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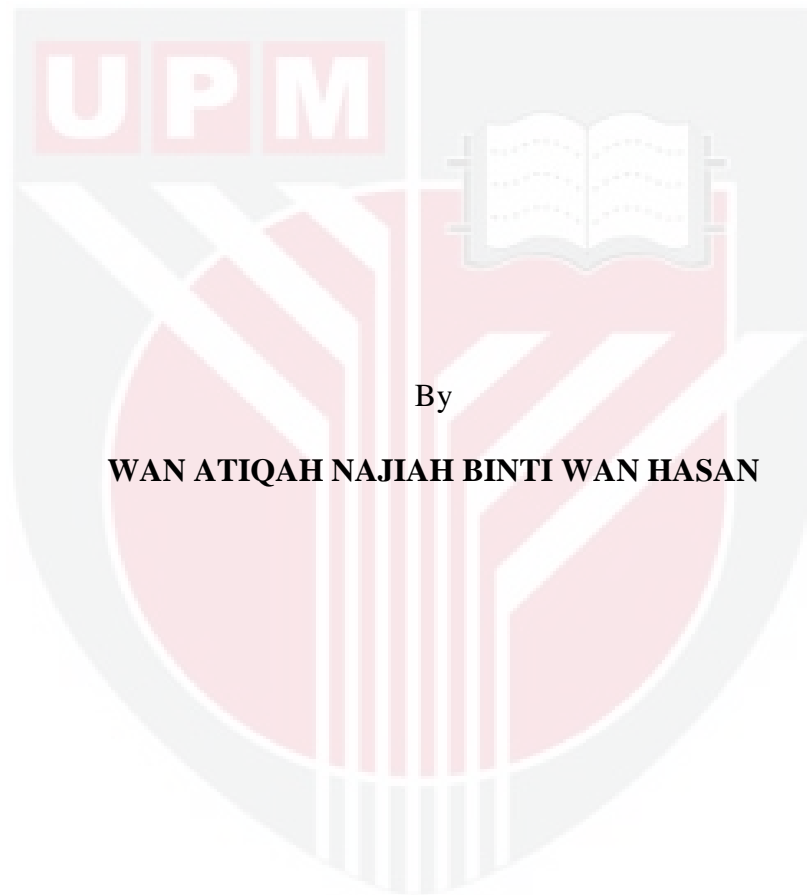
***CHIMERIC LIPASE FOR UNDERSTANDING THE ROLE OF KEY RESIDUES  
GOVERNING PH PROFILES***

**WAN ATIQAH NAJIAH BINTI WAN HASAN**

**FBSB 2014 31**



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GOVERNING PH PROFILES**



By

**WAN ATIQAH NAJIAH BINTI WAN HASAN**

**Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in  
Fulfilment of the Requirements for the Degree of Master of Science**

**December 2014**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia  
In fulfilment of the requirement for the degree of Master of Science

**CHIMERIC LIPASE FOR UNDERSTANDING THE ROLE OF KEY  
RESIDUES GOVERNING PH PROFILES**

By

**WAN ATIQAHA NAJIAH BINTI WAN HASAN**

**December 2014**

**Chairman: Adam Leow Thean Chor, PhD**

**Faculty: Biotechnology and Biomolecular Sciences**

The industrial processes often require enzymes to function under very specific reaction condition. Enzymes which basically are proteins depend greatly on the environmental pH and temperature in order for them to achieve their maximum activity. However, most enzymes from natural source do not meet the industrial processes requirements. Advanced technology and knowledge in protein engineering allow us to modify the natural source enzymes to suit with the industrial condition by changing some components of the protein structure. The study aims to identify key residues modulating the pH profile of lipase by generating chimeric lipases from homologous lipases with different pH profile and to purify and characterize the chimeric lipases. Using the gene shuffling method, fragments of lipase gene from *Staphylococcus hyicus* lipase and *Staphylococcus epidermidis* lipase were amplified and purified before being treated with restriction enzyme *MseI* which cut each lipase at several sites. The digested fragments were purified and assembled using Polymerase Chain Reaction (PCR). Specific primers for each gene were used to reamplify the assembled product. There was full length PCR product with the size of about 1.2 kbp produced when specific primers of *Staphylococcus epidermidis* lipase were used in the PCR reaction. pTrcHis were used as vector and *E. coli* TOP10 were used as the host cell to clone and express the chimeric genes. Three chimeric (Chimeric 1, chimeric 2 and chimeric 3) which possessed different optimum pH from the parent lipase were selected for further investigation after pre-liminary assay screening using *p*-nitrophenyl butyrate as substrate at pH 4 until 12. Analysis of gene revealed that several mutation sites had occurred in the lipase gene which may lead to the changes of optimum pH. The optimum pH for chimeric 1 (4 mutations), chimeric 2 (3 mutations) and chimeric 3 (2 mutations) were pH6, pH6 and pH8, respectively. The mutation between charged residues to other polar or non polar amino acids caused changes in the electrostatic surface of the protein thus affecting the pH profile of the protein. Site-directed mutagenesis were done for amino acid at point 212 in chimeric 1, 2 and 3 and point 359 in chimeric 3 proved that those amino acids effect the pKa of Histidine 346 which was the active site of the enzyme, possibly contributing to the changes in pH of the enzyme. For the sequence analysis, the chimeric lipases were compared with *Staphylococcus epidermidis* lipase structure

