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PRODUCTION, PURIFICATION, CHARACTERIZATION AND APPLICATION OF ORGANIC SOLVENT TOLERANT LIPASE FROM *TRICHODERMA* SP. BW45 IN PALM OIL HYDROLYSIS

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2010

PRODUCTION, PURIFICATION, CHARACTERIZATION AND APPLICATION OF ORGANIC SOLVENT TOLERANT LIPASE FROM *TRICHODERMA* SP. BW45 IN PALM OIL HYDROLYSIS

By

MUHAMAD ODEH ATYEH AL-LIMOUN

Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

2010

ACKNOWLEDGMENT

Alhamdulillah. Thanks to Allah SWT, whom with His willing giving me the opportunity to complete this PhD project.

First and foremost, I am deeply grateful to my supervisor, Prof Darah Ibrahim for her important support for submission of this thesis.

I would like to express my deep and sincere gratitude to my co-supervisor Prof Ibrahim Che Omar. His wide knowledge and his logical way of thinking have been of great value for me in correcting the thesis. His understanding, encouraging and personal guidance have provided a good basis for the present thesis

Besides, I also want to thanks the staffs of School of Biological Sciences for their cooperation on completing this project by providing the needed facilities.

Especially, I would like to give my special thanks to my wife Sajidah Pang Pei Kheng whose patient love enabled me to complete this work.

Finally, my special gratitude and thanks to my parents, brothers, sisters and their families for their loving support. They have lost a lot due to my research abroad. Without their encouragement and understanding it would have been impossible for me to finish this work.

TABLE OF CONTNETS

	Page
Acknowledgment…………………………………………………………..	**ii**
Table of Contents………………………………………………………..	**iii**
List of Tables…………………………………………………………….	**xiii**
List of Figures…………………………………………………………...	**xv**
List of Plates…………………………………………………………….	**xix**
Abstrak……………………………………………………………….....	**xxi**
Abstract………………………………………………………………	**xxv**
CHAPTER 1- INTRODUCTION………………………………………….	**1**
1.1 Rational and research objectives…………………………………….	**3**
1.2 Research scope……………………………………………………….	**5**
CHAPTER 2- LITERATURE REVIEW…………………………………..	**7**
2.1 Industrial enzymes…………………………………………………...	**7**
2.2 Microbial Lipases: Sources, production and purification……………	**10**
2.2.1 Sources of microbial lipases………………………………..	**10**
2.2.2 Lipase production…………………………………………..	**16**
2.2.3 Lipase purification Strategies………………………………	**26**
2.3 Lipase as biocatalysts………………………………………………..	**35**
2.3.1 Biocatalytic properties……………………………………..	**35**
2.3.2 Structure and mechanism of action in triglyceride hydrolysis…………………………………………………..	**41**
2.4 Application of lipases in triglycerides splitting for the production of fatty acids and glycerol………………………………………………………..	**44**

CHAPTER 3- MATERIALS AND METHODS………………………….. 60

3.1 Screening and selection of extracellular organic solvent tolerant lipase producing microorganisms from indigenous soil samples…..... 60

3.1.1 Preliminary screening for extracellular lipase producing microorganisms from indigenous soil samples…………… 60

3.1.1(a) Soil samples collection sites…………………… 60

3.1.1(b) Preliminary screening for lipase producing microorganisms using potato dextrose agar and nutrient agar plates supplemented with tributyrin..……………………………………… 60

3.1.1(c) Screening for extracellular lipase producing microorganisms using broth fermentation media. 61

3.1.1(d) Qualitative detection of extracellular lipase activity using Rhodamine B-olive oil, Rhodamine B-triolein, and tributyrin agar plates…………………………………………… 62

3.1.1(e) Quantitative analysis of lipase activity using copper soap colorimetry assay method………… 63

3.1.2 Selection of organic solvent tolerant lipase enzyme……..... 64

3.1.2(a) Screening for organic solvent tolerant lipase…… 64

3.1.2(b) Effect of different concentrations of various hydrophobic organic solvents on lipase hydrolysis activity of isolates BW16 and BW45. 64

3.1.2(c) Effect of different concentrations of various hydrophilic organic solvents on lipolytic enzyme hydrolysis activity of isolates BW16 and BW45.. 65

3.1.2(d) Organic solvent stability of lipase enzyme produced by isolate BW16 and BW45………….. 66

3.2 Identification of the selected organic solvent tolerant lipase producing microorganisms, BW16 and BW45……………………… 66

3.2.1 Identification of the bacterial isolate BW16……………….. 66

3.2.1(a) Scanning electron microscope………………….. 66

3.2.1(b) Transmission electron microscope……………… 67

3.2.1(c) Identification of the bacterial isolate BW16 using API kit…………………………………… 68

3.2.1(d) Identification of the bacterial isolate BW16 using 16S rRNA………………………………… **68**

3.2.2 Identification of the fungal isolate BW45………………........ **69**

3.2.2(a) Micromorphological characteristics of the fungal isolate BW45 using light and scanning electron microscope (SEM)... **69**

3.2.2(b) Identification of *Trichoderma* BW45 species using Biolog system..…………………………..... **71**

3.3 Comparative characterization of the lipolytic enzyme produced by *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45…………... **71**

3.3.1 Effect of temperature on activity and stability of crude lipases from *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45…………………………..…………………… **71**

3.3.2 Effect of pH on the activity and stability of crude lipases from *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45……………………………………….................. **72**

3.3.3 Substrate specificity of *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45 crude lipolytic enzyme…................ **73**

3.3.4 Time profile of lipase enzyme production by *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45…………... **73**

3.4 Sequential parametric optimization of extracellular organic solvent tolerant lipase production by *Trichoderma* sp. BW45……………… **74**

3.4.1 Inoculum preparation………………………….…………… **74**

3.4.2 Basal fermentation medium…..…………………………… **74**

3.4.3 Physical optimization of extracellular lipase production….. **75**

3.4.3(a) Effect of inoculum concentration on extracellular lipase production……………………………... **75**

3.4.3(b) Effect of incubation temperature on extracellular lipase production……………………………... **75**

3.4.3(c) Effect of agitation rate on lipase production……. **76**

3.4.3(d) Effect of initial pH of the fermentation media on lipase production……………………………... **76**

3.4.3(e) Time course of lipase production after physical parameters optimization………………………… **76**

3.4.4 Chemical optimization of extracellular lipase production from *Trichoderma* sp. BW45……………………………… **77**

3.4.4(a) Effect of different lipid nutrients on lipase production………………………………………. **77**

3.4.4(b) Effect of Tween 80 concentration on extracellular lipase production………………….. **77**

3.4.4(c) Effect of carbon sources on *Trichoderma* sp. BW45 lipase production……………………... **78**

3.4.4(d) Effect of starch concentration on lipase production... **78**

3.4.4(e) Effect of nitrogen sources on extracellular lipase production……………………………………… **78**

3.4.4(f) Effect of yeast extract concentration on extracellular lipase production………………….. **79**

3.4.4(g) Effect of metal salts on *Trichoderma* sp. BW45 extracellular lipase production………………….. **79**

3.4.4(h) Effect of NaCl concentration on extracellular lipase production……………………………... **80**

3.4.4(i) Tween 80 addition to the growth medium at different growth times………………………….. **80**

3.4.4(j) Time course of extracellular lipase enzyme production after physical and chemical optimization process…………………………… **80**

3.4.4(k) Extracellular lipase production by different species of *Trichoderma*………………..…...... **81**

3.5 Purification of extracellular lipase produced by *Trichoderma* sp. BW45 using aqueous two phase system coupled with Sephadex G-75 gel filtration……………………………...…………………… **82**

3.5.1 Partial purification of extracellular lipase using aqueous two phase system………………………………………... **82**

3.5.1(a) Binodal curve and critical point of the phase system…………………………………………... **84**

3.5.1(b) Aqueous-two phase systems preparation…...….... **85**

3.5.1(c) Effect of potassium phosphate concentration on partitioning and purification of *Trichoderma* sp. BW45 lipase enzyme………………………….. **85**

3.5.1(d) Effect of polyethylene glycol 6000 concentration on the partitioning and purification of lipase…… **87**

3.5.1(e) Effect of pH on partitioning and purification of lipase enzyme…………………………………… **87**

3.5.1(f) Addition of NaCl to the phase system…………. **88**

3.5.1(g) Calculations…………………………………... **89**

3.5.2 Purification using sephadex G-75 gel filtration……………. **90**

3.5.3 Polyacrylamide gel electrophoresis (SDS-PAGE)………… **91**

3.6 Characterization of pure lipase preparation of *Trichoderma* sp. BW45…………………………………………………………... **92**

3.6.1 Determination of Isoelectric point of lipase……………….. **92**

3.6.2 Immobilization of lipase enzyme on cellulose powder……. **93**

3.6.3 Lipase activity of *Trichoderma* sp. BW45 in hydrophobic and hydrophilic organic solvents…………………………... **93**

3.6.4 Stability of lipase of *Trichoderma* sp. BW45 in hydrophilic and hydrophobic organic solvents…………………………. **94**

3.6.5 Determination of the positional specificity of *Trichoderma* sp. BW45 lipase……………………………………………. **94**

3.6.6 The effect of temperature and pH on the activity of the purified lipase……………………………………………… **95**

3.6.7 Effect of temperature and pH on the stability of the purified lipase…………………………………………………….. **95**

3.6.8 Effect of metal salts on the purified enzyme activity……… **96**

3.6.9 Effect of various detergents and chemical agents on the purified lipase activity……………………………………... **96**

3.6.10 Substrate specificity……………………………………….. **96**

3.7 Enzymatic hydrolysis of palm oil in organic-aqueous system…....... **97**

3.7.1 Time course profile of palm oil hydrolysis by the lipase of *Trichoderma* sp. BW45…………………………………… **97**

3.7.2 Effect of isooctane to palm oil ratio on palm oil hydrolysis. **98**

3.7.3 Effect of organic solvents on hydrolysis degree of palm oil. **99**

3.7.4 Effect of pH on palm oil hydrolysis……………………….. **99**

3.7.5 Effect of aqueous to non aqueous phase ratio on palm oil hydrolysis degree…………………………………………... **100**

3.7.6 Effect of palm oil amount……..…………………………… **100**

3.7.7 Effect of enzyme loading on palm oil hydrolysis…………. **101**

3.7.8 Effect of agitation on palm oil hydrolysis degree………… **102**

3.7.9 Effect of temperature on palm oil hydrolysis……………… **102**

3.7.10 Effect of $CaCl_2$ on palm oil hydrolysis degree…………….. **102**

3.7.11 Recovery and reusability of the enzyme…………………... **103**

3.7.12 Time course profiles of palmitic acid and oleic acid production………………………………………………….. **103**

3.7.13 Crude palm oil hydrolysis………………………………… **103**

3.8 Analysis…………………………………………………………... **104**

3.8.1 Copper soap colorimetry lipase assay method…………….. **104**

3.8.2 *p*-nitrophenyl fatty acid ester lipase assay…………………. **104**

3.8.3 Biomass determination…………………………………….. **105**

3.8.4 Protein content determination in the aqueous two phase system…………………………………………………… **105**

3.8.5 Palm oil hydrolysis degree………………………………… **106**

3.8.6 GC analysis………………………………………………… **108**

CHAPTER 4- RESULTS AND DISCUSSION…………………………… **109**

4.1 Screening and selection of organic solvent tolerant extracellular lipase producing microorganisms from indigenous soil samples........ **109**

