3.5 Enhancement of thermostable lipase production by *G. thermodenitrificans* IBRL-nra in a stirred tank bioreactor 72

4.3.5.1 Effect of agitation rate on the production of thermostable lipase by *G. thermodenitrificans* IBRL-nra 72

4.3.5.2 Effect of aeration rate on the production of thermostable lipase by *G. thermodenitrificans* IBRL-nra 76

4.3.5.3 Effect of inoculum size on the production of themostable lipase by *G. thermodenitrificans* IBRL-nra 78

4.3.6 Final time-course profile for the production of thermostable lipase by *G. thermodenitrificans* IBRL-nra in a stirred tank bioreactor 79

4.4 CONCLUSION 81

CHAPTER 5 - PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR THERMOSTABLE LIPASE FROM *Geobacillus thermodenitrificans* IBRL-nra

5.1 INTRODUCTION 82

5.2 MATERIALS AND METHODS

5.2.1 Purification of thermostable lipase 83

5.2.1.1 Ultrafiltration 83

5.2.1.2 Affinity chromatography 84

5.2.1.3 Gel-filtration chromatography 84

5.2.1.4 Sodium Dodecyl Sulfate-Polyarcylamide Gel Electrophoresis (SDS-PAGE) 85

5.2.1.5 Determination of molecular weight of the purified thermostable lipase 85

5.2.2 Characterization of purified thermostable lipase 86

5.2.2.1 Effect of temperature on purified enzyme activity and stability 86

5.2.2.2 Effect of pH on purified enzyme activity and stability 87

5.2.2.3 Effect of effector molecules on purified enzyme activity 87

5.2.2.4 Substrate specificity of purified lipase 88

5.2.2.5 Effect of organic solvent on enzyme activity 88

5.2.2.6 Effect of surfactants on enzyme activity 89

5.3 RESULTS AND DISCUSSION

5.3.1 Purification of thermostable lipase from *G. thermodenitrificans* IBRL-nra 90

5.3.2 Effect of temperature on thermostable lipase activity and stability 96

5.3.3 Effect of pH on thermostable lipase activity and stability 100

5.3.4 Effect of effector molecules on thermostable lipase activity 103

5.3.5 Substrate specificity of thermostable lipase 107

5.3.6 Effect of organic solvent on thermostable lipase activity 112

5.3.7 Effect of surfactants on thermostable lipase activity 116

5.4 CONCLUSION 119

CHAPTER 6 – CLONING AND OVEREXPRESSION OF THERMOSTABLE LIPASE GENE FROM *Geobacilius thermodenitrificans* IBRL-nra

6.1 INTRODUCTION 120

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strains and plasmids 122

6.2.2 Culture preparation of *G. thermodenitrificans* IBRL-nra 122

6.2.3 Genomic DNA extraction 122

6.2.4 Cloning of thermostable lipase gene from *G. thermodenitrificans* IBRL-nra 122

6.2.4.1 PCR amplication of thermostable lipase gene 122

6.2.4.2 Ligation of purified LipGt with pGEM®-T easy cloning vector 124

6.2.4.3 Transformation of pGEM-LipGt into *E. coli* JM 109 124

6.2.4.3.1 Preparation of competent cells, *E. coli* JM 109 by using TSS method 124

6.2.4.3.2 Transformation of competent cells with recombinant plasmid 127

6.2.5 Colony PCR 128

6.2.6 Extraction of plasmid 128

6.2.7 Digestion of plasmid harbouring LipGt gene 128

6.2.8 Sequence analysis 130

6.2.9 Subcloning of thermostable lipase gene, LipGt into expression host cell *E. coli* BL21 (DE3) 130

6.2.10 Subcloning of thermostable lipase gene, LipGt into expression host cell OverExpress C43 (DE3) pLysS 132

6.2.11 Expression of the thermostable lipase gene 133

6.2.12 SDS-PAGE analysis 134

6.2.13 Effect of temperature, concentration of IPTG and the induction period on the expression of thermostable lipase in OverExpress C43 (DE3) pLysS 134

6.3 RESULTS AND DISCUSSION

6.3.1 Cloning of thermostable lipase gene 135

6.3.2 Analysis of sequence of LipGt 139

6.3.3 Overexpression of thermostable lipase gene, LipGt in 143

E. coli expression system

6.3.4 Effect of temperature, concentration of IPTG and time-course analysis on the expression of thermostable lipase in OverExpress C43 (DE3) pLysS 154

6.4 CONCLUSION 159

CHAPTER 7 – PURIFICATION, CHARACTERIZATION AND PRELIMINARY CRYSTALLIZATION OF RECOMBINANT THERMOSTABLE LIPASE FROM *Geobacillus thermodenitrificans* IBRL-nra

7.1 INTRODUCTION

7.1.1 Purification of thermostable lipase LipGt 160

7.1.2 Protein crystallization 161

7.2 MATERIALS AND METHODS

7.2.1 Purification of thermostable lipase LipGt 162

7.2.2 Characterization of thermostable lipase LipGt 163

7.2.2.1 Effect of temperature on purified thermostable lipase LipGt activity and stability 163

7.2.2.2 Effect of pH on purified thermostable lipase 164

LipGt activity and stability

7.2.2.3 Effect of organic solvent on thermostable lipase LipGt activity 164

7.2.3 Preliminary crystallization trial of thermostable lipase LipGt 165

7.2.3.1 Crystal Screen Cryo kit HR2-121 and HR2-122 (Hampton Research) 165

7.2.3.2 Hanging Drop Vapor Diffusion method 165

7.2.3.3 Microbatch method 166

7.2.4 Enhancement of the Crystallization Parameters 166

7.3 RESULTS AND DISCUSSION

7.3.1 Purification of thermostable lipase LipGt 167

7.3.2 Characterization of thermostable lipase LipGt 172

7.3.3 Screening of thermostable lipase LipGt for crystal formation 179

7.3.4 Enhancement of the crystallization parameters 182

7.4 CONCLUSION 185

CHAPTER 8 – GENERAL CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

8.1 GENERAL CONCLUSION 187

8.2 RECOMMENDATIONS FOR FUTURE RESEARCH 188

REFERENCES 190

APPENDICES 211

LIST OF PUBLICATIONS 232

LIST OF AWARDS 234

LIST OF TABLES

Table 2.1: Commercial lipases that are available in the market 15

Table 3.1: Preparations of separating and stacking gels of SDS-PAGE 56

Table 5.1: Summary of purification of thermostable lipase from *G. thermodenitrificans* IBRL-nra 95

Table 5.2: Stability of thermostable lipase in the presence of organic solvents 113

Table 6.1: The bacterial strains and plasmids used in this study 123

Table 6.2: PCR reaction mixture 125

Table 6.3: PCR amplication condition 125

Table 6.4: Ligation setup 126

Table 6.5: Digestion setup 129

Table 6.6: Digestion setup of insert and expression vector 131

Table 6.7: Thermostable lipase activity at different culture temperature 155

Table 6.8: Thermostable lipase activity at different concentration of IPTG 157

Table 6.9: Thermostable lipase activity at different induction time 158

Table 7.1: Summary of purification of thermostable lipase LipGt 173

LIST OF FIGURES

Figure 2.1:	The α/β hydrolase fold of lipase	8
Figure 2.2:	Structure of *Mucor miehei* lipase in closed and open form	9
Figure 2.3:	Diverse reactions catalyzed by lipase	12
Figure 2.4:	Catalytic mechanism of lipase-catalyzed ester hydrolysis	13
Figure 2.5	Schematic diagram of a stirred tank bioreactor	25
Figure 2.6:	Typical crystallization phase diagram of a protein	37
Figure 2.7:	Picture of thermostable lipase crystals	39
Figure 2.8:	Three-dimensional structures of thermostable lipase	43
Figure 4.1:	Time-course analysis of production of thermostable lipase and growth of *G. thermodenitrifications* IBRL-nra in a shake flasks system	69
Figure 4.2:	Intial time-course analysis of production of thermostable lipase and growth of *G. thermodenitrifications* IBRL-nra in a 5L- stirred tank bioreactor	71
Figure 4.3:	Effect of physical parameters on lipase production and all growth of *G. thermodenitrifications* IBRL-nra	74