using Yet Another Scientific Artificial Reality Application (YASARA) software. The root mean square deviation (RMSD) values for *Staphylococcus epidermidis* lipase compared with chimeric 1,2 and 3 were 0.451 Å, 0.528 Å and 0.460 Å, respectively with sequence identity of 97.87%, 96.82% and 97.66%, respectively. The characterization study revealed that chimeric 1 and 2 were able to hydrolyze long carbon chain length substrates up to carbon 18 but chimeric 3 preferred short carbon chain length. The optimum temperature for chimeric 1, 2 and 3 were 35 °C, 30 °C and 45 °C, respectively. As a conclusion, the knowledge of the effect of key residues in changing certain characteristics of an enzyme may be useful for further usage in protein engineering study, such as to create enzymes with specific optimum pH or temperature for various industrial applications.



Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia  
Sebagai memenuhi keperluan untuk ijazah Master Sains

## **LIPASE KIMERA BAGI MEMAHAMI PERANAN RESIDUE UTAMA DALAM MEMODULASI PROFIL pH**

Oleh

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Industri memerlukan enzim yang bertindak balas secara khusus. Enzim yang pada asasnya adalah protein banyak bergantung kepada pH dan suhu alam sekitar bagi membolehkan mereka untuk mencapai aktiviti maksimum. Walau bagaimanapun, kebanyakan enzim daripada sumber semula jadi tidak memenuhi keperluan proses perindustrian. Teknologi dan pengetahuan dalam bidang kejuruteraan protein membolehkan enzim dari sumber semula jadi diubahsuai untuk disesuaikan dengan keadaan industri dengan menukar beberapa komponen dalam struktur protein. Tujuan kajian ini adalah untuk mengenal pasti residue utama yang memodulasi profil pH lipase dengan menjana lipases kimera dari lipases homolog dengan profil pH yang berbeza. Dengan menggunakan kaedah gen shuffling, serpihan lipase gen dari *Staphylococcus hyicus* lipase dan *Staphylococcus epidermidis* lipase telah diamplifikasikan dan dituliskan sebelum dirawat dengan enzim *MseI* yang memotong setiap lipases di beberapa tempat. Gen yang telah dipotong dan dituliskan kemudian dicantumkan menggunakan reaksi rantai polimerase (PCR). Primer khusus bagi setiap gen digunakan untuk mengamplifikasikan produk yang telah dicantumkan. Produk PCR dengan panjang yang penuh kira-kira 1.2 kbp dihasilkan apabila primers spesifik *Staphylococcus epidermidis* lipase digunakan dalam tindak balas PCR. pTrcHis digunakan sebagai vektor dan *E. coli* TOP10 digunakan sebagai sel tuan rumah bagi mengklon dan mengekspreskan gen kimera. Tiga kimera (kimera 1, kimera 2 dan kimera 3) yang mempunyai pH optimum berbeza daripada lipase induk telah dipilih untuk kajian lanjut selepas pemeriksaan pra-pengasaan menggunakan *p*-nitrophenyl butyrate sebagai substrat pada pH 4 hingga pH 12 dilakukan. Analisis gen menunjukkan beberapa mutasi berlaku dalam gen lipase yang boleh membawa kepada perubahan pH optimum. pH optimum untuk kimera 1 (4 mutasi), kimera 2 (3 mutasi) dan kimera 3 (2 mutasi) masing-masing adalah pH6, pH6 dan pH8. Mutasi antara residue bercaj kepada asid amino berkutub atau tidak berkutub menyebabkan perubahan terhadap permukaan elektrostatik protein yang akan menjejaskan profil pH protein. Mutagenesis tapak tertentu dilakukan untuk asid amino di titik 212 di kimera 1, 2 dan 3 dan titik 359 dalam kimera 3 membuktikan bahawa mereka memberi kesan kepada pKa Histidine 346 yang merupakan tapak aktif enzim yang mungkin boleh menyumbang kepada perubahan pH enzim. Dalam