4.1.1 Preliminary screening for extracellular lipase producing microorganisms…………………………………………… **109**

4.1.1(a) Preliminary screening for lipase producing microorganisms using PDA and NA plates supplemented with tributyrin..………………….. **109**

4.1.1(b) Qualitative and quantitative analysis of extracellular lipase activity……………………... **110**

4.1.2 Selection of organic solvent tolerant lipase enzyme producing microorganism………………………………….. **114**

4.1.2(a) Screening for organic solvent tolerant lipase enzyme……………………………………….. **111**

4.1.2(b) Effect of different concentrations of various hydrophobic and hydrophilic organic solvents on lipase hydrolysis activity of the bacterial isolate BW16…... **120**

4.1.2(c) Effect of different concentration of various hydrophobic and hydrophilic organic solvents on lipase activity secreted by the fungal isolate BW45…………………………………………… **124**

4.1.2(d) Stability of the lipase enzyme secreted by isolate BW16 and BW45 after exposure to different hydrophilic and hydrophobic organic solvents…. **126**

4.2 Identification of the selected organic solvent tolerant lipase producing microorganisms BW16 and BW45………………………. **130**

4.2.1 Identification of the bacterial isolate BW16……………….. **130**

4.2.1(a) Morphological structures……………………….. **130**

4.2.1(b) Identification of the bacterial isolate BW16 using API kit……………………………….……… **130**

4.2.1(c) Identification of the bacterial isolate BW16 using 16S rRNA………………………………… **130**

4.2.2 Identification of the fungal isolate BW45 using micro-morphological and fungi colony morphological characteristics……………………………………………… **132**

4.2.2(a) Micromorphological characteristics of the fungal isolate BW45 using light and scanning electron microscope (SEM)……………………………… **132**

4.2.2(b) Identification of *Trichoderma* BW45 to the species level using biolog system……………… **133**

4.3 Comparative characterization of extracellular crude lipase produced by *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45……….. **138**

4.3.1 Effect of temperature on *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45 crude lipase activity and stability…………………………………………………….. **138**

4.3.2 Effect of pH on *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45 crude lipase enzyme activity and stability…………………………………………………….. **139**

4.3.3 Substrate specificity of *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45 crude lipase enzyme……............... **144**

4.3.4 Time course profile of lipase production by *Bacillus megaterium* BW16 and *Trichoderma* sp. BW45…………... **145**

4.4 Sequential parametric optimization of extracellular organic solvent tolerant lipase production by *Trichoderma* sp. BW45……………… **150**

4.4.1 Physical optimization of extracellular lipase enzyme production………………………………………………….. **150**

4.4.1(a) Effect of inoculum size on lipase production…... **150**

4.4.1(b) Effect of incubation temperature on lipase production………………………………………. **151**

4.4.1(c) Effect of agitation speed on lipase production…. **153**

4.4.1(d) Effect of the initial pH of the fermentation medium on lipase production…………………… **155**

4.4.1(e) Time course of lipase production after physical parameters optimization………………………… **156**

4.4.2 Chemical optimization of extracellular lipase enzyme production…………………………………………………. **158**

4.4.2(a) Effect of various lipase inducers on the enzyme production………………………………………. **158**

4.4.2(b) Effect of carbon sources and starch concentration on lipase production by *Trichoderma* sp. BW45….. **163**

4.4.2(c) Effect of nitrogen sources and yeast extract concentration on *Trichoderma* sp. BW45 lipase production.. **166**

4.4.2(d) Effect of metals and NaCl concentration on lipase production………………………………... **171**

4.4.2(e) Addition of Tween 80 to the growth medium at different growth time…………………………… **173**

4.4.2(f) Time profile for lipase production after the physical and chemical optimization process……. **174**

4.4.3 Lipase enzyme production by different strains of *Trichoderma* grown in the optimized fermentation medium **174**

4.5 Purification of extracellular lipase produced by *Trichoderma* sp. BW45 using aqueous two phase system coupled with Sephadex G-75 gel filtration……………………………………………………… **176**

4.5.1 Partitioning and purification of *Trichoderma* sp. BW45 lipase using aqueous two phase system (ATPS)……………………………………………………... **176**

4.5.1(a) Binodal curve and critical point of the phase system…………………………………………... **177**

4.5.1(b) Effect of potassium phosphate concentration on partitioning and purification of *Trichoderma* sp.

BW45 lipase enzyme…………………………… **178**

4.5.1(c) Effect of PEG 6000 polymer concentration on partitioning and purification of *Trichoderma* sp. BW45 lipase…………………………………….. **189**

4.5.1(d) Effect of pH on partitioning and purification of lipase enzyme…………………………………… **185**

4.5.1(e) Effect of NaCl addition on the enzyme partitioning and purification in the two phase system……………………………………... **184**

4.5.2 Purification of *Trichoderma* sp. BW45 lipase to the homogeneity level using Sephadex G-75 gel filtration……. **191**

4.5.3 Polyacrylamide gel electrophoresis (SDS-PAGE)………… **191**

4.6 Characterization of the purified extracellular lipase produced by *Trichoderma* sp. BW45……………………………………............. **196**

4.6.1 Isoelectric point……………………………………………. **196**

4.6.2 Immobilization of lipase enzyme on cellulose powder…… **196**

4.6.3 Activity and stability of *Trichoderma* sp. BW45 lipase enzyme in organic solvents………………………………... **198**

4.6.4 Positional specificity of *Trichoderma* sp. BW45 lipase enzyme……………………………………...…………… **203**

4.6.5 The effect of temperature and pH on pure lipase enzyme activity…………………………………………………... **205**

4.6.6 Effect of temperature and pH on the pure lipase enzyme stability…………………………………………………….. **208**

4.6.7 Effect of metals on the purified enzyme activity…………... **210**

4.6.8 Effect of various detergents and chemical agents on the purified lipase enzyme activity…………………………….. **210**

4.6.9 Substrate specificity……………………………………….. **212**

4.7 Enzymatic hydrolysis of palm oil in biphasic organic-aqueous system….. **215**

4.7.1 Initial time course profile of palm oil hydrolysis by *Trichoderma* sp. BW45 crude lipase enzyme……………..... **216**

4.7.2 Effect of isooctane to palm oil ratio on palm oil hydrolysis degree by *Trichoderma* sp. BW45 extracellular crude lipase enzyme……………………………………………… **219**

4.7.3 Effect of different organic solvent on hydrolysis degree of palm oil…………………………………………………….. **224**

4.7.4 Effect of pH on lipase catalysis of palm oil hydrolysis……. 227

4.7.5 Effect of aqueous to non aqueous phase ratio on palm oil hydrolysis degree……………………………………….... 232

4.7.6 Effect of palm oil concentration on palm oil hydrolysis degree…………………………………………………… 234

4.7.7 Effect of enzyme loading on palm oil hydrolysis degree….. 236

4.7.8 Effect of mixing speed on palm oil hydrolysis degree…….. 239

4.7.9 Effect of temperature on lipase catalysis of palm oil hydrolysis……………………………………………….. 243

4.7.10 Effect of $CaCl_2$ on palm oil hydrolysis degree……………. 246

4.7.11 Recovery and reusability of crude lipase enzyme…………. 249

4.7.12 GC time course of fatty acids production from palm oil hydrolysis……………………………………………….. 251

4.7.13 Hydrolysis of crude palm oil……………………………… 254

CHAPTER 5- CONCLUSION…………………………………………. 255

REFFERENCES……………………………………………………...... 258

APPENDICES

LIST OF PUBLICATIONS AND SEMINARS

LIST OF TABLES

		Page
2.1	Summary of enzyme market based on application sector ($Million)…………………………………………………………..	**10**
3.1	Location of the collected soil samples…………………………..	**60**
3.2	Fermentation media composition……………………………….	**62**
3.3	Phase system composition at different potassium phosphate concentration in the range of 8-12 % (w/w)………………………..	**86**
3.4	Phase system composition at different potassium phosphate concentration in the range of 8.2-9.4 % (w/w)……………………..	**86**
3.5	Phase system composition at different PEG6000 concentrations…	**87**
3.6	Phase system composition at different potassium phosphate pH value………………………………………………………………..	**88**
3.7	Phase system composition at NaCl concentration in the range of 1-5% (w/w)………………………………………………………...	**89**
3.8	Composition of the hydrolysis reaction mixture at different palm oil concentrations………………………………………………..	**101**
3.9	Hydrolysis reaction mixture composition at different enzyme concentrations……………………………………………………	**101**
3.10	Chemical composition of the solutions used in protein content determination…...	**106**
4.1	Growth and extracellular lipolytic activity of the selected isolates grown on three different types of fermentation media…………….	**112**
4.2	Organic solvent tolerance test for the selected lipolytic enzyme producing microorganisms………………………………………	**117**
4.3	The effect of different concentrations of various hydrophilic and hydrophobic organic solvents on *p*-nitrophenyl laurate hydrolysis activity of lipase produced by the bacterial isolate BW16…………	**123**
4.4	Relative activity of the lipolytic enzyme produced by the fungal isolate BW45 in presence of elevated concentration of both hydrophobic and hydrophilic organic solvents……………………	**125**
4.5	Lipase enzyme production by different strains of *Trichoderma* fungus grown on the optimized fermentation medium…………….	**176**
4.6	Purification table of lipase enzyme obtained at different ATPS compositions with the change in potassium phosphate concentration in the mixture in the range of 9-12% (w/w)………...	**181**

4.7 Purification table of lipase enzyme obtained at different ATPS compositions with the change in potassium phosphate concentration in the mixture in the range of 8.2-9.4% (w/w)…….. **181**

4.8 Purification table of lipase enzyme in the upper and lower phases obtained at different concentrations of PEG 6000 polymer ranging from 10.0 to 15.0 % (w/w)……………………………………… **184**

4.9 lipase enzyme purification in different aqueous two phase system composed of 8.6% of PO4 and 11.0% of PEG6000 adjusted to different pH values………………………………………………… **188**

4.10 Lipase enzyme partitioning and purification in different aqueous two phase system composed of 8.6% (w/w) of PO_4, 11.0% (w/w) of PEG6000 and different concentrations of NaCl in the range of 1.0 to 5.0% (w/w)……………………………………………….. **190**

4.11 Purification of the *Trichoderma* sp. BW45 lipase enzyme…………………………………………………………….. **193**

4.12 Relative activity of the pure lipase activity in the presence of 50% of various hydrophobic organic solvents…………………………... **201**

4.13 Lipase enzyme relative activity in presence of different concentrations of various hydrophilic organic solvents…………… **201**

4.14 Effects of different metal chloride on the hydrolysis activity of *Trichoderma* sp. BW45 pure lipase enzyme……………………… **213**

4.15 Effect of Tween 80 and Tween 20 on *Trichoderma* sp. BW45 lipase enzyme hydrolysis activity………………………………….. **213**

4.16 *p*-nitrophenyl Mono-acyl ester and triglyceride substrate specificity of *Trichoderma* sp. BW45 pure lipase enzyme.............. **214**

4.17 Effect of the aqueous phase pH on palm oil hydrolysis activity of *Trichoderma* BW45………………………………………………... **230**

4.18 Effect of aqueous phase to non-aqueous phase ratio in the reaction mixture on the hydrolysis degree of palm oil by *Trichoderma* sp. BW45 crude lipase enzyme……………………………………... **233**