Figure 4.4:	Time-course analysis of production of lipase and growth of *G. thermodenitrifications* IBRL-nra after enhancements of physical parameters in a stirred tank bioreactors	80
Figure 5.1:	Elution profile of thermostable lipase from *G. thermodenitrifications* IBRL-nra on hitrap heparin column affinity chromatography	91
Figure 5.2:	Elution profile of thermostable lipase from *G. thermodenitrifications* IBRL-nra on Sephadex G-100 gel filtration chromatography	93
Figure 5.3:	Determination of the molecular weight of thermostable lipase from *G. thermodenitrifications* IBRL-nra	95
Figure 5.4:	Effect of temperature on activity (A) and stability (B) of the purified thermostable lipase of *G. thermodenitrificans* IBRL-nra	97
Figure 5.5:	Effect of pH on thermostable lipase activity at 65˚ C	101
Figure 5.6:	Effect of metal ions on thermostable lipase activity	108
Figure 5.7:	Effect of oxidizing, reducing and chelating agent on	110

thermostable lipase activity

Figure 5.8: Relative activity of thermostable lipase from *G. thermodenitrificans* IBRL-nra on triglycerides of C2 to C18. 108

Figure 5.9: Relative activity of thermostable lipase from *G. thermodenitrificans* IBRL-nra on vegetable oil 111

Figure 5.10: Effect of surfactants on thermostable lipase activity 117

Figure 6.1: Nucleotide sequence and the deduced amino acid sequence of LipGt 140

Figure 6.2: Sequence alignment of GDSL family lipases 144

Figure 6.3: Lipase screening of cell lysate from C43 (DE3) pLysS harbouring pET-LipGt on Rhodamine B- olive oil plate at 65˚C 153

Figure 7.1: Thermostable lipase activity after heat treatment at 65˚C and 70˚C 170

Figure 7.2: Elution profile of thermostable lipase, LipGt after gel filtration 171

Figure 7.3: Effect of temperature on thermostable lipase, LipGt activity (A) and stability (B) 175

Figure 7.4: Effect of pH on thermostable lipase, LipGt activity (A) and stability (B) 176

Figure 7.5: Effect of organic solvent on thermostable lipase, LipGt activity 178

LIST OF PLATES

Plate 4.1: Growth of *G. thermodenitrifications* IBRL-nra on nutrient agar plates at different temperatures after 24 hours of incubation 65

Plate 4.2: Formation of hydrolysis zones on lipase screening agar plates after 24-48 hours of incubation 67

Plate 5.1: SDS-PAGE of thermostable lipase from *G. thermodenitrificans* IBRL-nra 94

Plate 6.1: Genomic DNA extraction from *G. thermodenitrificans* IBRL-nra 136

Plate 6.2: The PCR product (LipGt) using ARF and ARR primers on the agarose gel (1.0%) 136

Plate 6.3: Colony PCR products on agarose gel (1.0%) 137

Plate 6.4: Plasmid digestion of pGEM-LipGt using *Eco*R1 on the agarose gel (1.0%) 137

Plate 6.5: Lipase screening of transformed colonies on tributyrin plates and Rhodamine-B olive oil plates 138

Plate 6.6: PCR of clones, *E. coli* BL21(DE3) harbouring pET-LipGt 145

Plate 6.7: Restriction digestion of pET-LipGt 145

Plate 6.8: Expression of thermostable lipase in *E. coli* BL21 (DE3) harbouring pET-LipGt 147

Plate 6.9: SDS-PAGE of the solubility of LipGt expressed in *E. coli* BL21 (DE3) 150

Plate 6.10: Expression of thermostable lipase in OverExpress C43 (DE3) pLysS 152

Plate 6.11: SDS-PAGE of the solubility of LipGt expressed in OverExpress C43 (DE3) pLysS 153

Plate 6.12: SDS-PAGE of the expression of thermostable lipase, LipGt in OverExpress C43 (DE3) pLysS induced at different temperatures 155

Plate 6.13: SDS-PAGE of the expression of thermostable lipase, LipGt in OverExpress C43 (DE3) pLysS induced at different concentration of IPTG 157

Plate 6.14: SDS-PAGE of the expression of thermostable lipase, 158

LipGt in OverExpress C43 (DE3) pLysS induced at different incubation time

Plate 7.1: Crude thermostable lipase, LipGt after heat treatment at 65˚C (A) and 70˚C (B) 168

Plate 7.2: Purification of thermostable lipase, LipGt 173

Plate 7.3: Formation of microcrystal using crystal screen cryo 180

Plate 7.4: Formation of rod clusters using sodium acetate, pH 4.6 (A) and using Tris-Cl, pH 8.5 (B) as buffer component in Formulation 14 and formation of crystals using 0.28M (A) and 0.38M (B) of calcium chloride in the formulation 14 of HR2-122 at 20˚C 184

LIST OF SYMBOLS AND ABBREVIATIONS

%	-	percentage
˚C	-	degree Celsius
cm	-	centimeter
µm	-	micrometer
mm	-	millimeter
µL	-	microliter
mL	-	milliliter
L	-	Liter
µg	-	microgram
µg/mL	-	microgram per milliliter
mg	-	milligram
g	-	gram
g/L	-	gram per Liter
EC	-	Enzyme Commision
α	-	alpha
β	-	beta
DNA	-	deoksiribonucleic acid
rRNA	-	ribosomal ribonucleic acid
UV	-	ultraviolet
nm	-	nanometer
µmole	-	micromole
mM	-	millimolar
M	-	molar
w/v	-	weight per volume

v/v	-	volume per volume
U	-	Unit
min	-	minute
hr	-	hour
rpm	-	revolutions per minute
et al	-	and others
R_f	-	relative mobility
Da	-	Dalton
kDa	-	kiloDalton
bp	-	base pair
kb	-	kilobase pair
V	-	volt
mA	-	milliAmpere
AmpR	-	Ampicillin resistant gene
ddH20	-	double distilled water
EDTA	-	ethylene diaminetetraacetic
HCl	-	hydrochloric acid
IPTG	-	isopropyl β-thiogalactophyranoside
X-gal	-	5-bromo-4-chloro-3-indoyl-β-galactoside
PCR	-	polymerase chain reaction
TBE	-	tris boric EDTA
vvm	-	volume of gas per volume of liquid per minute
TSS	-	transport and storage solution

PENGHASILAN, PENGKLONAN DAN PENCIRIAN LIPASE STABIL HABA DARIPADA *Geobacillus thermodenitrificans* IBRL-nra

ABSTRAK

Sejak kebelakangan ini, lipase daripada mikroorganisma termofilik telah menjadi tumpuan yang istimewa disebabkan kepelbagaian aplikasinya dalam sektor industri. Ini disebabkan oleh cirinya yang mempunyai kestabilan yang tinggi pada suhu yang tinggi dan tahan penyahaslian oleh bahan kimia. *Geobacillus thermodenitrificans* IBRL-nra yang digunakan dalam penyelidikan ini telah dipencilkan dari kolam air panas di Labok, Kelantan, Malaysia dan mempunyai suhu pertumbuhan antara 45ºC ke 70ºC. Penghasilan lipase stabil haba daripada *G. thermodenitrificans* IBRL-nra pada suhu 65˚C telah dikaji secara kualitatif diatas plat agar penyaringan lipase. Penghasilan lipase stabil haba ekstrasel oleh *G. thermodenitrificans* IBRL-nra telah dijalankan di dalam sistem kelalang goncangan dan bioreaktor tangki teraduk 5L. Penghasilan lipase stabil haba dan pertumbuhan sel di dalam bioreaktor tangki teraduk telah meningkat sebanyak 5 kali ganda dan 3 kali ganda, masing-masing daripada penghasilannya di dalam sistem kelalang goncangan. Penghasilan enzim dan pertumbuhan sel meningkat sebanyak 30% dan 20%, masing-masing selepas pengoptimuman parameter fizikal dalam bioreaktor. Lipase daripada sumber yang berlainan mempunyai ciri-ciri yang unik dan berlainan. Oleh itu, lipase stabil haba daripada *G. thermodenitrificans* IBRL-nra telah ditulenkan dan dicirikan. Lipase stabil haba kasar telah ditulenkan sebanyak 34 ganda dengan 9% hasil dan aktiviti spesifik 73.4 mg/ml dengan menggunakan penurasan-ultra, kromatografi afiniti Heparin dan kromatografi penurasan gel Sephadex G-100. Berat molekul lipase stabil haba dianggarkan sebanyak 27.3 kDa