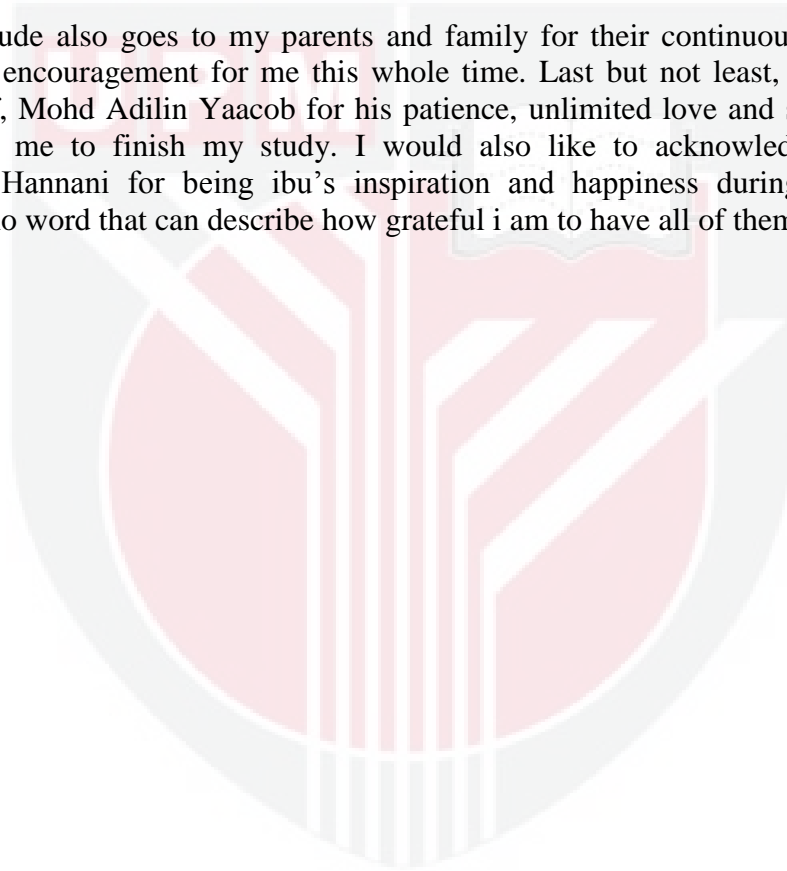
analisis penjujukan, lipases kimera dibandingkan dengan struktur lipase *Staphylococcus epidermidis* dengan menggunakan perisian 'Yet Another Scientific Artificial Reality Application' (YASARA). Nilai 'Root Mean Square Deviation' (RMSD) untuk *Staphylococcus epidermidis* lipase berbanding kimera 1,2 dan 3 masing-masing adalah 0,451 Å, 0.528 Å dan 0.460 Å dengan identiti jujukan masing-masing ialah 97.87%, 96.82% dan 97.66%. Kajian pencirian mendedahkan kimera 1 dan 2 dapat menghidrolisis substrat dengan karbon panjang sehingga karbon 18 tetapi kimera 3 memilih karbon berantai pendek. Suhu optimum untuk kimera 1, 2 dan 3 masing-masing ialah 35 °C, 30 °C dan 45 °C. Kesimpulannya, pengetahuan mengenai kesan residue utama dalam mengubah sesuatu ciri-ciri enzim mungkin berguna dalam bidang kejuruteraan protein seperti menghasilkan enzim dengan pH optimum dan suhu optimum yang khusus bagi pelbagai aplikasi dalam bidang industri.