4.19 Effect of various palm oil concentrations on the hydrolysis degre... **235**

4.20 Effect of *Trichoderma* sp. BW45 crude lipase enzyme loading into the reaction mixture on palm oil hydrolysis degree……………….. **238**

4.21 Enzyme recovery from the hydrolysis reaction mixture and reusability in another hydrolysis reaction………………………… **253**

4.22 Hydrolysis degree of crude palm oil and refined palm oil by *Trichoderma* sp. BW45 lipase enzyme under the optimum conditions obtained for palm oil hydrolysis……………………….. **254**

LIST OF FIGURES

		Page
2.1	Lipase mechanism in the hydrolysis of lipids………………………….	**43**
3.1	Flow chart of *Trichoderma* sp. BW45 purification process………..	**82**
3.2	Flow chart of the purification steps of *Trichoderma* sp. lipase using ATPS……………………………………………………....	**83**
4.1	Lipase enzyme stability of the bacterial isolate BW16 (a) and the fungal isolate BW45 (b) after exposure to various hydrophobic and hydrophilic organic solvents. …………………………………...	**129**
4.2	The effect of incubation temperature on lipase enzyme activity from *Bacillus megaterium* BW16…………………………………	**140**
4.3	Effect of incubation temperature on lipase enzyme activity from *Trichoderma* sp. BW45…………………………………………….	**140**
4.4	Thermostability of lipase enzyme produced by *Bacillus megaterium* BW16…………………………………………………	**141**
4.5	Thermostability of lipase enzyme produced by *Trichoderma* sp. BW45……………………………………………………………	**141**
4.6	pH effect on lipase enzyme activity produced by *Bacillus megaterium* BW16…………………………………………………	**142**
4.7	pH effect on lipase enzyme activity produced *Trichoderma* sp. BW45……………………………………………………………	**142**
4.8	pH stability of *Bacillus megaterium* BW16 lipase enzyme………..	**143**
4.9	pH stability of *Trichoderma* sp. BW45 lipase enzyme…………….	**143**
4.10	*p*-nitrophenyl fatty acid mono-ester substrate specificity of lipase enzyme secreted from *Trichoderma* sp. BW45 and *Bacillus megaterium* BW16…………………………………………………	**146**
4.11	Triglycerides substrate specificity of lipase enzyme produced by *Trichoderma* sp. BW45. and *Bacillus megaterium* BW16……………………………………………………………	**146**
4.12	Time course of growth and lipase enzyme production by *Bacillus megaterium* BW16…………………………………………………	**147**
4.13	Time course of growth and lipase enzyme production from *Trichoderma* sp. BW45…………………………………………….	**147**
4.14	Time course of lipase enzyme production obtained with 1% (v/v) of different spore suspension concentrations.................................	**152**

4.15	Effect of culture incubation temperature on lipase enzyme production by *Trichoderma* sp. BW45….......................................	**154**
4.16	Agitation rate effect on growth and lipase enzyme production by *Trichoderma* sp. BW45……………………………………………	**154**
4.17	Effect of the initial pH of the fermentation medium on lipase enzyme production of *Trichoderma* sp. BW45……………………	**157**
4.18	Time course of growth and lipase enzyme production by *Trichoderma* sp. BW45 sp. after physical parameters optimization of the fungal culture………………………………………………..	**157**
4.19	Effects of various lipase enzyme inducers (0.3% w/v) on the enzyme production by *Trichoderma* sp. BW45…………………....	**162**
4.20	Effect of Tween 80 concentration on lipase enzyme production…..	**162**
4.21	Effect of various carbon sources (1% w/v) on lipase production…..	**165**
4.22	Effect of starch concentration on lipase production………………..	**165**
4.23	Effect of various organic and inorganic nitrogen sources (1% w/v) on lipase enzyme production by *Trichoderma* sp. BW45………….	**168**
4.24	Effects of yeast extract concentration on lipase enzyme production	**168**
4.25	Effect of various metals on biomass and lipase enzyme production.	**172**
4.26	Effect of NaCl concentration on lipase enzyme production………..	**172**
4.27	Addition of 0.1 % (w/v) of Tween 80 at different time intervals to *Trichoderma* sp. BW45 growth medium…………………………..	**175**
4.28	Time course of biomass and lipase enzyme production by *Trichoderma* sp. BW45 after physical and chemical optimization process………………………………………………………...	**175**
4.29	Binodal curve for PEG6000/Phosphate salt aqueous two phase system at 25 ±1°C…………………………………………………	**180**
4.30	Partitioning of lipase enzyme and contaminates proteins in the two phase system at different concentration of PO_4 ranging from 9.0 to 12.0 % (w/w)……………………………………………………	**182**
4.31	Partitioning of lipase enzyme and contaminates proteins in the two phase system at different concentrations of PO_4 ranging from 8.2 to 9.4 % (w/w)…………………………………………………...	**182**
4.32	Partitioning of lipase enzyme and the contaminate proteins in the phase system at different concentrations of PEG 6000 polymer…..	**184**
4.33	Lipase enzyme and protein partitioning in different aqueous two phase systems composed of 8.6% PO4 and 11.0% PEG6000 adjusted to various pH value………………………………………	**188**

4.34 Lipase enzyme and proteins contaminate partitioning in different aqueous two phase systems composed of 8.6% PO4 and 11.0% PEG6000 and different concentrations (w/w) of solid NaCl……… **190**

4.35 Chromatography of lipase from *Trichoderma* sp. BW45 on Sephadex G-75………………………………………………….. **193**

4.36 Molecular weight estimation of *Trichoderma* sp. BW45 lipase by SDS-PAGE electrophoresis………………………….................. **195**

4.37 Relative activities of lipase enzyme in the presence of different ratio of benzene…………………………………………………… **202**

4.38 Residual enzyme activities after exposure of the immobilized pure enzyme to various hydrophilic and hydrophobic organic solvents under neat conditions…………………………………………… **202**

4.39 Effect of pH on *Trichoderma* sp. BW45 pure lipase enzyme………………………………………………………….. **207**

4.40 Effect of incubation temperature on p-nitrophenyl laurate hydrolysis activity of *Trichoderma* sp. BW45 pure lipase enzyme.. **207**

4.41 Pure lipase enzyme stability pre-incubated at different pH values... **209**

4.42 Pure lipase enzyme stability pre-incubated at different temperatures…………………………………………………….. **209**

4.43 Pure lipase enzyme relative activities in presence of 1.0, 5.0 and 10 mM of 2-Mercaptoethanol, EDTA and SDS…………………… **214**

4.44 Initial time profile of palm oil hydrolysis using crude lipase enzyme produced by *Trichoderma* sp. BW45……………………... **218**

4.45 Palm oil hydrolysis course time in presence of 0.25:1 (v/w) ratio of isooctane to palm oil…………………………………………… **221**

4.46 Palm oil hydrolysis course time in presence of 0.5:1 (v/w) ratio of isooctane to palm oil……………………………………………. **221**

4.47 Palm oil hydrolysis course time in presence of 0.75:1 (v/w) ratio of isooctane to palm oil…………………………………………. **222**

4.48 Palm oil hydrolysis course time in presence of 1:1 (v/w) ratio of isooctane to palm oil……………………………………………… **222**

2.49 Palm oil hydrolysis in presence of 0.5:1 (v/w) ratio of isooctane to palm oil……………………………………………………… **225**

2.50 Palm oil hydrolysis in presence of 0.5 (v/w) ratio of cyclohexane to palm oil…………………………………………………… **225**

4.51 palm oil hydrolysis in presence of 0.5 (v/w) ratio of n-hexane to palm oil……………………………………………………….. **226**

4.52 palm oil hydrolysis in presence of 0.5 (v/w) ratio of benzene to palm oil………………………………………………………….. **226**

4.53 Effect of mixing speed on lipase enzyme catalyze hydrolysis of palm oil (a) 400 rpm; (b) 600 rpm; (c) 800 rpm; (d) 1000 rpm…… **242**

4.54 Effect of operation temperature on *Trichoderma* sp. BW45 crude lipase enzyme hydrolysis catalytic activity of palm oil (a) 25 ±1; (b) 30; (c) 35 and (d) $40^{o}C$………………………………………… **245**

4.55 Effect of Ca^{+2} ion on lipase enzyme hydrolysis activity of palm oil (a) 1.0 mM; (b) 2.5 mM; (c) 5.0 mM……………………………… **248**

4.56 Gas chromatography time course of palmitic acid and oleic acid release in the hydrolysis reaction of palm oil……………………… **253**

LIST OF PLATES

		Page
4.1	Hydrolysis zone formed by the lipolytic enzyme action of the fungal isolate BW45 grown on PDA supplemented with 3% (w/v) tributyrin…………………………………………………………	**111**
4.2	Hydrolysis zone formed by the lipolytic enzyme action of the bacterial isolate BW16 grown on NA supplemented with 3% (w/v) tributyrin…………………………………………………………	**111**
4.3	The hydrolysis zone (HZ) in the tributyrin agar plate supplemented with tributyrin due to the action of BW45 isolate lipase enzyme………………………………………………………..	**115**
4.4	The Florescence zone (FZ) in the rhodamine B agar plates supplemented with (a) Olive oil and (b) Triolein due to the action of BW45 isolate lipase enzyme…………………………………….	**115**
4.5	Scanning electron micrograph of the bacterial isolate BW16 showing the rode shape and the length (2.918 µm) and width (1.137 µm) of the bacterial cell…………………………………….	**131**
4.6	Transmission electron micrograph of negatively stained cell of the bacterial isolates BW16…………………………………………….	**131**
4.7	Fungal isolate BW45 colony growing on PDA plates. The picture shows the growth characteristics………………………………...	**134**
4.8	Light micrograph showing the mycelium of the fungal isolate BW45 bearing repeatedly branched conidiophores………………...	**134**
4.9	Flask-shaped phialides of the conidiophores of the fungal isolate BW45………………………………………………………………	**135**
4.10	Electron micrographs showing phialides of the fungal isolate BW45………………………………………………………………	**135**
4.11	The green globse or sub-globose conidia of the fungal strain BW45………………………………………………………………	**136**
4.12	Light micrograph showing the extensive re-branching of the conidiophores………………………………………………………	**136**
4.13	Electron micrograph showing the conidiophore bearing 4-5 phialides and the cluster of conidia at the tip of the phialides……..	**137**
4.14	Electron micrograph showing the wide main branching of the conidiophores > 10 µm wide…………………………………….	**137**
4.15	Upper phase, interface, and lower phase of the two phase system...	**187**
4.16	SDS-PAGE of the lower phase enzyme solution obtained from the optimized aqueous two phase system………………………………	**194**

4.17 SDS-PAGE of the collected fractions with lipase activity………… **194**

4.18 *Trichoderma* sp. BW45 lipase enzyme focused position on the IPG ready strip……………………………………………………………….. **197**

4.19 Thin layer chromatography of the hydrolyzate of triolein obtained through the action of the pure lipase enzyme……………………… **204**

Penghasilan, penulenan, pencirian dan aplikasi lipase toleran pelarut organik daripada *Trichoderma* sp. BW45 di dalam hidrolisis minyak kelapa sawit

ABSTRAK

Demi menentukan kejayaan proses hidrolisis minyak kelapa sawit secara enzimatik bagi penghasilan asid palmitik dan asid oleik, objektif berikut telah dikenalpasti: Penyaringan mikroorganisma yang berpotensi menghasilkan ekstraselular lipase bertoleransi pelarut organik yang signifikan daripada sampel tanah tempatan, pengoptimuman parameter fisikokimia yang diperlukan untuk mencapai penghasilan lipase yang maximum dalam sistem kelalang bergoncang, penulenan enzim menggunakan sistem dua fasa akueous ditambah dengan gel filtrasi Sephadex G-75, pencirian sifat enzim yang tulen dan aplikasi lipase kasar dalam hidrolisis minyak kelapa sawit bagi penghasilan asid palmitik dan oleik.