pada SDS-PAGE. Gen lipase stabil haba, LipGt daripada *G. thermodenitrificans* IBRL-nra telah diklon dan diekspres di dalam sistem *Escherichia coli* untuk penghasilan enzim yang tinggi (pukal). Gen yang mengkodkan LipGt telah diamplikasi daripada DNA genom *G. thermodenitrificans* IBRL-nra dengan menggunakan PCR. Gen yang diamplikasi telah diklon ke dalam pGEM-T Easy dan diekspreskan dalam vektor pengekspresan pET-15b. Kehadiran gen lipase stabil haba di dalam plasmid rekombinan diperiksa dengan mengunakan penjujukan DNA dan penghadaman sekatan dan ditransformasikan ke dalam *E. coli* BL21 (DE3) dan OverExpress C43 (DE3) pLysS. Lipase rekombinan telah ditulenkan dengan menggunakan rawatan haba, penurasan-ultra dan kromatografi gel. Enzim yang telah ditulenkan diskrin untuk penghasilan hablur dengan menggunakan 'Hampton Research Crystal Screen Cryo', HR2-121 dan HR2-122 dengan menggunakan kaedah 'penyebaran wap titisan gantung' dan 'kumpulan mikro'. Penemuan kajian ini menunjukkan bahawa gen lipase stabil haba daripada *G. thermodenitrificans* IBRL-nra telah diklon and diekspresskan dalam sistem *E. coli* dan lipase stabil haba rekombinan mempunyai ciri-ciri yang sama dengan lipase stabil haba ekstrasel asli daripada *G. thermodenitrificans* IBRL-nra. Didapati ciri-ciri enzim ini adalah unik dan berpotensi memainkan peranan yang penting dalam aplikasi bioteknologi dan industri.

PRODUCTION, CLONING AND CHARACTERIZATION OF THERMOSTABLE LIPASE FROM *Geobacillus thermodenitrificans* IBRL-nra

ABSTRACT

Lipases from thermophiles have gained interest in recent years as they have various applications in industries. They play significant roles in industries as they have high stability at elevated temperatures and resistant to chemical denaturation. *Geobacillus thermodenitrificans* IBRL-nra exploited in this study was originally isolated from a hot spring in Labok, Kelantan, Malaysia with growth temperatures ranging from 45ºC to 70ºC. The production of thermostable lipase by *G. thermodenitrificans* IBRL-nra at 65˚C was checked qualitatively by streaking the bacteria on lipase screening agar plates. The production of extracellular thermostable lipase by *G. thermodenitrificans* IBRL-nra was carried out in a shake flask system and 5L stirred-tank bioreactor. The production of thermostable lipase in stirred-tank bioreactor was improved five fold compared to the production in shake flask system and an increment of three fold was observed in the cell growth. The enzyme activity was improved by 30% while the cell growth was also increased approximately 20% after the enhancement of physical parameters in the bioreactor. Lipases isolated from different sources exhibit diverse and unique characteristics. Therefore, thermostable lipase from *G.thermodenitrificans* IBRL-nra was purified and characterized to determine its properties. The extracellular crude thermostable lipase was purified to homogeneity by using ultrafiltration, Heparin affinity chromatography and Sephadex G-100 gel-filtration chromatography by 34 fold with a final yield of 9% and specific activity of 73.4 U/mg. The molecular weight of the purified enzyme was estimated to be 27.3 kDa on SDS-PAGE. Thermostable lipase gene, LipGt from *G.*

thermodenitrificans IBRl-nra was cloned and over-expressed in *Escherichia coli* system for bulk enzyme production. Gene coding for LipGt from *G. thermodenitrificans* IBRL-nra was amplified from the genomic DNA, cloned into pGEM-T Easy and then expressed in expression vector pET-15b. The plasmid harbouring the thermostable lipase gene was verified for the presence of insert by DNA sequencing and restriction enzyme digestion and transformed in *E. coli* BL21 (DE3) and OverExpress C43 (DE3) pLysS. The recombinant protein was purified by employing heat treatment, ultrafiltration and gel-filtration chromatography. The purified recombinant thermostable lipase, LipGt was screened for crystal formation using Hampton Research Crystal Screen Cryo, HR2-121 and HR2-122 using hanging drop vapour diffusion and microbatch methods. The findings of the study reveal that the recombinant thermostable lipase gene from *G. thermodenitrificans* IBRL-nra was cloned and overexpressed in *E. coli* system and the recombinant thermostable lipase exhibits similar characteristics with the wild-type extracellular thermostable lipase. The properties of the enzyme are unique and therefore it holds a promising role in the biotechnological and industrial applications.

CHAPTER 1

INTRODUCTION

Lipolytic enzymes play vital role in the turnover of lipids. Lipases have been used in 'in situ' lipid metabolism and 'ex-situ' industrial application which contribute towards the lipid technology bio-industry (Verma, 2012). Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), catalyze the hydrolysis of long-chain triglycerides into diacylglycerols, monoacyglycerols, fatty acids and glycerols as well as the reverse reaction of the synthesis of esters formed from fatty acids and glycerols (Leow *et al*., 2004; Li and Zhang, 2005). It acts only on lipid-water interfaces (Patil *et al*., 2011). They are ubiquitous whereby they are present in diverse organisms including animals, plants, fungi and bacteria (Thakur, 2012). However, only microbial lipases are commercially significant for their promising usage in industries.

Lipases are known for its versatility and find diverse applications in industries which include flavour enhancement in food and dairy processing industry, as detergent additives in detergent industry, hydrolysis of fats and oils in fat and oil industry and removing pitch from pulp in paper industry (Jaeger and Reetz, 1998; Gupta *et al*., 2004; Royter *et al*., 2009). Besides that lipases are also used to enrich the polyunsaturated fatty acids (PUFAs) from animal and plant lipids for nutraceuticals and pharmaceutical purposes and in transesterification for biodiesel production (Jaeger and Eggert, 2002). This is due to its characteristics; whereby it is stable and active in organic solvents (Niehaus *et al*., 1999), it does not need cofactors

to catalyse the reactions (Rubin and Dennis, 1997), it exhibits exquisite chemoselectivity, stereoselectivity and regioselectivity (Jaegar and Eggert, 2002) and possess a broad range of substrate specificity for the conversion of several unnatural substrates (Sheikh *et al*., 2003).

All the enzymatic reactions in industrial processes are executed at elevated temperatures. This is to boost the conversion rates of the substrates, for improved substrate solubility, to diminish the microbial contamination and to decrease the viscosity of the reaction media (Li and Zhang, 2005). Therefore, the key requirement for commercial lipases is thermal stability. This prerequisite has drawn the interest towards thermophiles in both research and industry as thermophiles are superior sources for thermostable enzymes. Lately, lipases cloned or isolated from extreme thermophiles have led to a special focus due to their higher thermodynamics both at elevated temperatures and organic solvents (Li and Zhang, 2005). Although many lipases from mesophiles are stable at elevated temperatures, lipases from thermophiles exhibit higher activity with prolonged thermostability at elevated temperatures (Sheikh *et al*., 2003).

