UNIVERSITI PUTRA MALAYSIA

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

WAN ATIQAH NAJIAH BINTI WAN HASAN

FBSB 2014 31

CHIMERIC LIPASE FOR UNDERSTANDING THE ROLE OF KEY RESIDUES 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

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 ATIQAH 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 *Mse*I 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 futher 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 preffered 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

WAN ATIQAH NAJIAH BINTI WAN HASAN

Disember 2014

Pengerusi: Adam Leow Thean Chor, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

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 ditulenkan sebelum dirawat dengan enzim *Mse*I yang memotong setiap lipases di beberapa tempat. Gen yang telah dipotong dan ditulenkan 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 mengekspresskan 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-pengasaian 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.

ACKNOWLEDGEMENT

Alhamdullillah, thank to God for giving me the strength and health for me to be able to finish up my master study. My deepest appreciation goes to my supervisor, Dr. Adam Leow Thean Chor for his advice, motivation and kindness in supervising me throughout this project. Next, special thanks to my research group (EMTECH) especially to Prof. Raja Noor Zaliha Raja Abd Rahman, Prof Dato' Abu Bakar Salleh and Dr. Mohd Shukuri Bin Mohamad Ali for their assistance and ideas which helps me a lot in completing the project. I would also like to thank each and everyone of my labmates in IBS and Lab 140 (Biotech 2) whose guidance and support make me manage to successfully finish up my project. I have gained lots of new knowledge and experienced while working together with them throughout the project.

My gratitude also goes to my parents and family for their continuous support and words of encouragement for me this whole time. Last but not least, I thanked my other half, Mohd Adilin Yaacob for his patience, unlimited love and support which motivates me to finish my study. I would also like to acknowledge my baby, Aamilah Hannani for being ibu's inspiration and happiness during hard times. There is no word that can describe how grateful i am to have all of them in my life.

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:

Adam Leow Thean Chor, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raja Noor Zaliha Raja Abdul Rahman, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Abu Bakar Salleh, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____

BUJANG KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

- This thesis is my original work;
- Quotations, illustrations and citations have been duly referenced;
- This thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- Intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- Written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- There is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _____ Date: _____

Name and Matric No.: Wan Atiqah Najiah Binti Wan Hasan GS28922

Declaration by Members of Supervisory Committee

This is to confirm that:

- The research conducted and the writing of this thesis was under our supervision;
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____
Name of
Chairman of
Supervisory
Committee: _____

Signature: _____
Name of
Member of
Supervisory
Committee: _____

Signature: _____
Name of
Member of
Supervisory
Committee: _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg/mL	Microgram per mililiter
µM	Micromolar
A	Alanine
Å	Amstrong
C	Celsius
dH_2O	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	*Staphylococcus epidermidis* lipase
SHL	*Staphylococcus hyicus* lipase
sp.	Species
T	Threonine
U/mL	Unit per mililiter
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 theirs 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, alicylic, 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 fullfill 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, α-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.

REFERENCES

Abdou, A.M. (2003). Purification and partial characterization of psychrotrophic *Serratia marcescens* lipase. *Journal of Dairy Science*, 86:127–132.

Acharya, P., Rajakumara, E., Sankaranarayanan, R., and Rao, N.M. (2004). Structural basis of selection and thermostability of laboratory evolved *Bacillus subtilis* Lipase. *Journal of Molecular Biology*, 341: 1271-1281.

Akbuluta, N., Ozturka, M.T., Pijning, T., Saliha¸ I., Ozturka, S. and Gumus, F. (2013). Improved activity and thermostability of Bacillus pumilus lipase by directed evolution. *Journal of Biotechnology*, 164: 123– 129.

Aravindan, R., Anbumathi, P. and Viruthagiri, T. (2007). Lipase applications in food industry. *Indian Journal of Biotechnology*, 6 :141-158.

Bassegoda, A., Cesarini, S., and Diaz, P. (2012). Lipase improvement: goals and strategies. *Computational and Structural Biotechnology Journal*, 2: 1-8.

Berg, J.M., Tymoczko, J.L. and Stryer, L. (2002). Biochemistry 5th edition, New York: W H Freeman.