Mikroorganisma penghasil lipase dipencilkan daripada sampel tanah tempatan menggunakan agar NA dan PDA yang disuplementasikan dengan tributirin. Kemudian, sejumlah 21 coloni penghasil lipase dikulturkan menggunakan media pemfermentasian kultur tenggelam dan ekstrak kasarnya digunakan untuk penyaringan lipase bertoleransi pelarut organik. Dua pencilan dipilih iaitu pencilan bakteria BW 16 dan pencilan kulat BW 45 berdasarkan kestabilan lipase mereka dalam kehadiran pelbagai pelarut organik hidrofobik. Pencilan bakteria BW 16 dikenal pasti sebagai spesies *Bacillus megaterium* dan pencilan kulat BW 45 pula dikenal pasti sebagai spesies *Trichoderma*. Pencirian perbandingan dari kedua-dua sifat enzim ini mendedahkan bahawa lipase daripada pencilan *Bacillus megaterium* adalah enzim bertoleransi pelarut organik larut air. Sementara itu, enzim lipase yang

dihasilkan oleh *Trichoderma* sp. BW45 adalah enzim bertoleransi pelarut organik yang tidak bercampur dengan air. Aktiviti maksimum lipase daripada *Bacillus megaterium* diperolehi pada 45°C dan pH 9.0. Lipase daripada *Trichoderma* sp. BW45 mempamerkan julat suhu optimum yang lebar untuk aktiviti hidrolisis yang maksimum iaitu di antara 30 hingga 45°C pada pH 6.0. Kedua-dua enzim menunjukkan kestabilan termo yang sederhana. Enzim lipase daripada *Trichoderma* sp. BW45 menunjukkan kestabilan pH pada julat yang lebih lebar iatiu dari pH 4.0 hingga 9.0 berbanding dengan lipase daripada *Bacillus megaterium* BW16 yang menunjukkan kestabilan pH pada julat 7.0 hingga 10.0. Selain daripada ciri-ciri yang dinyatakan di atas untuk *Trichoderma* sp. BW45 dan *Bacillus megaterium* BW16, produktiviti awal yang tinggi bagi enzim lipase daripada *Trichoderma* sp. BW45 (138.8 U/mL) berbanding dengan *Bacillus megaterium* (7.8 U/mL), kecenderungan yang tinggi kepada rantai karbon panjang trigliserida dan kestabilan dalam pelarut organik hidrofobik menjadikan lipase yang dihasilkan daripada *Trichoderma* sp. BW45 sesuai dipilih untuk hidrolisis minyak kelapa sawit. Kesan parameter fizikal dan kimia ke atas penghasilan lipase daripada *Trichoderma* sp. BW45 menggunakan sistem kelalang bergoncang dikaji melalui pengoptimuman berlangkah. Aktiviti lipase yang maksimum sebanyak 621.0±1.3 U/ml dalam kultur filtrat diperolehi selepas 60 jam pengkulturan apabila media fermentasi (pH 8.0) dengan komposisi 0.5% (b/i) kanji, 1% (b/i) ekstrak yis, 0.3% (b/i) Tween 80 dan 0.5% (b/i) NaCl diinokulasikan dengan 1.4 x 10^5 ampaian spora dan dieramkan pada 25°C dan 150 rpm kadar goncangan.

Larutan enzim separa tulen dengan peningkatan 2.78 kali ganda tahap ketulenan dan pemulihan sebanyak 56.4% diperolehi pada fasa bawah kaya garam sistem ATPS yang terdiri daripada 11% (b/b) PEG 6000 dan 8.6% (b/b) penimbal kalium fosfat

(pH 6.0). Penulenan enzim seterusnya ke tahap homogen dicapai melalui kromatografi filtrasi menggunakan Sephadex G-75. Elektrophoresis SDS-PAGE menunjukkan jalur tunggal protein dengan jisim molekul relatif 61 kDa. Jalur kecerunan pH bergerak menunjukkan enzim tulen yang diperolehi mempunyai anggaran nilai *pI* sebanyak 6.2 dan menghidrolisiskan triolein secara rawak. Enzim tulen ini mampu mengekalkan aktiviti asalnya di dalam pelarut organik sikloheksana, n-heksana, n-heptana dan isooktana. Akan tetapi, kehadiran pelarut organik bercampur air mengakibatkan pengurangan aktiviti enzim. Walau bagaimanapun, enzim tulen ini menunjukkan aktiviti maksimum pada julat suhu 30 hingga 40°C dan pH 6.5 dan mampu mengekalkan 100% aktiviti asalnya pada julat pH 4.0 hingga 7.0 dan sehingga 40°C. Tiada kesan penghambatan pada aktiviti enzim diperhatikan ke atas pelbagai logam dan 1.0 mM Mercaptoethanol, EDTA dan SDS. Akan tetapi, penambahan Tween 20 dan Tween 80 pada kepekatan yang berlainan ke dalam campuran reaksi asei menunjukkan kesan penghambatan dan kesan ini bertambah dengan peningkatan kepekatan. Enzim lipase ini menunjukkan keutamaan pada kumpulan asil C4 daripada p-nitrophenyl monoesters. Di samping itu, enzim ini menunjukkan spesifikasi keutamaan yang lebih tinggi terhadap trigliserida, triolein sintetik berbanding dengan tributirin.

Namun, kadar hidrolisis minyak kelapa sawit yang maksimum iaitu sebanyak 94.86±0.58% diperolehi selepas 48 jam apabila 1:1 fasa akueus kepada fasa bukan akueus dikendalikan pada 25±1°C dan 600 rpm. Kepekatan enzim yang optimum pada fasa akueus adalah 434 U/g apabila enzim kasar ini disesuaikan kepada pH 6.0 menggunakan penimbal fosfat dan keadaan optimum pada fasa bukan akueus mengandungi 50 g minyak kelapa sawit yang dilarutkan dalam isooktana pada nisbah 1:0.5 (b/i). Namun demikian, selepas pengoptimuman proses hidrolisis, 233.34 dan

195.02 mg asid palmitat dan asid oleik masing-masing dikesan pada setiap ml fasa bukan akueus menggunakan analisis Kromatografi Gas. Penggunaan semula enzim lipase ini juga diuji dan didapati hampir 93.7% hidrolisis minyak kelapa sawit diperolehi selepas kitar semula pertama.

Production, purification, characterization and application of organic solvent tolerant lipase from *Trichoderma* sp. BW45 in palm oil hydrolysis

ABSTRACT

In order to establish a successful process of enzymatic hydrolysis of palm oil, for the production of palmitic acid and oleic acid, the following objectives have been designated: Screening for potential microorganisms producing a significant amount of extracellular organic solvent tolerant lipase from indigenous soil samples, optimization of the physicochemical parameters required to achieve maximum lipase production in shake flask system, purification of the enzyme using aqueous two phase system coupled with Sephadex G-75 gel filtration, characterization of the pure enzyme properties and application of the crude lipase preparation in palm oil hydrolysis for the production of palmitic and oleic acids.

Microorganisms producing lipase were isolated from indigenous soil samples on NA and PDA plates supplemented with tributyrin. A total of 21 lipolytic colonies were further cultivated in submerged fermentation media and their crude extracts were screened for organic solvent tolerant lipase. Two isolates were selected, bacterial isolate BW16 and fungal isolate BW45, based on their lipase stability in presence of broad range of hydrophobic organic solvents. The bacterial isolate BW16 was identified as *Bacillus megaterium* species and the fungal isolate BW45 was identified as *Trichoderma* species. The comparative characterization of both enzyme properties revealed that isolate *Bacillus megaterium* BW16 lipase as water miscible organic solvent tolerant enzyme. Meanwhile, lipase enzyme produced by *Trichoderma* sp. BW45 as water immiscible organic solvent tolerant enzyme. Maximum activity of

Bacillus megaterium lipase was obtained at 45°C and pH 9.0. *Trichoderma* sp. BW45 lipase enzyme exhibited broader range of optimum temperature for maximum hydrolytic activity in the range of 30 to 45°C at pH 6.0. Both enzymes were revealed a moderate thermostability. *Trichoderma* sp. BW45 lipase enzyme exhibited broader range of pH stability ranging from pH 4.0 to pH 9.0 compared to *Bacillus megaterium* BW16 lipase which exhibited pH stability in the range of 7.0 to 10.0. In addition to the characteristics mentioned above for *Trichoderma* sp. BW45 and *Bacillus megaterium* BW16, high initial productivity of *Trichoderma* sp. BW45 lipase enzyme (138.8 U/mL) compared to *Bacillus megaterium* (7.8 U/mL), the high preference for long carbon chain triglycerides and the stability in the hydrophobic organic solvents made the suitable choice for palm oil hydrolysis is the lipase produced by *Trichoderma* sp. BW45. The physical and chemical parameters effect on lipase production by *Trichoderma* sp. BW45 in shake flask system using stepwise optimization approach was investigated. Maximum lipase activity of 621.0 ± 1.3 U/ml in the culture filtrate was obtained after 60 hr cultivation when the fermentation medium (pH 8.0) composed of 0.5% (w/v) starch, 1% (w/v) yeast extract, 0.3% (w/v) Tween 80 and 0.5% (w/v) NaCl was inoculated with 1.4 x 10^{5} spore suspension and incubated at 25°C and 150 rpm agitation speed.

Partially pure enzyme solution with 2.78 folds enhancement in lipase purity and total recovery of 56.4% was obtained in the bottom salt-rich phase using ATPS composed of 11% (w/w) PEG 6000 and 8.6% (w/w) potassium phosphate buffer (pH 6.0). Further purification of the enzyme to homogeneity level was achieved by filtration chromatography using sephadex G-75. SDS-PAGE electrophoresis showed a single band protein with a relative molecular mass of 61 KDa. The purified enzyme exhibited a *pI* value approximately equal to 6.2 using immobilized pH gradient strip

and the enzyme displayed random regio-specificity in triolein hydrolysis. The pure enzyme maintained the original activity after exposure to cyclohexane, n-hexane, n-heptane and isooctane. Meanwhile, with water miscible organic solvents the enzyme exhibited reduction in the activity. However, the pure enzyme showed maximum activity in the temperature range of 30 to 40°C and pH 6.5 and the enzyme maintained 100% of the original activity in pH range of 4.0 to 7.0 and up to 40°C. No inhibitory effects on the enzyme activity were observed when various metals and 1.0 mM of 2-Mercaptoethanol, EDTA and SDS were added. On the other hand, addition of different concentrations of Tween 20 and Tween 80 to the assay reaction mixture showed inhibitory effects on the enzyme activity and this inhibition in the enzyme activities was increased with an increase in the concentration. The lipase showed a preference to C4 acyl group of the *p*-nitrophenyl monoesters. On the other hand, the enzyme showed higher preference specificity towards the synthetic triglyceride triolein compared to tributyrin.