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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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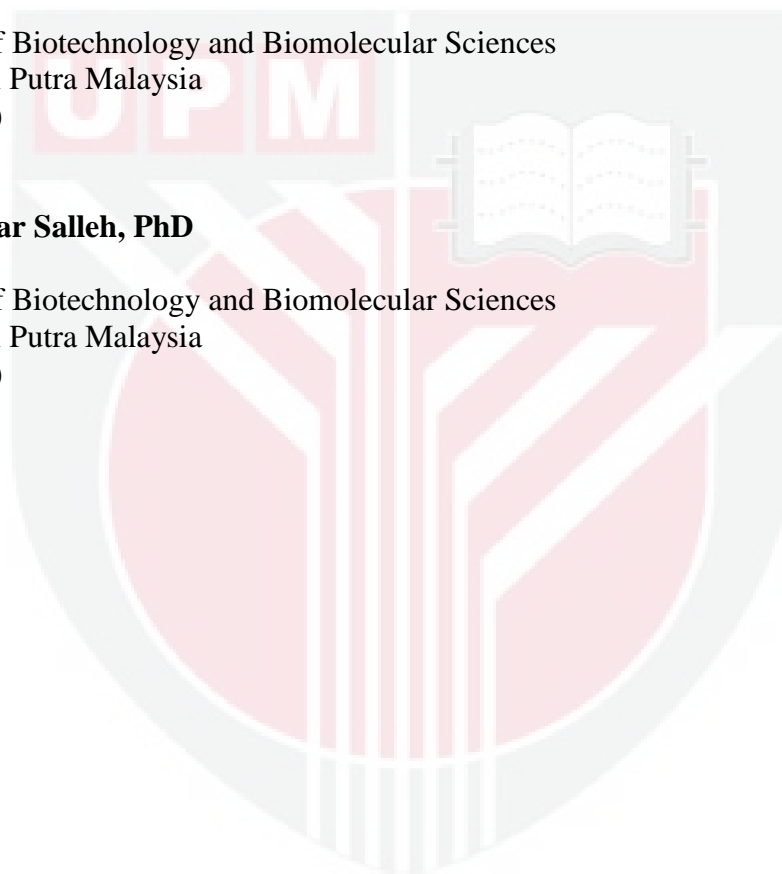
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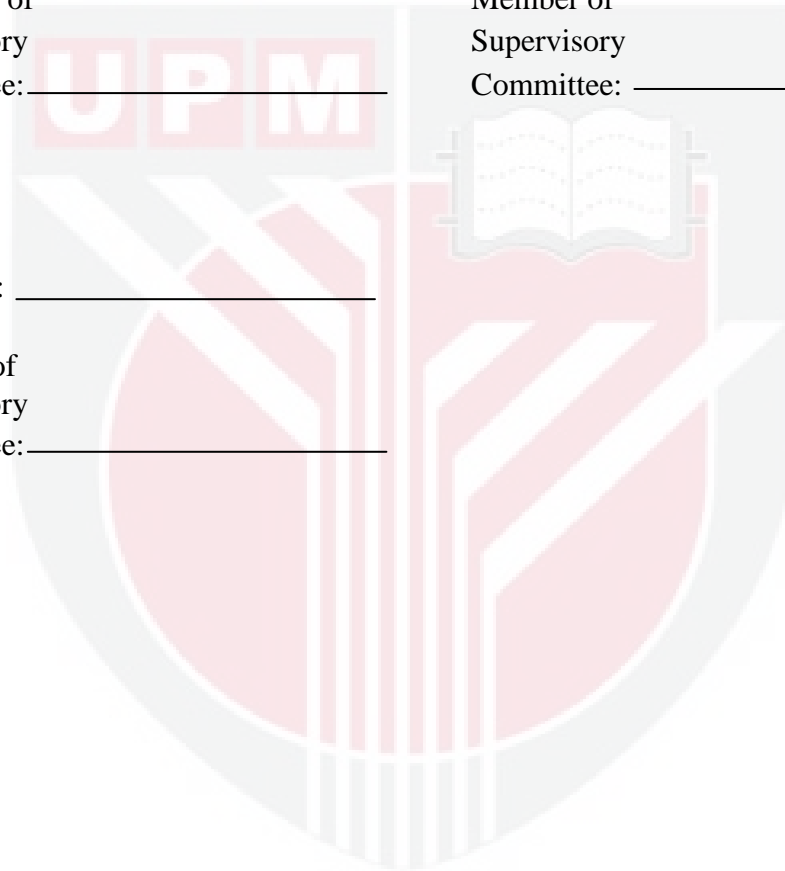
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## LIST OF ABBREVIATIONS