Thermophiles are reliable sources of thermostable lipases but they produce small volume of biomass which results in low yield of lipase. Besides, high temperature fermentations may need specialized equipment (Leow *et al*., 2004) and substrate decomposition reactions may result in formation of toxic and inhibitory compounds (Dominguez *et al*., 2005). As a consequence, foreign protein expression in prokaryotic systems has been introduced to achieve high-level expression of lipase for bulk production cost-effectively (Leow *et al*., 2007; Abdel-Fattah and Gaballa, 2008). In addition, the fast growth rate and ease of cultivation technology for *Escherichia coli* make it appropriate for industrial application. Li and Zhang (2005)

reported that a lipase gene was successfully cloned from a novel thermophile *Geobacillus* sp. TW1 and expressed in *E. coli* and the recombinant lipase displayed similar characteristics when compared with the native lipase.

1.1 Rational and Research Objectives

The world market for enzymes is growing rapidly and estimated to reach $7 billion in 2013, with 6.3% increase per annum (Hasan *et al.*, 2010). The demand is mainly in the food and beverages, diagnostic, pharmaceutical, animal feed and biotechnological enzymes. In Malaysia, the vast application of enzymes is in food and dairy industries, oleochemical industries, detergent industries, animal feed industries and baking industries (Ibrahim, 2008). However these enzymes are imported from other major enzyme producing countries like USA, Europe and Japan and millions of dollars are spent for this purpose. Therefore government is now looking into developing its own industrial enzyme production technologies using its natural resources (Ibrahim, 2008).

Lipases have unique catalysing capability which draws the interest in industries. Lipase as a biocatalyst is a good alternative for chemical reactions as lipase-catalysed process similar to natural metabolism pathway of living things (Sangeetha *et al.*, 2011). They are more safe and environment-friendly than the chemical syntheses (Sheikh *et al.*, 2003). In general, enzymes have lower activation energy and manufacture products with high quality and they do not change the equilibrium of the reactions it catalyzes. Hasan *et al.* (2006) stated that lipases isolated from different sources possess diverse properties and characteristics which could be employed for a range of biotechnological applications. Thermostable

lipases which retain high stability at elevated temperatures and resistance to chemical denaturation play a significant role in the industrial application (Sharma *et al*., 2001). Thermal stability of an enzyme is influenced by environmental factor and also related to its structure (Zhu *et al*., 2001). It is reported that the variation in the architecture of substrate binding-site influence the catalytic properties of lipase (Schmidt and Verger, 1998). Therefore, by determining the three dimensional structure of lipase, insights into the mechanisms used to enhance the thermal stability will be learned. Besides that, the structure-function relationship will also be elucidated and this will enable the researchers to tailor new lipases for rapidly growing biotechnology industry. Therefore, there is a continuous demand for screening, isolation and protein engineering of lipases which will lead to the discovery of new and desired properties. Thus the objectives of this study are:

1. To produce thermostable lipase from *Geobacillus thermodenitrificans* IBRL-nra (*G. thermodenitrificans* IBRL-nra) in a shake flask system and in a 5-L laboratory scale bioreactor.
2. To purify and characterize the thermostable lipase from *G. thermodenitrificans* IBRL-nra.
3. To clone and overexpress the thermostable lipase gene from *G. thermodenitrificans* IBRL-nra.
4. To purify and characterize the recombinant thermostable lipase, LipGt from *G. thermodenitrificans* IBRL-nra.
5. To screen for formation of thermostable lipase, LipGt crystals and enhancement of the crystallization conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Lipases

Lipase is a class of hydrolases and the numerical classification of lipase or triacylglycerol acylhydrolase is EC 3.1.1.3, where components indicate the following; EC 3 enzymes are hydrolases (class 3), EC 3.1 hydrolases that act on ester bonds, EC 3.1.1 it is carboxylic ester and finally EC 3.1.1.3 it is triacylglycerol lipase. To date there are 119 entries for triacylglycerol lipase in Protein Data Bank (PDBe). As implied by the classification, lipase plays major role in hydrolyzing triacylglycerides, the major component of fats and oils into free fatty acids and glycerols at water-lipid interface, between the insoluble substrate phase and the aqueous phase (Leow *et al*., 2004). Besides, lipase is also capable of catalyzing the esterification and transesterification reactions in water restricted conditions (Reetz, 2002).

2.1.1 Sources of lipases

Lipase can be found extensively in nature which includes bacteria, fungi, plants and animals. However, only microbial lipases are used vastly for biotechnological purposes nowadays (Saxena *et al*., 2003b). This is due to uncomplicated mass cultivation of microorganisms as the source of lipases, the microorganisms could be genetically modified and microbial lipases are more stable compared to lipases isolated from plants and animals (Hasan *et al*., 2010). Based on the review made by Patil *et al*. (2011), the biodiversity of lipase can be classified as 45% of lipases

isolated from bacteria, 21% from fungus, 18% from animals, 11% from plants and 3% from algae.

Bacterial lipases were widely studied compared to other groups of lipases. Lipases from *Pseudomonas* were the first studied lipases, followed by *Alcaligenes* sp., *Staphylococcus* sp., *Chromobacterium* sp., *Bacillus* sp., *Acinetobacter* sp., and *Pyrococcus* sp. (Patil *et al*., 2011). Several lipases have been isolated, purified and characterized from thermophilic isolates, mainly from *Bacillus* (Luisa *et al*., 1997; Nagarajan, 2012). Fungal lipases were isolated mainly from *Aspergillus* sp., *Candida* sp., *Mucor* sp., *Fusarium* sp., *Trichosporon* sp., *Rhizopus* sp., *Geotrichum* sp., and *Penicillium* sp. (Saxena *et al*., 2003a; Hasan *et al*., 2006; Thakur, 2012). Fungal lipases were exploited due to its thermal stability, pH stability, substrate specificity and activity in organic solvents (Saxena *et al*., 2003b).

In plants, lipases are found in coconut seeds, *Carissa carandas* fruit, castor bean, cucumis melo, and rice bran (Patil *et al*., 2011; Ejedegba *et al*., 2007), but the availability of lipases from plants is seasonal due to the weather influence (Smith, 2004). Animal lipases were mainly isolated from human pancreatic, pig pancreatic, insects, and fishes (Patil *et al*., 2011). In 1850s, the first lipase was discovered from pancreatic juice which was used to hydrolyse insoluble oil droplets (Hasan *et al*., 2006). Traditionally, lipase from animal pancreases has been used to aid in the digestion of human. But over the time, the shortage of pancreases has lead to the isolation of lipase from microorganisms. Besides that, lipase from pancreas lacks purity as it contains trypsin, animal viruses and hormones (Vakhlu and Kour, 2006).

2.1.2 Structure of lipases

Lipases are, in general, highly variable in size and the sequence similarity between them is limited to short spans located around the active-site residues (Kim *et al*., 1997). The molecular size of lipases ranges from 19kDa to 60 kDa. It belongs to the α/β hydrolase family with the active site is formed by a catalytic triad of Ser-Asp/Glu-His residues (Jaeger *et al*., 1999). The active site serine residue is located in a β-turn-α motif (hairpin turn). The motif is composed of a central β sheet with eight different β-strands, β 1- β 8 which are connected by six α-helices, A-F as depicted in Figure 2.1 (Jaeger and Reetz, 1998). The consensus sequence of lipase is a 'nucleophilic elbow' which is at the end of α sheet (Schmidt and Verger, 1998). The consensus sequence is Gly/Ala-X-Ser-X-Gly, where X could be any amino acid residue. Lipase exhibits interfacial activation whereby it is activated when absorbed to the water-lipid interface (Nagarajan, 2012). Lately, the elucidation of three-dimensional structure has provided a rationalization for the interfacial activation of lipases (Jaeger and Reetz, 1998). The active site of lipase is covered by a lid-like α-helical structure. The lid undergoes a conformational rearrangement and moves away upon binding to a lipid-water interface (Figure 2.2), causing the active site of lipase to be fully accessible, enhancing hydrophobic interaction between the enzyme and lipid surface (Schmidt and Verger, 1998; Jaeger *et al*., 1999).