Maximum hydrolysis degree of palm oil of 94.86% ±0.58 was achieved after 48 hr hydrolysis when 1:1 aqueous to non-aqueous phase was operated at 25 ±1°C and 600 rpm stirring speed. The optimum concentration of enzyme in the aqueous phase was found to be 434 U crude enzyme per gram palm oil adjusted to pH 6.0 using phosphate buffer and the optimum non-aqueous phase contained 50 g of palm oil dissolved in isooctane in the ratio of 1:0.5 (w/v). Nevertheless, after optimization of the hydrolysis process, 233.34 and 195.02 mg of palmitic acid and oleic acid, respectively were detected in each mL of the non-aqueous phase using Gas Chromatography analysis. The reusability of lipase was tested and it was found that nearly 93.7% palm oil hydrolysis was achieved after the first recycle.

CHAPTER ONE

INTRODUCTION

Enzymes are novel biocatalysts; represent the key tool in industrial biotechnology and always contributes to clean industrial products and processes (Drepper *et al*., 2006). Compared to chemicals, enzymes are specific in their action, selective to their substrate, often carry out reactions which are not even possible with conventional chemistry and they are compatible with the environment (Schäfer *et al*., 2007). Among the most important biocatalysts carrying out novel reactions in both aqueous and non aqueous media, lipases stand out due to their versatility, regio and enantioselectivity, wide spectrum of substrates specificity, high stability towards extreme temperatures, pH and organic solvents (Ota *et al*., 2000; Bruno *et al*., 2005; Torres-Gavilán *et al*., 2006; Freitas *et al*., 2007).

Lipases (carboxyl ester hydrolases E.C. 3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of carboxyl ester bonds in triacylglycerols at the oil-water interface (Cygler and Schrag, 1997)**,** and more specifically defined as long-chain fatty acid ester hydrolases, with "long-chain fatty acid" meaning aliphatic acids, saturated or unsaturated, with twelve or more carbon atoms with glycerol as alcohol moiety. They are usually distinguished from carboxyl esterases (EC 3.1.1.1) by their substrate spectra, i.e., esterases prefer water-soluble substrates and lipases show significantly higher activity towards their natural substrates, triglycerides (Ghanem and Aboul-Enein, 2005).

Lipases may be isolated from animals, plants and microorganisms. Plant lipases are not used commercially while lipases originated from animals and microorganisms are

used extensively (Savitha *et al*., 2007). Microorganisms are considered a good source of industrial enzymes for the great diversity of enzymes that have been found (Alves *et al*., 2002) and their potential for large scale production (Hasanuzzaman *et al*., 2004). A considerable number of lipases produced by bacteria, yeast and fungi have been isolated, the latter being preferable because fungi generally produce considerable amounts of extracellular enzymes, which facilitates recovery of the enzyme from the fermentation broth (Lima *et al*., 2004a). Most fungi are known to produce several extracellular enzymes simultaneously as survival tools in extreme environments (Dalbøgea, 2006). They are known to be more potent extracellular lipase producers compared to other species of bacteria and yeast (Choo *et al*., 1998). *Candida cylindracea*, *Aspergilus niger*, *Humicola lanuginose*, *Mucor miehei* and several *Rhizopus* species were reported as major commercial producers of lipases (Saxena *et al*., 2004). Commercial production of lipolytic enzymes from microorganisms have been performed in submerged fermentation usually by batch or fed batch fermentation (Babu and Rao, 2007).

Lipases constitute a very important group of biocatalysts for biotechnological applications in the detergent formulation (Saisubramanian *et al*., 2006), food processing (Olempska-Beer *et al*., 2006), flavour esters synthesis (De los Ríos *et al*., 2008), biocatalytic resolution of pharmaceuticals (Yadav and Dhoot, 2009), bioactive fatty amide derivative synthesis (Khare *et al*., 2009), as biosensor (Fernandez *et al*., 2008), in biodiesel production (Shah and Gupta, 2007), bioremediation of hydrocarbons (Gaur *et al*., 2008a), cosmetics industry (Yadav and Dhoot, 2009), perfumery (Fujiwara *et al*., 2006), etc. However, lipids constitute a large part of organic materials, and lipolytic enzymes play an important role in the turnover of these water-insoluble substrates (Hasan *et al*., 2006). The rapid increase in palm oil

production in the last 20 years has made palm oil the most important oil in the world (Rupilius and Ahmad, 2007). Both Malaysia and Indonesia are the world's largest exporters of palm oil, commanding more than three-quarters of the world market and Malaysia herself accounted for 47.9% of the production and 57.5% of the trade (Simeh and Kamarudin, 2009). The high productivity and the low cost of production of palm oil and palm kernel oil are moving the centre of gravity of the oleochemicals industry towards South East Asia (Rupilius and Ahmad, 2007). The oleochemical industry in Malaysia started in the early 1980s and it comprises of the basic oleochemicals and oleochemical derivatives. The basic oleochemicals produced are fatty acids, fatty alcohols, methyl esters and glycerine [Chemical Industries Council of Malaysia (CICM), 2009]. The hydrolysis of triglycerides to yield free fatty acids and glycerol represents an important group of chemical reactions (Liu *et al.*, 2008a). Hydrolysis is the principle reaction for the production of free fatty acids, one of the basic oleochemicals that may then be inter-esterified, trans-esterified, or converted into high-value fatty alcohols (Rooney and Weatherley, 2001).

1.1 Rational and research objectives

Industrial production of fatty acids from oils and fats are continuously carried out by conventional chemical splitting which is environmental unfriendly. It also needs high pressure and energy (Albasi and Riba, 1997). In addition, the resultant fatty acids consist of undesirable compounds. Therefore, an alternative method is needed. Enzymatic hydrolysis of fats using lipases offers an alternative method for fatty acid production. Lipases have distinct advantages compared to classical chemical catalysts because they function under mild reaction conditions. Thus, the formation of side product is minimum, little or no thermal degradation of the products and yielding products of higher quality in terms of colour and flavour (Rakshit *et al.*, 2000; H-

Kittikun *et al*., 2000; Albasi and Riba, 1997). In tropical countries, the preferred raw material for basic oleochemicals production is palm oil (Rupilius and Ahmad, 2007). Fu *et al*. (1995) reported that the solidifying point of palm oil is relatively high (47°C) because it contains large amounts of saturated long-chain fatty acids and only when dissolved in organic solvents could be enzymatically hydrolyzed at 37°C. Therefore, organic solvents are needed in the enzymatic hydrolysis process of palm oil. Hence, in order to establish a successful process of enzymatic hydrolysis of palm oil in the presence of organic solvent, organic solvent tolerant lipase enzyme is needed. According to Hermansyah *et al*. (2007), in order for industrial utilization of enzymes for hydrolysis reaction of triglycerides, it is important to elucidate that: screening of lipases for high activity, selection of solvents which enhance enzyme activity and the factors affecting the hydrolysis behavior and kinetic studies of the hydrolysis process. Thus, the objectives of the current research are as follows:

i. Screening for potential microorganisms producing a significant amount of extracellular organic solvent tolerant lipase from indigenous soil samples

ii. Optimization of the physicochemical parameters required to achieve maximum lipase production in shake flask system

iii. Purification of the enzyme using aqueous two phase system coupled with Sephadex G-75 gel filtration and characterization of the pure enzyme properties

iv. Application of the crude lipase preparation in the hydrolysis of palm oil in organic-aqueous system for the production of palmitic and oleic acids

1.2 Research scope

Soil samples were collected from different locations in Penang, Malaysia and screened for organic solvent tolerant extracellular lipase producing microorganisms in two steps protocol. In the first step, tributyrin agar plates were used to select the potential extracellular lipase producers based on the qualitative observations on the hydrolysis zones around the microbial colonies. In the second step, the selected microorganisms were further grown in liquid media, and the supernatant was used as the source of the crude enzyme preparation. Using the crude enzyme solution, the activity and stability of lipases produced by the selected isolates were investigated in the presence of 20% (v/v) of various hydrophobic organic solvents. The activity and stability of the lipases produced by the selected isolates obtained from the first organic solvent tolerance experiments were further investigated using different concentrations of various hydrophobic and hydrophilic organic solvents. The potential producers were then identified to the genus and species level.

Comparative characterization between the two crude lipases obtained from BW16 and BW45 isolates was conducted, including the stability and activity of both enzyme preparations as functions of temperature and pH, substrate specificity and time course profile to evaluate the enzyme production level of both isolates. Sequential parametric optimization of lipase production by the selected isolate was carried out in shake flask system to evaluate the effect of various physical and chemical parameters on the enzyme production. Purification of the lipase was carried out using the aqueous two phase system (ATPS) composed of polyethylene glycol 6000 polymer (PEG6000) and potassium phosphate salt, followed by gel filtration chromatography using Sephadex G-75. Characteristics of the purified enzyme: *pI* value, enzyme activities and stabilities in the presence of various concentrations of different hydrophilic and

hydrophobic organic solvents were determined. Positional specificity of the enzyme was determined by analyzing the hydrolyzate of triolein by TLC. Relative activity and stability of the pure enzyme was also studied as functions of temperature and pH. Various divalent cations were tested for their effect on the catalytic activities of the pure enzyme. The relative activities of lipase in the presence of 1.0 mM of different chemicals and surfactants namely, 2-Mercaptoethanol, EDTA, SDS, Tween 80 and Tween 20 were also evaluated. Substrate specificity of the pure lipase was also investigated with various *p*-nitrophenyl fatty acid monoesters and triglycerides.

The crude lipase preparation was used in palm oil hydrolysis process in biphasic organic-aqueous system. Hydrolysis profiles of palm oil were carried out for five days without the addition of organic solvents. In order to enhance the hydrolysis degree, palm oil hydrolysis was investigated in the presence of different volume to weight ratio of isooctane to palm oil. Four organic solvents namely isooctane, cyclohexane, n-hexane, and benzene were tested for their effect on the enzymatic hydrolysis of palm oil. In addition, the pH value of the aqueous phase, effect of aqueous to non-aqueous ratio of the reaction mixture, palm oil concentration, enzyme concentration, mixing speed, temperature and the addition of Ca^{2+}, recovery and reusability of the crude lipase were also investigated. The hydrolytic products, mainly the content of the total oleic and palmitic acid, were analyzed at different time intervals using gas chromatography. Comparison on the hydrolysis degree of crude palm oil and refined palm oil under the optimum conditions was also studied.

CHAPTER TWO

LITERATURE REVIEW

2.1 Industrial enzymes

The practical uses of industrial enzymes during the ancient civilizations were established from their historical records. These practical uses include conversion of alcohol to acetic acid (vinegar) for food preservation and preparation as well as for medical purposes, in wine making, cheese preparation by the action of number of enzymes in the extract from fig trees (ficin) and from the lining of the fourth stomach of multiple-stomach animals (rennin), leavening of bread by the action of the yeast and meat tenderizing by the papaya fruit extract (Copeland, 2002).

Even though the action of enzymes has been recognized and used throughout history, it was quite recently that their importance was realized. In the nineteenth and twentieth centuries, scientists began to study the action of enzymes in more systematic fashion. In 1833, an aqueous solution from malt extract contain a working principle that could convert starch into sugar was obtained (Copeland, 2000; Buchholz and Poulsen, 2000). In 1857, the work by Pasteur showed that the fermentation of sugar is closely associated with live yeast by the action of the soluble ferments, later were labeled enzymes by Kuhne in 1878. Concrete evidence for this assumption was provided by Buchner and his brother Hans in 1897, as they showed that the cell free extract from yeast cells could also produce alcohol from sugar. In 1926, the enzyme urease was purified by James Sumner in America and showed that it was a protein. During the next few years, more enzymes were purified and crystallized by another American biochemist, Northrup and showed them likewise to be proteins (Aehle, 2004).