µg/mL	Microgram per milliliter
µM	Micromolar
A	Alanine
Å	Amstrong
C	Celsius
dH <sub>2</sub> O	Distilled water
DNA	Deoxyribonucleic acid
E	Glutamic acid
G	Gram
g/L	Gram per Litre
H	Hour
H	Histidine
IPTG	Isopropyl β-D Thiogalactoside
K	Lysine
Kbp	Kilobasepair
kDa	KiloDalton
L	Leucine
Mg	Milligram
Min	Minit
mL	Milliliter
mL/min	Mililiter per minute
mM	Millimolar
Ng	Nanogram
OD	Optical density
PCR	Polymerase chain reaction
R	Arginine
RMSD	Root Mean Square Deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEL	<i>Staphylococcus epidermidis</i> lipase
SHL	<i>Staphylococcus hyicus</i> lipase
sp.	Species
T	Threonine
U/mL	Unit per milliliter
w/v	Weight per volume
Y	Tyrosine
YASARA	Yet Another Scientific Artificial Reality Application
µg	Microgram

# CHAPTER 1

## INTRODUCTION

The demand for industrial enzymes, particularly of microbial origin, is increasing due to their applications in a variety of industrial processes. Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. Lipases are enzymes which hydrolyze long chain triacylglyceride to form diacylglyceride, monoacylglyceride, glycerol and fatty acids at the interface between aqueous and organic phase (Thomson *et al.*, 1999). These enzymes are versatile enzymes as they can accept broad range of substrates such as aliphatic, alicyclic, bicyclic, aromatic esters, thioesters and activated amine while still maintaining high regioselectivity, chemoselectivity and enantioselectivity (Lotti and Alberghina, 2000). Lipases from microbial play a vital role in various industrial applications such as food, dairy, detergent and pharmaceutical industry (Gupta *et al.*, 2004).

The application of enzyme in industrial processes often requires highly specific condition such as specific pH and temperature. The most favourable pH value is the point at which the enzymes are at its most active state or also known as optimum state. Most lipases used in biocatalysis have neutral or alkaline pH optima. These lipases showed profound stability around pH 6.0 to 7.5 with considerable stability at acidic pH up to 4 and at alkaline pH up to 9. Since industrial applications requires enzyme to works at certain extreme pH and temperature, the lipases that can fulfill these requirements are in great advantage. For example, lipase from *Aspergillus niger* was able to retain 75% of its activity at very extreme pH value as low as pH 1.5 (Mahadik *et al.*, 2002). In the case of Staphylococcal lipases, the optimum pH varies from 6 to 9 despite having high homology between the species and even between strains. Lipase from *S.aureus* strain NCTC 8530 was most active at pH 6 while lipase from different strain of *S.aureus* works optimally at pH 9.5 (Horchani *et al.*, 2012)

Advanced technology and knowledge in protein engineering had broadened the perspective in the designing and creating of desired enzyme. In the last decade, artificial activity was designed on mini scaffolds by transferring previously identified protein active sites into structurally compatible region via site-directed mutagenesis. For example, a CD4 mimic as a powerful inhibitor of HIV-1 entry (Vita *et al.*, 1999). Besides that, despite requiring big screening efforts, directed evolution seems to be the most commonly used approach for lipase improvement. Lipases that evolved for higher thermostability and high-temperature activity using directed evolution methods such as error-prone Polymerase chain reaction (ep-PCR) were reported previously (Acharya *et al.*, 2004; Suen *et al.*, 2004; Niu *et al.*, 2006; Bordes *et al.*, 2011). In addition, directed evolution has also been used to improve or change the pH profile of enzymes such as endoglucanase,  $\alpha$ -amylase and phytase (Wang *et al.*, 2005; Jones *et al.*, 2008; Shivange *et al.*, 2014). However, only few reports on directed evolution aimed at improving pH profiles of lipases were reported (Colin *et al.*, 2010; Jia *et al.*, 2010) and none is derived from Staphylococcal lipases.

Therefore, the aim of this study is to create chimeric lipases of *Staphylococcus* sp. lipases with different pH profile using directed evolution approaches in

understanding the key residues that govern the shifting of optimum pH of the chimeric lipases. The main objective of this project is to determine the key residue governing the optimum pH of Staphylococcal lipase by constructing novel chimeric lipases via directed evolution. Besides that, the characteristics of the chimeric lipases were also identified.



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