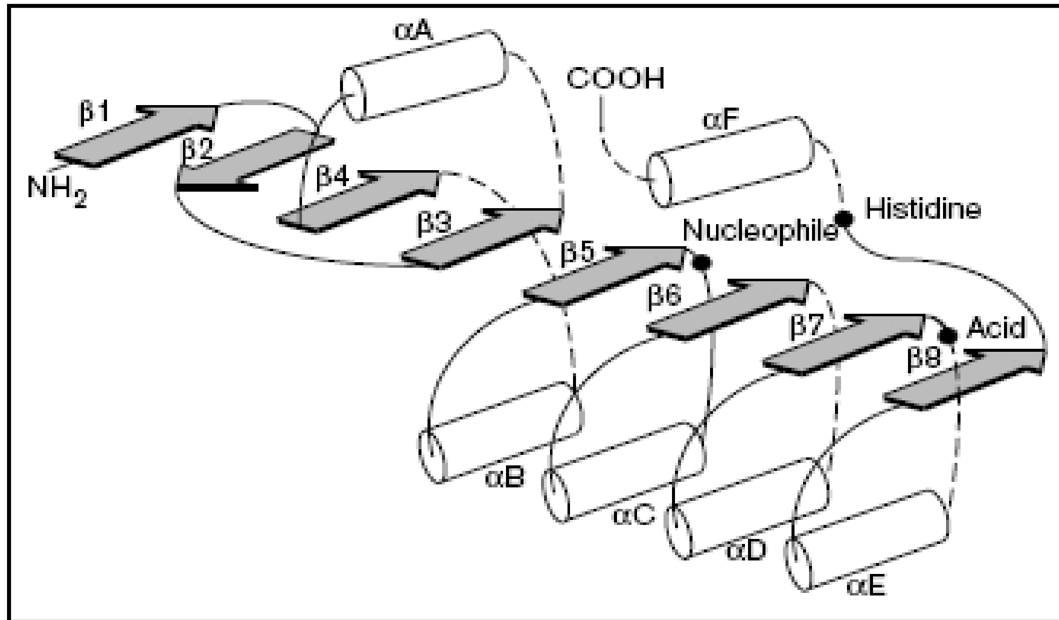


Figure 2.1: The α/β hydrolase fold of lipase (Jaeger *et al*., 1999)

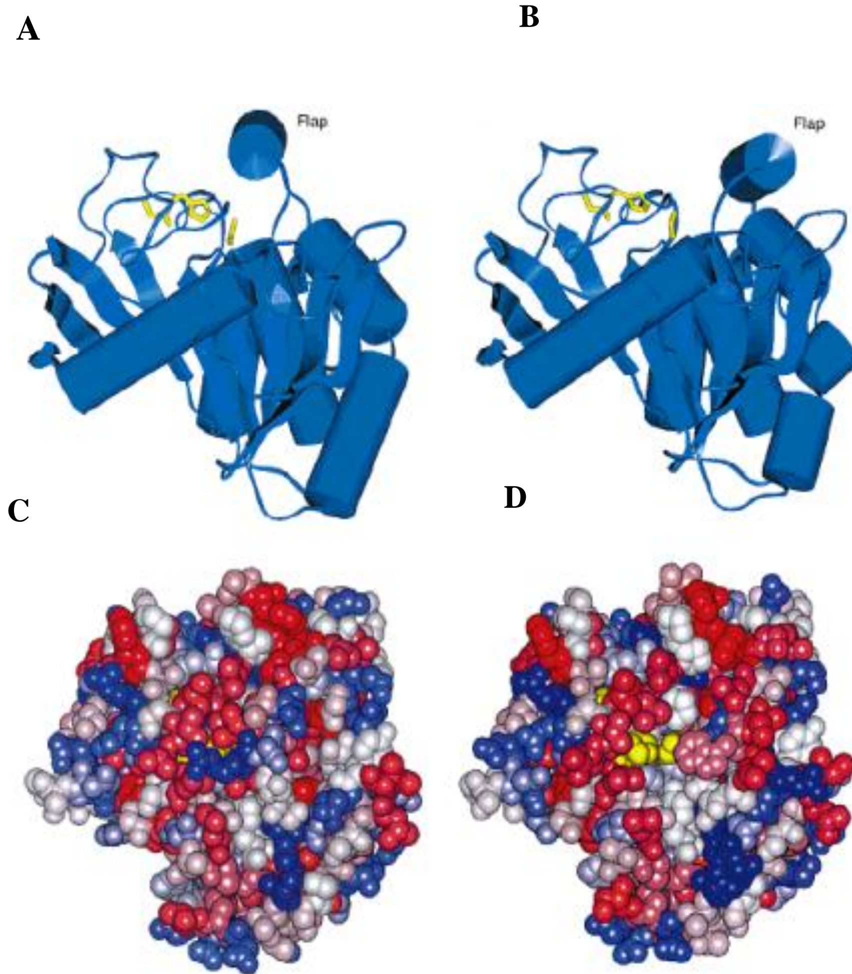


Figure 2.2: Structure of *Mucor miehei* lipase in closed (A and C) and open form (B and D). A and B are the side view of the secondary structure of lipase showing the α/β hydrolase fold with the catalytic triad in yellow. C and D are the top view of space-filling model coloured with decreasing polarity (dark blue>light blue>white>light red>dark red). Once the lid opens, the catalytic triad (yellow) becomes accessible for the substrate, and the region binding to the interphase becomes significantly more apolar (D) (Schmidt and Verger, 1998).

2.1.3 Classification of lipases

Lipase can be classified into eight families based on its biological properties and its conserved sequence motifs (Arpigny and Jaeger, 1999). Family I consist of true lipases which belong to *Pseudomonas*, *Bacillus*, and *Staphylococcus* with conventional catalytic pentapeptide (Gly-Xaa-Ser-Xaa-Gly) motif. Lipases with Gly-Asp-Ser-Leu motif and esterases of *Streptococcus*, *Aeromonas* and *Salmonella* are grouped in Family II. Family III consist of extracellular lipases of *Streptomyces*. Mammalian hormone sensitive lipases are grouped in Family IV while lipases of mesophilic bacteria like *Pseudomonas oleovorans* and *Haemophilus influenza* are in Family V. Family VI consist of the smallest esterases with dimeric active enzymes while larger esterases with amino acid sequence homologous to eukaryotic's acetyl choline esterases are grouped in Family VII. Lipases similar to β-lactamases are in Family VIII. Lipases which could not be grouped in the super eight families are arbitrarily classified into new families (Family IX and X) (Sangeetha *et al*., 2011). Cold active lipases which do not fit to the traditional classification are reported to belong to a novel lipolytic family (de Pascale *et al*., 2008).

2.1.4 Lipase-catalyzed reactions

Lipases are most versatile enzymes which are known to catalyze diversified reactions and they have wide substrate specificity. The reactions catalysed by lipases can be divided into hydrolysis and synthesis as described below:

1. Hydrolysis

   Lipase degrades triglycerides into fatty acid and glycerol.

2. Synthesis

(a) Esterification: Lipase catalyses the reaction of alcohols with acids to produce esters and water. Interesterification is a process whereby hydrolysis and esterification occurs simultaneously (Sharma *et al.*, 2001).

(b) Transesterification: Triglycerides are hydrolysed into methylester and glycerol in the presence of a catalyst.

(i) Acidolysis: It is a process of reacting acids with esters in the presence of organic solvents.

(ii) Aminolysis: Lipase catalyses the conversion of amines and alcohols into amides and esters.

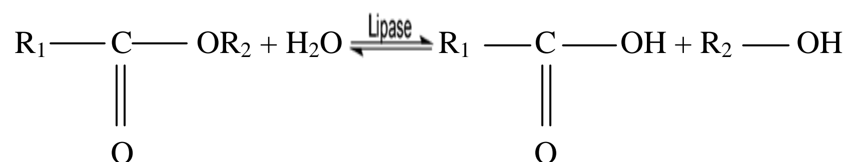
(iii) Alcoholysis: Lipase catalyses the reaction between tryglycerides and alcohols to produce esters.