Over the following decades, many enzymes were isolated from microorganisms, plants and animals and their characteristics were extensively studied. The growing fundamental knowledge about the mechanism of action, structure, substrate specificity and many other biochemical characteristics of enzymes have attracted attention from researchers all over the world and new ideas about their practical applications have emerged. Till 2003, more than 3000 different enzymes have been identified and many of them found their way into certain industrial applications (Burg, 2003). In industrial use, by far the most important group of enzymes is the hydrolases. For example, amylases are important enzymes in starch processing industries for the hydrolysis of polysaccharides (Baks *et al*., 2008) and played a significant role in the beverage and paper industries (Schwab *et al*., 2007), in detergent formulation (Mitidieri *et al*., 2006) and textile de-sizing (Fan *et al*., 2008). Proteases were involved in laundry detergent formulation (Rao *et al*., 2009), as bating agent for producing high quality leather (Zambare *et al*., 2007) and peptide synthesis (Kumar and Bhalla, 2005). The major current industrial applications of cellulases and xylanases is in the pulp and paper industry, as animal feed additives, in food processing, laundry detergent formulation, bioethanol production and in the textile industry (Hasan *et al*., 2006). The main application of phytases is still as a feed supplement (Cheng and Hardy, 2004; Cao *et al*., 2007) to improve phosphorus bioavailability in plant feed of pigs, poultry and fish. Some practical applications of laccases includes dechlorination of bleached kraft pulp (Ünal and Kolankaya, 2001), removal of phenolic compounds from wine, decolorization of dyes, drug analysis and ethanol production (Cavallazzi *et al*., 2004). Tannases have wide applications as a clarifying agent in fruit juices industry and coffee-flavored soft drinks, manufacture of instant tea, production of gallic acid and treatment of wastewater contaminated with polyphenolic compounds (Belmares *et al*., 2004).

Lipolytic enzymes represent one of the most important groups of the hydrolytic enzymes as catalysts in several industries. Lipolytic activity was detected nearly 109 years ago (1901) by C Eijkmann (Jaeger *et al*., 1999; Hasan *et al*., 2006). Since then, research on lipolytic enzymes has been driven by their central roles in lipid metabolism and signal transduction (Lotti and Aiberghina, 2007). After proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volume (Liu *et al*., 2008b). Microbial lipases have already set up their huge potential uses in various industries with the detergent industry as the biggest market for their application (Bora and Kalita, 2009). In addition, more than 20% of the bio-transformations processes in organic synthesis are performed with lipases (Kashmiri *et al*., 2006). Lipases are unique as they hydrolyze fats into fatty acids and glycerol at the water-lipid interface and can reverse the reaction in non-aqueous media. Due to their versatile novel characteristics, lipases found wide spectrum of industrial applications in oleochemical industry, in detergent formulation, leather and textile industry, pharmaceutical industry, fine chemical production, food processing and as ingredient in the animal feed (Jaeger and Reetz, 1998; Hasan *et al*., 2006; Schäfer *et al*., 2007).

Industrial enzymes are now offered by many manufacturers all over the world. In 2008, Battery Control Centre (BCC, USA) published a report entitled "Enzymes for Industrial Applications" on the global industrial enzymes market. According to the report, the global market for industrial enzymes increased from $2.2 billion in 2006 to an estimated $2.3 billion by the end of 2007. It should reach $2.7 billion by 2012. The greatest growth rate is expected to be used in the animal feed formulation particularly by the increased use of phytase enzymes (Table 2.1). In 1991, the worldwide lipase market was reported to be about 3% of the total enzyme market

(Moses *et al*., 1999). In 2006, lipase market was increased and reached about 5% of the market and is expected to increase rapidly in the face of a growing number of commercial applications (Vakhlu and Kour, 2006).

Table 2.1 Summary of enzyme market based on application sector ($ Million).

Application sector	2005	2006	2007	2012	CAGR% 2007-2012
Technical Enzymes	1,075	1,105	1,140	1,355	3.5
Food Enzymes	775	800	830	1,010	4.0
Animal Feed Enzymes	240	260	280	375	6.0
Total	2,090	2,165	2,250	2,740	4.0

CAGR; Compound Annual Growth Rate.

2.2 Microbial lipases: Sources, production and purification

2.2.1 Sources of microbial lipases

Lipases are diverse and ubiquitous group of enzymes widely distributed in nature and produced almost by all living organisms. Even though most of the developed work has focused on microbial lipases (De María *et al*., 2006), lipases from non-microbial sources such as plant and animal origins were also been isolated, characterized and described as useful biocatalysts for several applications (Abdelkafi *et al*., 2009). Lipases from plants have been obtained from various plant tissues and extracts (Fiorillo *et al*., 2007). On the other hand, lipases from animal origin have been produced and used more largely in various industrial applications compared to the plant lipases (Kilcawley, 2006). In 1960, about 70% of the enzymes were extracted from plant tissues or exudates and animal organs and the remaining percentage originated from microbial sources. Twenty years later the situation had reversed and over 85% of the industrial enzymes in the market were produced from microbial sources. Today, animal derived enzymes represent about 10% of the total enzyme

market while plant derived enzymes roughly represent 5% of total market (Illanes, 2008). Most lipases extensively used in laboratory, pilot or industrial scale are almost exclusively from microbial origin mainly bacterial and fungal lipases (Joshi *et al*., 2006; Dutta and Ray, 2009). The majority of known lipolytic enzymes are from bacterial origin (Meilleur *et al*., 2009). Most bacterial lipases are sourced from *Pseudomonas*, *Burkholderia*, *Alcaligenes*, *Acinetobacter*, *Bacillus* and *Chromobacterium* species (Gupta *et al*., 2007), while widely used fungal lipases are produced by *Candida*, *Humicola*, *Penicillium*, *Yarrowia*, *Mucor*, *Rhizopus* and *Aspergillus* sp. (Colen *et al*., 2006; Vakhlu and Kour, 2006). Several lipase preparations are commercially available and can be purchased from several manufacturers such as Novozyme, Amano Enzyme, Altus, Biocatalytics, Fluka and Sigma, which is suitable for many industrial applications (Kademi *et al*., 2006).

The increasing potential of the industrial applications of lipases resulted in an increasing demands for new lipolytic enzymes with different characteristics, which in place stimulate the isolation and selection of new novel lipases. Recently, the search for enzymes from organisms adapted to life in extreme environments has enriched the spectrum with thermostable, cold active, alkalophilic, acidophilic, halophilic and organic solvent tolerant lipases. Thermostable lipases are usually derived from thermophilic and mesophilic microbial strains and expected to produce intrinsically more heat-stable enzymes than their mesophilic counterparts. Thermostable lipases were reported from *Rhizopus oryzae* (Hiol *et al*., 2000), *Aspergillus carneus* (Saxena *et al*., 2003a), *Aspergillus niger* (Namboodiri and Chaltopadhyaya, 2000), *Geotrichum-like strain* (Ginalska *et al*., 2004), several *Bacillus* species (Ghanem *et al*., 2000; Sinchaikul *et al*., 2001; Kambourova *et al*., 2003; Bora and Kalita, 2008; Dutta and Ray, 2009), *Pseudomonas* (Kulkarni and Gadre, 1999; Rathi *et al*., 2000;

Sharma *et al*., 2001a) and Actinomyces (Al-khudary *et al*., 2004). In contrast, cold active lipases are enzymes produced by a psychrotroph or mesophilic microorganism which exhibit higher catalytic activities at low temperatures than their mesophilic and thermophilic counterparts do (Joseph *et al*., 2008; Cai *et al*., 2009). Compared to other lipases, relatively smaller number of cold active bacterial and fungal lipases was well studied (Joseph *et al*., 2007 and 2008). Several examples of lipases from cold adapted microorganisms are *Aspergillus nidulans* (Mayordomo *et al*., 2000), *Pseudomonas fluorescens* (Preuss *et al*., 2001), *Candida Antarctica* (Zhang *et al*., 2003) *Aeromonas* sp. LPB4 (Lee *et al*., 2003), *Staphylococcus epidermidis* (Joseph *et al*., 2006), *Microbacterium phyllosphaerae* (Joseph *et al*., 2008) and *Geotrichum* sp. SYBC WU-3 (Cai *et al*., 2009). Cold active alkaline lipases were also isolated from *Pseudomonas* sp. Strain B11-1(Choo *et al*., 1998) and marine *Pseudomonas* sp. MSI057 (Kiran *et al*., 2008).

Most of the lipases used in biocatalysis have neutral or alkaline pH optima and less common are acidic lipases, however some lipases from *Bacillus* sp. are active over a broad pH range (Gupta *et al.*, 2004; Lotti and Alberghina, 2007). Alkalophilic lipases were produced by moderate and extreme alkalophiles (Sidhu *et al*., 1998; Ghanem *et al*., 2000; Sharma *et al*., 2002; Joshi *et al*., 2006; Dutta and Ray, 2009) as well as neutrophiles (Chen *et al*., 1998; Rathi *et al*., 2000; Rathi *et al*., 2001; Gupta *et al*., 2007). The production of thermo-active alkaline lipase have been reported from *Bacillus coagulans* BTS-3 (Kumar *et al*., 2005), *Staphylococcus aeureus* (Horchani *et al*., 2009), *Bacillus* sp. (Wang *et al*., 1995; Sidhu *et al*., 1998; Sharma *et al*., 2002; Bora and Kalita., 2008), *Bacillus alcalophilus* (Ghanem *et al*., 2000), *Pseudomonas* sp. (Kulkarni and Gadre, 1999; Rathi *et al*., 2000), *Bacillus cereus* C7 (Dutta and Ray, 2009), *Pseudomonas aeruginosa* (Karadzic *et al*., 2006) and *Burkholderia*

cepacia (Rathi *et al*., 2001). Alkaline lipases with optimal activity at moderate temperature were also isolated from *Acinetobacter radioresistens* (Chen *et al*., 1998), *Corynebacterium paurometabolum* (Joshi *et al*., 2006) and *Pseudomonas mendocina* PK-12CS (Jinwal *et al*., 2003). On the other extreme, acidic lipases which are active at extremely acidic pH (pH 1.5-2.0) were usually reported from mammalian sources such as gastric lipase (Miled *et al*., 2000). There are not many reports on microbial lipases that are active at extremely acidic pH because most of the research efforts have been focused on alkaline lipases (Sharma *et al*., 2001a). To date, only a few articles can be found regarding acidic lipases. Bradoo *et al*. (1999a) reported the isolation and characterization of two acido-thermo tolerant lipases from new variants of *Bacillus* spp. Mahadik *et al*. (2002) also reported the production of acidic lipase active at pH 2.5 by *Aspergillus niger* in solid state fermentation. Nevertheless, another group of lipases produced by extremophiles is the halophilic lipases. The existence of halophilic lipases from halophilic bacteria was reported as early as 1970 by González and Gutiérrez. Recently, a few halophilic lipases were isolated from halophilic bacteria (Birbir *et al*., 2004; Boutaiba *et al*., 2006; Amoozegar *et al*., 2008; Rohban *et al*., 2009; Moreno *et al*., 2009).