Bissaro, B.M., Surel, O., Jaziri, F.A., Saulnier, L., Milon, A., Tenkanen, M., Monsan, P., O'Donohue, M.J., and Fauré, R. (2014). Mutation of a pH-modulating residue in a GH51α-L-arabinofuranosidase leads to a severe reduction of the secondary hydrolysis of transfuranosylation products. *Biochimica et Biophysica Acta*, 1840: 626–636.

Bloom, J.D., Meyer, M.M., Meinhold, P., Otev, C.R., MacMillan, D., and Arnold, F.H. (2005). Evolving strategies for enzyme engineering. *Current Opinion in Structural Biology*, 15: 447–452.

Bommarius, A.S., Blum, J.K., and Abrahamson, M.J. (2011). Status of protein engineering for biocatalysts: how to design an industrially useful biocatalyst. *Current Opinion in Chemical Biology*, 15:194–200.

Bordes, F., Tarquis, L., Nicaudd, J.M., and Marty, A. (2011). Isolation of a thermostable variant of Lip2 lipase from *Yarrowia lipolytica* by directed evolution and deeper insight into the denaturation mechanisms involved. *Journal of Biotechnology*, 156: 117-124.

Bornscheuer, U. and Pohl, M. (2001). Improved biocatalysts by directed evolution and rational protein design. *Current Opinion in Chemical Biology*, 5:137–143.

Bouaziz, A., Horchani, H., Salem, N.B., gargouri, Y., and Sayari, A. (2011). Expression, purification of a novel alkaline *Staphylococcus xylosus* lipase acting at high temperature. *Biochemical Engineering Journal*, 54: 93–102.

Bradford, N.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-54.

Brod, F.C.A., Pelisser, M.R., Bertoldo, J.B., Vernal, J., Bloch Jr. C., Terenzi, H. And Arisi, A.C.M. (2010). Heterologous expression and purification of a heat-tolerant *Staphylococcus Xylosus* lipase. *Molecular Biotechnology*, 44: 110–119.

Brune, A.K. and Gotz, F. (1992). Degradation of lipids by bacterial lipases. In: Winkelman, G. (ed). (pp 243-266). Microbial degradation of natural products. Wiley-VCH, Weinhein, Germany.

Bujnicki, J. M. (2009). Prediction of Protein Structures, Functions and Interactions. John Wiley & Sons, Ltd.

Casas-Godoy, L., Duquesne, S., Bordes, F., Sandoval, G. Marty, A., and Sandoval, G.(ed) (2000). Lipases and phospholipase: Methods and Protocols, Methods in Molecular Biology, Springer New York.

Caspeta, L., Flores, N., Pérez, N.O., Bolívar, F. and Ramírez, O.T. (2009). The effect of heating rate on *Escherichia coli* metabolism, physiological stress, transcriptional response, and production of temperature induced recombinant protein: a scale-down study. *Biotechnology and Bioengineering*, 102: 468-482.

Chang, R.C., Chou, S.J., and Shaw, J.F. (2000). Site-directed mutagenesis of a highly Active *Staphylococcus epidermidis* lipase fragment identifies residues rssential for catalysis. *Journal of American Oil Chemist's society*, 77: 1021-1026.

Colin, D.Y., Deprez-Beauclair, P., Silva, N., Infantes, L., and Kerfelec, B. (2010). Modification of pancreatic lipase properties by directed molecular evolution. *Protein Engineering Design and Selection*, 23 : 365-373.

Colovos, C. and Yeates, T.O. (1993). Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Science*, 2(9): 1511-9.

Dalby, P.A. (2003). Optimising enzyme function by directed evolution. *Current Opinion in Structural Biology*, 13: 500–505.

Dalby, P.A. (2011). Strategy and success for the directed evolution of enzymes. *Current Opinion in Structural Biology*, 21:473–480.

Daoud, L., Kamoun, J., Ali, M.B., Jallouli, R., Bradai, R., Mechichi, T., Gargouri, Y., Ali, Y.B., and Aloulou, A.(2013). Purification and biochemical characterization of a halotolerant *Staphylococcus* sp. extracellular lipase. *International Journal of Biological Macromolecules*, 57: 232– 237.

Dong, H., Gao, S., Han, S. and Cao, S. (1999). Purification and characterization of a *Pseudomonas* sp. lipase and its properties in non-aqueous media. *Biotechnology and Applied Biochemistry*, 30: 251–256.