All the processes are depicted in Figure 2.3.

2.1.5 Catalytic mechanism of lipases

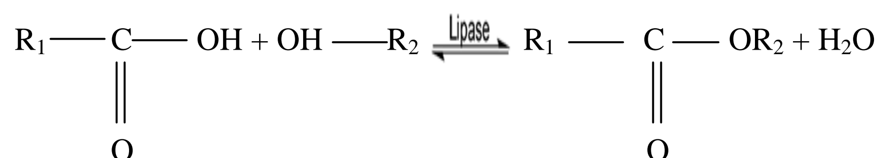
Different lipases have distinct preferences for substrates with different chain length. This is related to its size and structure of the substrate binding site (Pleiss *et al.*, 1998). The hydrolysis of lipids by lipase follows acylation-deacylation mechanism (Figure 2.4). Acylation is the prosess of formation of acyl-enzyme intermediate. Firstly, nucleophilic attack of the oxygen at the serine side chain of carbonyl carbon atom occurs. This forms the tetrahedral intermediate (Figure 2.4-1) (Jaeger *et al.*, 1994).

1. Hydrolysis



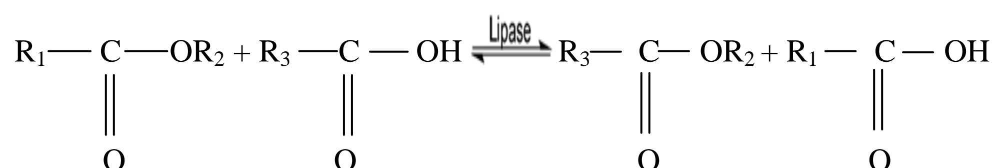
2. Synthesis

(a) Esterification

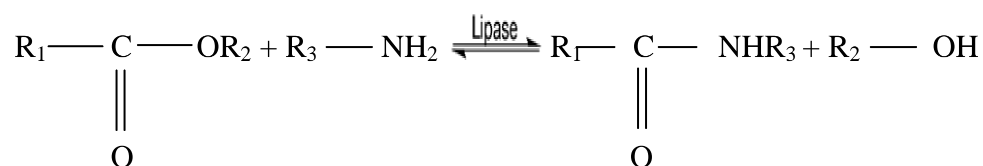


(b) Transesterification

(i) Acidolysis



(ii) Aminolysis



(iii) Alcoholysis

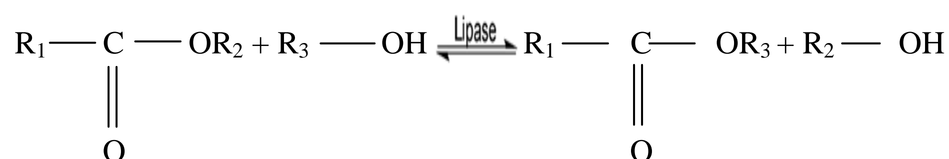


Figure 2.3: Diverse reactions catalysed by lipases (Patil *et al*., 2011; Casas-Godoy *et al*., 2012)

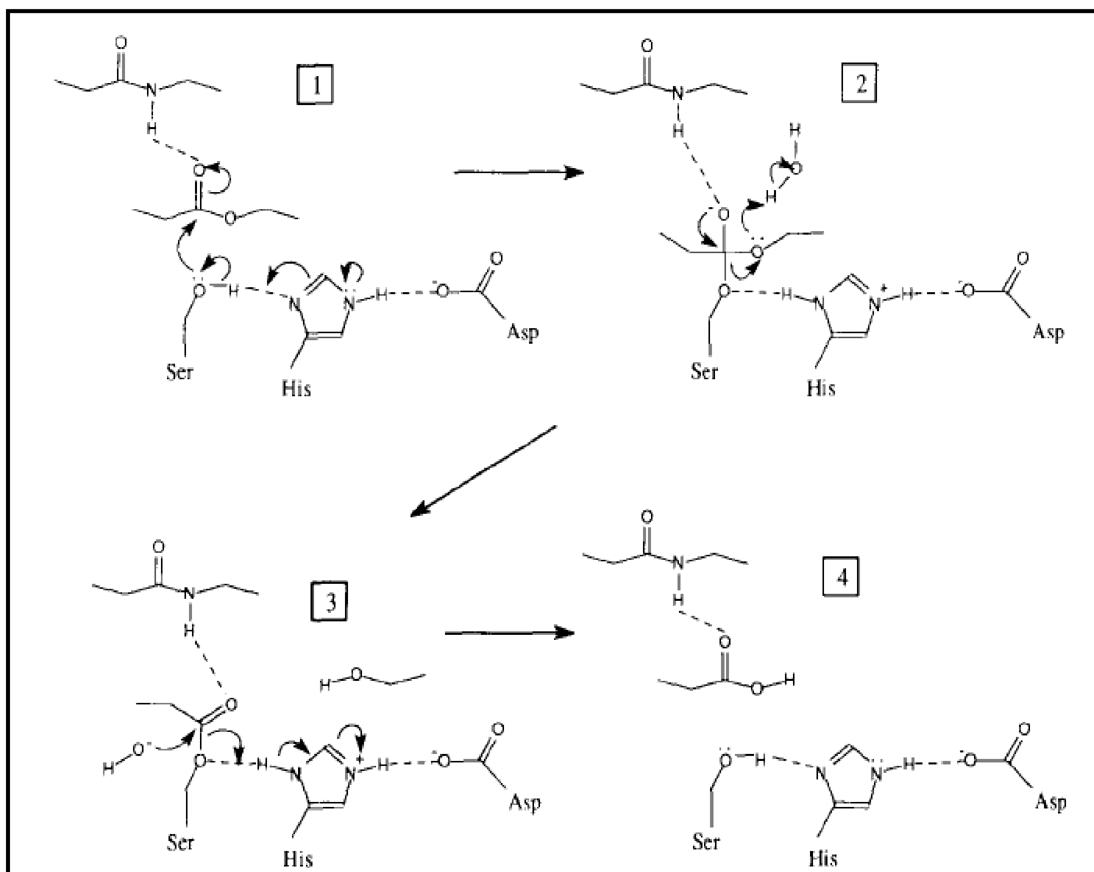


Figure 2.4: Catalytic mechanism of lipase-catalysed ester hydrolysis (Jaeger *et al*., 1994)

The nucleophilicity of serine hydrogen group is increased by the hydrogen bond from histidine assisted by the aspartate or glutamate residue. The imidazole ring of histidine becomes positively charged as a proton is abstracted from it. The negative charge of the acid residue stabilizes the positive charge (Figure 2.4-2). Two hydrogen bonds of the oxyanion hole stabilizes the tetrahedral intermediate (Casas-Godoy *et al*., 2012) and alcohol is liberated as shown in Figure 2.4-3 leaving behind acyl-enzyme complex. Finally the addition of water releases the acyl from the acyl-enzyme complex (deacylation) and the enzyme is regenerated for another round of catalysis (Figure 2.4-4).

2.2 Applications of lipase

Lipases have transpired as key enzymes in the field of biotechnology due to their multifaceted properties. Lipases employed in the enzymatic reactions in industrial application are easily recovered after the process and can be recycled for other reactions which are cost saving and also could be employed in continuous operations (Sheikh *et al*., 2003). Selected lipases which are commercially available and its sources are listed in Table 2.1. (Hasan *et al*., 2006; Casas-Godoy *et al*., 2012). Lipases are employed in several industries like detergent, food and diary processing, fats and oils modification, paper and pulp, pharmaceuticals, nutraceuticals, cosmetics, tea processing and organic synthesis. Besides that, lipases are also used as biosensors, in waste and sewage treatments, as diagnostic tools and for biodiesel production. The applications of lipase in some of the major industries are described further.