In nature, enzymes function in aqueous solutions. Therefore, it is not surprising that all enzymatic studies were predominantly performed in water or buffers as the reaction medium. However, this believe has been changed when Zaks and Klibanov (1985) pointed out that enzymes can also be active in organic solvents. The advantages of conducting enzymatic conversions in organic solvents can be seen in the high solubility of most organic compounds in non-aqueous media, the ability to carry out new reactions impossible in water, the greater stability of enzymes and the relative ease of product and enzyme recovery (Zaks and Klibanov, 1985). Therefore,

organic solvent tolerant enzymes become essential requirement in organic synthesis industry. The major limitation in carrying out the enzymatic reactions under water-restricted environment is the tendency of hydrophilic and hydrophobic organic solvents to strip off the essential water molecules from the enzyme surface especially near or at the enzyme active site leaving the enzyme inactive (Yang *et al*., 2004). One of the most biotechnologically important properties found in lipases are their high stability in presence or even in neat organic solvents (Hasan *et al*., 2006). Unfortunately the commercially available lipase preparations were always suffered from limited stability in organic solvents. Due to the limited stability (Ogino, 2008) several attempts have been proposed to improve the performance and stability of this enzymes in organic solvents such as immobilization (Knezevic *et al*., 2002), physical and chemical modifications of the enzymes by amphipathic molecules, lipids and/or surfactants (Okahata and Mori, 1997; De Santis and Jones, 1999; Koops *et al*., 1999; Ogino and Ishikawa, 2001), protein engineering (Magnusson *et al*., 2005) and co-lyophilization with non-buffer salts (Mine *et al*., 2003).

Alternatively, it has been proposed that instead of modifying the enzyme for increasing solvent stability, it would be more desirable to screen for naturally evolved solvent tolerant lipases which can be obtained from the natural biodiversity (Ogino, 2008). Organic solvent stable lipases have been found among the enzymatic systems of many microorganisms including bacteria and fungi. Most of the reported naturally occurring organic solvent tolerant lipases were derived from non screening programmes and few attempts have been made to screen for organic solvent tolerant bacteria producing organic solvent tolerant lipases (Ogino *et al*., 1994; Fang *et al*., 2006; Dandavate *et al*., 2009; Shu *et al*., 2009). Solvent-tolerant microbes are a newly emerging class that posses the unique ability to thrive in the presence of organic

solvents. Their enzymes are adapted to mediate cellular and metabolic processes in a solvent-rich environment and are logically stable in the presence of organic solvents (Gupta and Khare, 2009). Organic solvent tolerant bacteria such as *Pseudomonas aeruginosa* LST-03, *Bacillus sphaericus* 205y, *Staphylococcus saprophyticus* M36 and *Pseudomonas aeruginosa* strain PseA were found to be potential producers of the organic solvent stable lipases (Hun *et al*., 2003; Fang *et al*., 2006; Mahanta *et al*., 2008). So far, most of bacterium-produced organic solvent tolerant lipases belong to the genus *Pseudomonas* and *Bacillus* (Hun *et al*., 2003; Fang *et al*., 2006; Zhang *et al*., 2009).

In general, the isolated organic solvent tolerant lipases were characterized to be either stable in presence of water-immiscible, water-miscible or both organic solvents. Lipases produced by *Pseudomonas* sp. strain S5; thermophilic *Bacillus* sp. strain 42, *Staphylococcus saprophyticus* strain M36, *Pseudomonas aeruginosa* strain PseA, *Burkholderia multivorans* V2 (BMV2) were found to be stable in several hydrophobic organic solvents such as benzene, diethyl ether, isooctane, toluene, n-hexane, cyclohexane, 1-octanol, hexadecane, tetradecane and decanol (Baharum *et al*., 2003; Rahman *et al*., 2006; Eltaweel *et al*., 2005; Fang *et al*., 2006; Mahanta *et al*., 2008; Dandavate *et al*., 2009) and sometime showed certain degree of stimulation in their lipolytic activity (Rahman *et al*., 2006). Several examples were also exist in literatures regarding water-miscible organic solvent tolerant lipases originated from bacteria such as the lipase produced by *Pseudomonas* sp. AG-8 and *Bacillus thermoleovorans* CCR11 which showed good stability after incubation in strong dehydrating solvents like acetone, methanol, dimethylsulphoxide, 2-propanol and ethanol (Sharma *et al*., 2001a; Castro-Ochoa *et al*., 2005). Furthermore, immediate increase in the lipolytic activity of the lipase from *Bacillus thermoleovorans* CCR11

was observed when 70% (v/v) methanol was added to the lipase mixture (Castro-Ochoa *et al*., 2005). Other bacterial lipases showed stability in the presence of both polar and non-polar organic solvents. For example, lipase preparations from *Bacillus megaterium*, *Cryptococcus* sp. S-2, *Pseudomonas aeruginosa*, *Staphylococcus caseolyticus* EX17, *Serratia marcescens* ECU1010 and *Pseudomonas fluorescens* JCM5963 were found to be highly tolerant to water-immiscible and water-miscible organic solvents (Lima *et al*., 2004b; Kamini *et al*., 2000; Karadzic *et al*., 2006; Volpato *et al*., 2008; Xu *et al*., 2008; Zhao *et al*., 2008; Zhang *et al*., 2009). In addition, several fungal species were also reported to produce organic solvent stable lipases such as the lipase from *Rhizopus oryzae*, *Fusarium solani* FS1, *Penicillium aurantiogriseum* and *Penicillium chrysogenum* 9' which was more stable in hydrophobic solvents than hydrophilic ones (Essamri *et al*., 1998; Hiol *et al*., 2000; Maia *et al*., 1999; Lima *et al*., 2004a; Bancerz *et al*., 2005). On the other hand, the mycelium-bound lipase produced by *Aspergillus niger* MYA 135 showed high stability in the presence of water-miscible organic solvents such as methanol, ethanol, propanol, and acetone and in contrast, butanol, hexanol, n-hexane, and heptane caused a reduction in the enzymatic activity (Romero *et al*., 2007). Meanwhile, the purified lipase of *Galactomyces geotrichum* Y05 exhibited significant tolerance in presence of both hydrophilic and hydrophobic organic solvents such as glycerol, methanol, isooctane and n-hexane (Yan *et al*., 2007).

2.2.2 Lipase production

Most studies on lipolytic enzymes production by bacteria, fungi and yeasts have been performed in submerged fermentation (Babu and Rao, 2007) and less reports were found on lipase synthesis mainly by fungus in solid state fermentation (Babu and

Rao., 2007; Vardanega *et al*., 2008; Sun *et al*., 2009a) and even lesser regarding bacterial lipases production by SSF (Alkan *et al*., 2007; Mahanta *et al*., 2008; Bora and Kalita, 2009). Since the late 1980s lipases have been commercially available in the market for detergent formulation. Lipolase from Novo Nordisk was the first lipase to be produced by recombinant DNA technology. The lipase gene was isolated from a strain of the filamentous fungi *Humicola* and then transferred to *Aspergillus oryzae*, which is more readily cultivated in submerged fermentation (Waites, 2005). Nowadays, lipase enzyme preparations are commercially available from different manufacturers such as Novozyme, Amano Enzyme, Altus, Biocatalytics, Fluka and Sigma-Aldrich and are suitable for many industrial applications (Hayes, 2004). Microbial lipases are the most used as biocatalysts in biotechnological applications and organic chemistry. Fungal lipases from *Candida rugosa*, *Candida antarctica*, *Geotrichum*, *Mucor*, *Rhizopus*, *Thermomyces lanuginosus* and *Rhizomucor miehei* and bacterial lipases from *Burkholderia cepacia*, *Pseudomonas mendocina*, *Pseudomonas alcaligenes* and *Chromobacterium viscosum* are examples of commercially available lipases (Jaeger and Reetz, 1998; Illanes, 2008). Fungal and bacterial lipases as well as most other industrial enzymes can be obtained either by submerged (SmF) or by solid-state (SSF) fermentations. However, most of the studies conducted for the production of microbial lipases used submerged fermentation process (Luciana *et al*., 2007; Babu and Rao, 2007), while fed-batch is the preferred operational mode for getting high lipase productivity (Illanes, 2008).

The productions of lipases were often found to be growth related and reached the peak in the late stage of growth (Lotti and Alberghina, 2007; Camacho *et al*., 2009). The production of microbial lipases were reported to be intracellular (Ertuğrul *et al*., 2007; Teng *et al*., 2009), extracellular (Kumar and Gupta, 2008; Krügener *et al*.,

2009), mycelium (Loo *et al*., 2007) or cells bound lipase (Gandolfi *et al*., 2000) and recently it is also found in the spore coat of *Bacillus subtilis* (Masayama *et al*., 2007). However, from the industrial point of view the secreted extracellular lipases were studied more extensively compared to the others (Gandolfi *et al*., 2000). Therefore, many studies have been undertaken to define the optimal culture and nutritional requirements for extracellular lipase production in submerged fermentation by step to step or statistical optimization strategy (Eltaweel *et al*., 2005; Teng and Xu, 2008). Generally, lipase production is strongly induced by a wide range of fermentation parameters such as nitrogen and carbon sources, inoculum size, pH, temperature, agitation, dissolved oxygen concentration and presence of lipids and their derivatives as inducers as well as additional carbon source (Gupta *et al*., 2004). Lipases were found to be greatly induced by the presence of lipids and their derivatives as carbon sources (Bradoo *et al*. 1999a; Rathi *et al*. 2001). For example, olive oil was found to be the best carbon source that supports the highest lipase production from *Yarrowia lipolytica* 681, *Bacillus* sp. strain 42, *Metarhizium anisopliae*, *Corynebacterium paurometabolum* and *Burkholderia multivorans* V2 (Corzo and Revah, 1999; Eltaweel *et al*., 2005; Silva *et al*., 2005; Joshi *et al*., 2006; Dandavate *et al*., 2009). Other natural triglycerides such as corn oil, vegetable oil refinery, sesame oil, groundnut oil, mustard oil, soybean oil and sunflower oil when used as both carbon sources and inducers in the fermentation medium of *Yarrowia lipolytica* 681, *Aspergillus terreus*, *Penicillium citrinum*, *Fusarium solani* FS1, *Candida rugosa*, *Pseudomonas fluorescens* NS2W, *Bacillus coagulans* BTS-3 and *Corynebacterium paurometabolum* MTCC 6841 gave the highest lipase production (Corzo and Revah, 1999; Gulati *et al*., 1999; Miranda *et al*., 1999; Maia *et al*., 2001; Kulkarni and Gadre, 2002; Kumar *et al*., 2005; Joshi *et al*., 2006; Savitha *et al*., 2007). On the other hand, when synthetic triglycerides such as tributyrin and triolein used as the carbon

source in the fermentation medium of *Candida rugosa* with oleic acid as the control, the results showed that the short-chain glycerol tributyrate was not a good carbon source for growth and gave lower level of lipase activity, while glycerol trioleate gave the highest extracellular lipase activity (Wei *et al*., 2004). In addition, when compared to other lipidic carbon sources, surfactants and fatty acids were also found to support the highest lipase production from several microorganisms. For example, maximum lipase production by *Acinetobacter radioresistens* and *Alcaligenes* sp. (ATCC 31371) were obtained using Tween 80 and Polyoxyethylene (20) sorbitan tristearate, respectively (Li *et al*., 2001; Mori *et al*., 2009). Among all the fatty acids, oleic acid usually was found to be the best fatty acid to support the highest lipase production by *Candida rugosa*, *Yarrowia lipolytica* and *Rhizopus arrhizus* as compared to other fatty acids (Gordillo *et al*., 1998; Montesinos *et al*., 2003; Fickers *et al*., 2004; Li *et al*., 2006). Besides, other fatty acids were also reported to be efficient carbon sources for the production of lipases; for example, palmitic acid gave the highest lipase production from *Candida rugosa* (Dalmau *et al*., 2000). Ito *et al*. (2001) observed that the addition of long chain saturated fatty acids such as, stearic acid and arachidic acid into the lipase production medium of *Pseudomonas aeruginosa* LST-03 effectively induced lipase production.

Non-lipidic carbon sources such as carbohydrates and hydrocarbons were also reported to induce the highest lipase production from several microbial isolates. For example, the production of lipase from *Candida rugosa* was found to be the best when maltose was used in the fermentation media instead of glucose and starch (Benjamin and Pandey, 1996). Kamini *et al*. (2000) noticed an increase in the lipase production from the yeast *Cryptococcus* sp. S-2 after the addition of lactose to the basal medium, while the activity was low in glucose and galactose medium. In

contrast, lipase production from *Antrodia cinnamomea* and *Bacillus cereus* C7 was found to be stimulated upon the addition of glucose and starch, respectively to the fermentation media (Lin and Ko, 2005; Dutta and Ray, 2009). Research conducted by Lin *et al*. (2006) found that glycerol gave the highest lipase production from *Antrodia cinnamomea* among all the tested carbon sources and surprisingly no lipase activity was detected when lipids or fatty acids was used. In addition, it was reported that hydrocarbons showed stimulatory effect on lipase production when used as the sole carbon source in the fermentation media. For example, Chen *et al*. (1993) recorded that the mutant strain *Trichosporon fermentans* 2PU-18 was found to be superior in both the total lipase productivity and permeability of lipase compared to the parental strain *Trichosporon fermentans* WU-C12 when petroleum products were used as carbon sources. Mala *et al*. (2001) reported kerosene as the best carbon source that supported the highest lipase production from mutated strain of *Aspergillus niger*. Furthermore, Kanwar *et al*. (2002) also found that among the various lipidic and non-lipidic substances, normal alkanes within the chain lengths of C-12 to C-20 served as the best carbon substrates for the production of extracellular lipase from *Pseudomonas* species G6 with the highest production using n-hexadecane.

Nitrogen sources in either organic or inorganic forms were also found to modulate lipase production in several microbial strains and the response were found to be strain dependent. For example, the presence of organic nitrogen source in the fermentation medium of *Bacillus* sp. strain 42, *Pseudomonas flouresence* and *Candida rugosa* supported the highest lipase production (Eltaweel *et al*., 2005; Al-Saleh and Zahran, 1999; Montesinos *et al*., 1996) and in their absence no lipase production was detected (Montesinos *et al*., 2003). On the other hand, organic nitrogen sources were shown not essential for lipase production by *Burkholderia cepacia*, *Pseudomonas* species G6

and *Aspergillus terreus* (Rathi *et al*., 2001; Kanwar *et al*., 2002; Gulati *et al*., 1999). Furthermore, some of the organic nitrogen sources such as yeast extract decreased the enzyme production, while casein, casamino acid and molasses completely inhibited the enzyme production by *Bacillus* sp strain 42 (Eltaweel *et al*., 2005). However, it was found that organic nitrogen sources such as yeast extract, casein, corn steep liquor, combination of yeast extract and peptone, tryptone N1, peptone and 1-asparagine were preferably used for lipase production by *Bacillus alcalophilus*, *Cryptococcus* sp. S-2, *Bacillus* sp. strain 42, *Bacillus coagulans* BTS-3, *Aspergillus terreus*, *Rhizopus oryzae*, *Candida rugosa*, *Yarrowia lipolytica*, *Aspergillus* sp. and *Antrodia cinnamomea* (Ghanem *et al*. 2000; Kamini *et al*., 2000; Eltaweel *et al*., 2005; Kumar *et al*., 2005; Gulati *et al*., 1999; Hiol *et al*., 2000; Fadiloğlu and Erkmen, 2002; Fickers *et al*., 2004; Cihangir and Sarikaya, 2004; Lin *et al*., 2006). According to Turki *et al*. (2009) the molecular size of the peptides of tryptone and peptone from casein digests play an important role as inducers in the lipase production of *Yarrowia lipolytica* LgX64.81.

Inorganic nitrogen sources which are commonly available as either NH_4^+ or NO_3^- were investigated by several researchers for their effect on lipase production using several microbial strains. It was observed that inorganic nitrogen sources such as ammonium-ions based additives have been reported to be effective for lipase production from *Penicillium citrinum* and *Pseudomonas fluorescens* NS2W (Miranda *et al*., 1999; Kulkarni and Gadre, 2002). Meanwhile, the best nitrogen source for lipase production by *Yarrowia lipolytica* 681, *Candida rugosa* and *Kluyveromyces marxianus* were reported to be urea (Corzo and Revah, 1999; Benjamin and Pandey, 1996; Deive *et al*., 2003). Nitrate salts, sodium nitrate or potassium nitrate, supported the highest lipase production from *Antrodia cinnamomea* and *Corynebacterium*

paurometabolum MTCC 6841 (Lin *et al*., 2006; Joshi *et al*., 2006). However, the inhibitory effect of several inorganic nitrogen sources such as sodium nitrate and di-ammonium orthophosphate, ammonium nitrate and urea on lipase production from *Bacillus* sp. strain 42, *Cryptococcus* sp. S-2 and *Aspergillus* sp, respectively were also reported (Eltaweel *et al*., 2005; Kamini *et al*., 2000; Cihangir and Sarikaya, 2004) which might be due to their toxicity to the culture.

Microorganisms produce lipases constitutively (Messias *et al*., 2009) or inducibly (Berekaa *et al*., 2009). In the case of the inducible lipases, inducers were usually supplemented to the fermentation media to induce the enzyme production. Natural triglycerides and their chemical derivatives were predominantly used to induce higher production of lipases. For example, sardine oil and triolein, corn oil, tributyrin, olive oil, Tween 85 and Tween 80 were reported as the most effective inducers for lipase production from *Cryptococcus* sp. S-2, *Rhizopus arrhizus*, *Pseudomonas* species G6 and *Kluyveromyces marxianus*, *Aspergillus niger* and *Bacillus* sp strain 42 and *Yarrowia lipolytica* 681, respectively (Kamini *et al*., 2000; Elibol and Özer, 2000; Kanwar *et al*., 2002; Deive *et al*., 2003; Ellaiah *et al*., 2004; Eltaweel *et al*., 2005; Corzo and Revah, 1999). Two possible mechanisms were reported by Boekema *et al* (2007) as the explanation for the induction of lipase production by Tween 80 and hexadecane of *Burkholderia glumae*. Tween 80 appeared to stimulate extracellular lipase secretion as well as induce lipase gene expression while hexadecane, however appeared to enhance lipase secretion only without affecting the lipase gene expression at the transcriptional level.

Besides carbon, nitrogen and inducers, metal ions especially divalent cations, mainly Ca^{2+} and/or Mg^{2+}, in many studies were found to have a stimulatory effect on lipase

production of *Pseudomonas fluorescens* 2D, *Aspergillus terreus*, *Bacillus* sp. RSJ1, *Pseudomonas fluorescens* NS2W and *Burkholderia multivorans* (Makhzoum *et al*., 1995; Gulati *et al*., 1999; Sharma *et al*., 2002; Kulkarni and Gadre, 2002; Gupta *et al*., 2007). It was reported that exocytosis of proteins in eukaryotes occurs through 'regulated secretion' pathways controlled by free Ca^{+2} or Mg^{+2} concentration (Gulati *et al*., 1999). Iron was also reported to play a critical role in the lipase production from *Pseudomonas* species G6 (Kanwar *et al*., 2002). In contrast, the addition of Mg^{+2}, Ca^{+2} and Fe^{+3} into the fermentation medium of *Bacillus* sp. strain 42 resulted in the reduction of lipase enzyme production (Eltaweel *et al*., 2005). Other metal ions such as mercury, caseium, zinc and lithium showed inhibitory effect on lipase production from *Pseudomonas fluorescens* 2D, *Antrodia cinnamomea* and *Bacillus* sp. RSJ1 (Makhzoum *et al*., 1995; Lin *et al*., 2006; Sharma *et al*., 2002). Meanwhile, lipase production by *Bacillus* sp. A- 301 and *Antrodia cinnamomea* required a complex medium that contained different divalent cations such as Ca^{2+},Mg^{2+}, Na^{+}, or K^{+} Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and Mo^{2+} (Wang *et al*., 1995; Lin *et al*., 2006).

The age and size of the inoculum may also affect the production of lipases, for example, lipase from *Burkholderia multivorans* and *Bacillus cereus* C7 was produced at the maximum level when inoculum density of 3 and 2% (v/v), respectively was used (Gupta *et al*., 2007; Dutta and Ray, 2009), while no significant effect was observed on the lipolytic activity of *Rhizopus arrhizus* when the inoculum was varied (Elibol and Özer, 2000). The effect of the age of the inoculum on lipase production from *Bacillus cereus* C7 were also found to be significant on lipase production and the most suitable age of inoculum was found to be 24 hr (Dutta and Ray, 2009). Operational parameters such as temperature, pH, agitation and aeration rates were also important in microbial lipase production. The effects of these parameters were

also found to be strain dependent. Certain microorganisms produced maximum lipase at acidic pH value; such as the lipase production from *Cryptococcus* sp. S-2, *Yarrowia lipolytica* 681, *Antrodia cinnamomea*, *Rhizopus arrhizus* and *Lactobacillus plantarum* DSMZ 12028 recorded the highest production in the medium with pH value of 5.6, 4.7, 5.5, 6.0 and 5.5, respectively (Kamini *et al*., 2000; Corzo and Revah, 1999; Lin *et al*., 2006; Elibol and Özer, 2000; Lopes *et al*., 1999). Meanwhile, maximum lipase production by other strains was observed at neutral pH environment such as the production of lipase from *Geotrichum candidum*, *Pseudomonas aeruginosa* MB 5001, *Geotrichum candidum* (ATCC 34614, NRRL Y-552 and NRRL Y-553), *Staphylococcus epidermidis* and *Burkholderia cepacia* which was found to be at pH 7.0 (Gopinath *et al*., 2003; Marcin *et al*., 1993; Baillargeon *et al*., 1989; Joseph *et al*., 2006; Rathi *et al*., 2001). On the other hand, maximum production of some lipases were found to be preferably produced in alkaline environments such the lipase produced by *Geobacillus thermoleovorans*, *Aspergillus terreus*, *Bacillus coagulans* BTS-3, *Bacillus cereus* C7 and *Corynebacterium paurometabolum* MTCC 6841 which was found to be produced at the optimum pH value of 9.0, 9.0, 8.5, 8.0 and 8.5, respectively (Abedl-Fattah, 2002; Gulati *et al*., 1999; Kumar *et al*., 2005; Dutta and Ray, 2009; Joshi *et al*., 2006).

Temperature is yet another critical parameter in determining the maximum lipase production and its optimum temperature was reported to be strain dependent and may also correspond to the optimum growth of the microorganisms. According to Sharma *et al*. (2002), maximum lipase production from microorganisms usually occurs at optimum temperature in the range of 20-45°C, such as the lipase production by *Pseudomonas fluorescens* strain 2D, *Cryptococcus* sp. S-2 *Antrodia cinnamomea*, *Geotrichum candidum*, *Lactobacillus plantarum* DSMZ 12028, *Corynebacterium*