Table 2.1: Commercial lipases that are available in the market (Hasan *et al*., 2006; Casas-Godoy *et al*., 2012)

Lipase	Microbial sources	Manufacturer
Lipolase	*Thermomyces lanuginosus* expressed in *Aspegillus oryzae*	Novozymes, Denmark
Lipomax	*Pseudomonas alcaligens*	Genencor International, USA
Lumafast	*Pseudomonas mendocina* expressed in *Bacillus* sp.	Genencor International, USA
Lip PS	*Burkholderia cepacia*	Amono Enzyme Inc., Japan
Lip TL	*Pseudomonas srutzeri*	Meito Sangyo, Japan
Lipozyme	*Mucor miehei*	Novozymes, Denmark
Novozymes 435	*Candida antartica*	Novozymes, Denmark
Lipex	*Thermomyces lanuginosus*	Novozymes, Denmark
Palatase	*Rhizomucor miehei*	Novozymes, Denmark
Chirazyme	*Candida cylindracea*	Boehringer Mannheim, Germany

2.2.1 Lipase in detergent industry

Lipase is mainly used as additives in the household and industrial laundry detergents and household dishwashers (Jaeger and Manfred, 1998). 1000 tons of lipases are estimated to be added into 13 billion tons of detergents every year (Hasan *et al*., 2010). This is due to their ability to decompose fatty materials and to remove lipid and oil substances on the fabric during the cleaning processes (Godtfredsen, 1990). Bacterial lipases dominated the detergent market due to its stability in harsh washing conditions (alkaline pH and high temperatures), tolerant to other additives in detergent such as proteases and surfactants and has wide substrate specificity. Besides that, lipase has been used to clean the drains which had been clogged with food and non-food materials. The first commercial recombinant lipase 'Lipolase' was introduced by Novozymes (formely known as Novo Nordisk) in 1992 followed by Lumafast and Lipomax in 1995 by Genencor International (USA). These lipases are commercially used in the detergent industries. Rathi, *et al*. (2002) also isolated a detergent stable lipase from *Burkholderia cepacia*, which exhibits all the criteria as detergent additives and had better stability than Lipolase.

Wang *et al*. (2009) also isolated alkaline lipases from *Burkholderia cepacia* which were identified as suitable for applications in detergent industry. Bayoumi *et al*. (2007) reported on extracellular alkaline lipase for bio-detergent industry from *Bacillus licheniformis* B-42 and *Geobacillus stearothermophilus*. Lipex which was commercialized by Novozymes, performs deep cleaning interiorly whereby it breaks the fat trapped inside the fibres into glycerine and fatty acids (Hasan *et al*., 2010). Microbial lipases were also isolated and applied in detergent industry for fat removal, dish washing, dry cleaning solvents and in contact lens cleaner (Hasan *et al*., 2006).

2.2.2 Lipase in food and dairy processing industry

In food processing industry, lipase has been widely used in fat modifications, to modify flavours, to enhance food quality and produce fragrance compounds (Godtfredsen, 1990). Lipases were added to food to modify the flavour. This can be achieved by synthesis of esters of short chain fatty acid and alcohols (Macedo *et al*., 2003; Aravindran *et al*., 2007). Besides that, lipase is also actively used to produce lean meat (Andualema and Gessesse, 2012). This product is achieved by bio-lipolysis whereby the fats from meat and fish were removed by addition of lipase during the process (Patil *et al*., 2011). There are also reviews on microbial lipases used in refining rice flavour, enhancing the aroma and accelerating the fermentation of apple wine and finally altering soybean milk (Hasan *et al*., 2006). Moreover, lipase has been used in the quality improvement of food dressings like mayonnaise, dressings and whippings (Aravindran *et al*., 2007; Patil *et al*., 2011). Lipase is also utilized in the making of fermented soybean food like Koji and Tempeh. These traditional Asian foods are valuable and affordable source of protein (Aravindan *et al*., 2007).

In dairy industry, lipases are used for the hydrolysis of milk fat, the enhancement of flavour in cheese, acceleration of cheese ripening and lipolysis of cream and butter fat. A review by Casas-Godoy *et al*. (2012) reports on lipase from *Aspergillus niger* and *Aspergillus oryzae* which has been used for cheese flavouring and ripening. Lipases modify the fatty acid chain lengths which enhance the flavour of several cheeses while lipases used for hydrolysis of milk fat generate free fatty acids which produce the fragrance agent in cheese, milk and butter (Aravindran *et al*., 2007). Lipases are also used in flavour enhancement, shelf-life prolongation and improvement of texture and softness of bakery products.

2.2.3 Lipase in fats and oils processing industry

The utilization of lipase in fats and oils industry had provided solutions to overcome some industrial setback and consequently produced novel fats and oils (Andualema and Gessesse, 2012). Some lipids are more valuable than others due to the variation in their structures. These less desired fats can be converted into value added fats by using chemical methods but this processes results in random products (Hasan *et al*., 2006). Instead, lipase has been used to modify the fats which is more economical and environmentally safe. Modification of fats and oils is the major process in food industry which demands novel green technologies (Gupta *et al*., 2003). Lipase modifies the properties of fats and oils by altering its fatty acid chain locations (Ray, 2012). This process can modify an inexpensive and less desirable lipid into a higher value added fat in a more natural way (Sharma *et al*., 2001).

The de-gumming process (removal of phospholipids in oils) using microbial lipases have been introduced lately (Clausen, 2001). Lipase catalyses the transesterification of palm mid-fraction to produce cocoa butter substitute (Hasan *et al*., 2006). This process had overcome the shortage of cocoa butter fat for the production of chocolate (Andualema and Gessesse, 2012). An immobilized lipase from *Rhizomucor miehei* had been used for transesterification reaction which replaces the palmitic acid with stearic acid in palm oil (Undurraga *et al*., 2001). Lipases from *Pseudomonas* sp., *Rhizomucor miehei* and *Rhizopus oryzae* had been used for hydrolysis of lipids to produce glycerides for butter and margarine, concentrate or purified fatty acids and diglycerols for cooking oils (Casas-Godoy *et al*., 2012). Polyunsaturated fatty acids (PUFA) have gained attention due to its metabolic effect and pharmaceutical importance. Lipases are utilized to obtain PUFAs from animal lipids (like tuna oil) and plant lipids (like palm oil).

2.2.4 Lipase in pulp and paper industry

In paper making process, the hydrophobic components of wood, pitch emerge as sticky deposits in the paper machines which cause holes and smudges in the final paper (Andualema and Gessesse, 2012). Therefore, lipases are used to eliminate the pitch from the pulp generated during paper making process (Jaeger and Reetz, 1998). Once the tryglycerides had been hydrolysed by lipase, the pitch is far less sticky and more hydrophilic (Jaeger and Reetz, 1998). Besides that, lipases in paper industry increase the pulping rate of pulp, intensify the whiteness of paper, protects the equipment, diminish the chemical usage, overcome the waste water pollution and save power and time (Hasan *et al*., 2006; Andualema and Gessesse, 2012). The lipases from *Candida rugosa* has been used for pitch control, increase paper whiteness and reduce waste water pollution in Japan (Casas-godoy *et al*., 2012). A lipase from *Pseudomonas* species (KWI-56) was used to enhance the whiteness of paper and lessen the residual ink spots on final papers (Hasan *et al*., 2006).

2.2.5 Lipase in pharmaceuticals

At present, lipases are being employed by several international pharmaceutical companies in the preparation of optically active intermediates (Hasan *et al*., 2010). Some biotechnological companies like Enzymatix in United Kingdom offers a variety of intermediates prepared through lipase mediated resolution. Chirality is the main factor in determining the efficiency of many drugs; thus importance has been given in the production of enantiomers of drug intermediates. Biocatalytic processes had been employed in the preparation of chiral intermediates for pharmaceuticals (Hasan *et al*., 2006). Profens (2-aryl propinoic acids), are nonsteroidal anti-inflammatory drugs which are active in the (*S*)-enantiomer form

(Sharma *et al.*, 2001). Lee *et al.* (1995) described the production of pure (*S*)-ibuprofen by lipase-catalyzed kinetic resolution. Besides that, lipases are also used to modify the monoglycerides to function as emulsifiers in pharmaceutical applications (Sharma *et al.*, 2001). Lovastatin, a drug which lowers the serum cholesterol level has been synthesized using lipase from *Candida rugosa* (Andualema and Gessesse 2012). In a review by Hasan *et al.* (2006), it is stated that lipases have been efficiently used in the regioselective modification of castanospermine which is the potential drug in the treatment of AIDS.

2.2.6 Lipase as biosensors

Lipase has discovered new application in food and medical industries as biosensors. Lipase is used as biosensors to generate glycerol from triacylglycerol in analytical samples (Pandey *et al.*, 1999) and to determine the lipids in clinical purposes (Verma *et al.*, 2012). Immobilized lipases have been used as lipid biosensors to determine triglycerides and blood cholesterol (Hasan *et al.*, 2006). Besides that, a method has been developed to determine the organophosphorous pesticides with surface acoustic wave impedance sensor by lipase hydrolysis (Wei *et al.*, 1997). Lipase from *C. rugosa* has been detected for its rapid liberation of glycerol from triacylglycerols (Verma *et al.*, 2012). Pandey *et al.* (1999) also reported that a probe has been developed using lipase from *C. rugosa* which conjugates with biorecognition group in DNA. A potentiometric biosensor was fabricated using immobilized lipase from *Candida rugosa* on porous silica matrix for detection of tryglycerides (Setzu *et al.*, 2007).

2.2.7 Lipase in biodiesel production

The depletion of fossil fuels, the hike up in crude oil prices and environmental awareness to diminish pollutions are the factors contributing for the intensive research in biodiesel production (Bajaj *et al*., 2010). Biodiesel is alkyl esters of long chain fatty acids and short chain alcohols and expected to substitute the conventional diesel fuel (Iso *et al*., 2001). Biodiesel is synthesized through transesterification of vegetable oils with short chain alcohols like methanol and ethanol with the help of appropriate catalyst (Vicente *et al*., 2004). Biodiesel fuel originating from vegetable oil does not generate sulphur oxide compared to petroleum, which has environmental advantage (Hasan *et al*., 2006). It is reported that the chemically transesterified reaction (conventional method) of biodiesel production can give high yield but it often results in extreme consumption of energy and incurs extra cost in downstream processing (Shah *et al*., 2004). Therefore, to overcome these drawbacks, the usage of biocatalyst (lipase) in transesterification reactions for biodiesel production has been introduced. Immobilized lipase from *P. cepacia* was used in transesterification of soybean oil with the addition of methanol and ethanol (Noureddini *et al*., 2005). The commercial lipases, Novozyme 435 and Lipozyme IM have been used as the catalyst in the preparation of ethyl esters from castor oil using n-hexane as the solvent (de Oliveira *et al*., 2004). Fatty acid esters were also produced from palm kernel oil and coconut oil by using lipase PS30 as the catalyst (Hasan *et al*., 2006). However, biodiesel produced using enzymes as catalyst has not been commercialized due to high reaction time, high cost of enzyme and the need of organic solvents (Bajaj *et al*., 2010).

2.3 Production of lipase via submerged fermentation

Fermentation is a process of converting complex substrates into simple compounds utilizing diverse microorganisms. During this metabolic breakdown, they release bioactive compounds which are the secondary metabolites (Subramaniyam and Vimala, 2012). Secondary metabolites can be enzymes, antibiotics, peptides, sugars, organic acids and growth factors (Williams, 2002). Submerged fermentations are carried out using free flowing liquid substrates such as broths or molasses and the bioactive compounds are secreted into the fermentation broth (Subramaniam and Vimala, 2012). Around 75% of enzymes used in industrial application are produced using submerged fermentation since this technique supports the cultivation of genetically modified organisms. Microbial lipases are mostly produced using submerged fermentations (Sharma *et al*., 2001). Although there are few reports on lipase production via solid-state fermentation, submerged cultivation is preferred. This is due to its uncomplicated sterilization procedure and the process control is easier to engineer (Vidyalakshmi *et al*., 2009). Besides that, the recovery and purification of product from this technique is uncomplicated (Subramaniam and Vimala, 2012). Submerged fermentation can be carried out in small scale utilizing shake flasks system or in a large scale employing bioreactors.

2.3.1 Production of lipase in a shake flask system

In a shake flask system, the fermentation process is carried out in simple equipments for various purposes particularly for laboratory level researches. It is extensively used due to its low cost and simple operation and it has been the easier way to cultivate small amount of microbes (Vasala *et al*., 2006). Besides that, this system requires low energy, can be easily sterilized and is suitable for small scale

researches. However it is not feasible to be employed in big scale processes especially for commercial purposes. It is commonly used for the bioprocess optimization and to produce starter culture for bioreactor cultivation (Vasala *et al*., 2006). Shake flasks come in diverse shapes and sizes which includes test tubes, universal or Scott bottles, conical or Erlenmeyer flasks. All these are made of glass as it is cheaper, cleaner and can be easily sterilized. Kader *et al*. (2007) reported on lipase production by *Rhizopus* MR12 in a shake flasks system and the parameters like composition of carbon sources, pH, agitation rates and addition of metal ions were investigated to enhance the lipase activity. Extracellular lipase by *Bacillus megaterium* AKG-1 was produced by submerged fermentation using 250 ml Erlenmeyer flasks (Sekhon *et al*., 2006). Bonala and Mangamoori (2012) investigated the production of extracellular lipase production by *Bacillus tequilensis* and employed shake flasks submerged fermentation system to enhance the culture conditions for higher lipase production.

2.3.2 Production of lipase in a bioreactor system

Bioreactor is a vessel used for the cultivation of microorganisms in a controlled manner to convert the raw materials into desired products through specific reactions (Williams, 2002). Bioreactors differ from the typical chemical reactors as it is designed to support and control biological entities. Basically, laboratory scale bioreactors are made of glass and can support liquid 5 to 20 litres whereas commercial vessels are huge (up to 500 000 litres) and are made of stainless steel (Madigan and Martinko, 2006). The reactors are supported with mechanical components to provide controlled aeration, agitation, pH and temperature during the fermentation process. The mode of operation for bioreactors can be batch, fed batch and continuous and several designs are available for both laboratory and commercial

purposes which include stirred tank bioreactor, bubble column bioreactor, air-lift bioreactor, fluidized bioreactor and packed bed bioreactor (Williams, 2002).

Stirred tank bioreactor is the most commonly used fermenter for the production of bacterial enzymes. It features specific internal configuration for circulation purpose. The tank is built in with impellers for optimal mixing and baffles to prevent the whirlpool effect as illustrated in Figure 2.5 (Puthli *et al.*, 2006). The operating principle is relatively straightforward whereby the sterile medium and inoculum of microorganisms are introduced into the sterilized vessel then followed by the air supply which enters at the bottom and the fermentation proceeds with agitation (Williams, 2002). Bioreactors are commonly used to produce enzymes with enhanced activity. This is because the controlled aeration and agitation in the vessel provides a well mixed system for the microorganisms (Shukla *et al.*, 2001).

Production of extracellular lipase by *Rhizopus oligosporus* was carried out in a stirred tank bioreactor (Iftikhar *et al.*, 2010). The authors reported that the lipase production in bioreactor was enhanced compared to in a shake flask system and the fermentation period was reduced. Olusesan *et al.* (2011) also reported on the enhancement of lipase production by *Bacillus subtilis* NS 8 by employing continuous bioreactor. Krastanov *et al.* (2008) also investigated the production of lipase by *Candida cylindracea* NRRL Y-17506 via submerged fermentation in a stirred tank bioreactor. In a different study, lipase production by *Bacillus multivorans* was enhanced 12-fold in a 14-liter bioreactor (Gupta *et al.*, 2007). Kar *et al.* (2008) used a 20-L batch bioreactor for the production of extracellular lipase by *Yarrowia lipolytica* and the induction of LIP2 gene encoding for lipase of *Y. lipolytica*. The