Dong, H., Gao, S., Han, S., and Cao, S. (1999). Purification and characterization of *Pseudomonas sp*. lipase and its properties in non-aqueous media. *Applied Microbiology and Biotechnology*, 30: 251–256.

Dunhaupt, A., Lang S., Wagner, F. (1991). Properties and partial purification of a *Pseudomonas cepacia* lipase. In: Alberghina L, Schmid RD, Verger R (eds) Lipases: structure, mechanism and genetic engineering (pp 389-392). Wiley-VCH Weinheim Germany.

Engler, C., Gruetzner, R., Kandzia, R., and Marillonet, S. (2009). Golden Gate Shuffling: A One Pot DNA Shuffling Method Based on Type IIs restriction enzymes. *Public Library of Science*, 4: 1-9.

Eisenberg, D., Luthy, R. and Bowie, J.U. (1997). VERIFY3D: assessment of protein models with three-dimensional profiles. *Methods of Enzymology*, 277: 396-404.

Fox, R.J., Davis, S.C., Mundorff, E.C., Newman, L.M., Gavrilovic, V., Ma, S.K., Chung, L.M., Ching, C., Tam, S., Muley, S.,Grate, J., Gruber, J., Whitman, J.C., Sheldon, R.A., and Huisman, G.W. (2007). Improving catalytic function by ProSAR-driven enzyme evolution. *Nature Biotechnology*, 25:338-344.

Gao, C.G., Lan, D., Liu, L., Zhang, H., Yang, B., and Wang, Y. (2014). Site-directed mutagenesis studies of the aromatic residues at the active site of a lipase from *Malassezia globosa*. *Biochimie*, 102: 29-36.

Ghanem, E.H., Al-Sayeed, H.A., and Saleh, K.M. (2000). An alkalophilic thermostable lipase produced by a new isolate of *Bacillus alcalophilus*. *World Journal of Microbiology and Biotechnology*, 16:459–464.

Gibbs, M.D., Reeves, R.A., Choudry, P.R., and Bergquist, P.L. (2010). Alteration of the pH optimum of a family 11 xylanase, XynB6 of *Dictyoglomus thermophilum*. *New Biotechnology*, 27: 803-809.

Gormsen E., Aaslyng D. and Malmos H. (1991). Mechanical studies of proteases and lipases for detergent industry. *Journal of Chemical Technology and Biotechnology*, 50: 321 330.

Gotor-Fern´andez, V., Brieva, R. and Gotor, V.(2006). Lipases: Useful biocatalysts for the preparation of pharmaceuticals. *Journal of Molecular Catalysis B: Enzymatic*, *40* :111- 120.

Grimsley, G.R., Scholtz, M., and Pace, C.N. (2009) .A summary of the measured pK values of the ionizable groups in folded proteins. *Protein Science*, 18: 247-251.

Gupta, R., Gupta, N. and Rathi, P. (2004). Bacterial Lipases: An overview of production, Purification and Biochemical Properties. *Applied Microbiology and Biotechnology*, 64:763-781.

Hasan, F., Shah, A.A., Javed, S. and Hameed, A.(2010). Enzymes used in detergent : Lipases. *African Journal of Biotechnology*, 9(31): 4836-4844.

Haude, A., Kademi, A. and Leblanc, D.(2003). Lipases and Their Industrial Applications : An Overview. *Applied Biochemistry and Biotechnology*, 118(1-3):155-70.

Heinzelman, P., Komor, R., Kanaan, A., Romero, P., Yu, X., Mohler, S., Snow, C., and Arnold, F. (2010). Efficient screening of fungal cellobiohydrolase class I enzymes for thermostabilizing sequence blocks by SCHEMA structure-guided recombination. *Protein Engineering Design and Selection*, 23:871-880.

Heinzelman, P., Snow, C.D., Smith, M.A., Yu, X.L., Kannan, A., Boulware, K., Villalobos, A., Govindarajan, S., Minshull, J., and Arnold, F.H. (2009). SCHEMA recombination of afungal cellulase uncovers a singlemutation that contributes markedly to stability. *Journal of Biological Chemistry*, 284: 26229-26233.

Hiraga, K., and Arnold, F.H.(2003). General method for sequence-independent site-directed chimeragenesis. *Journal of Molecular Biology*, 330: 287-296.

Hooft.R.W.W.,Vriend.G..Sander.C.andAbola.E.E.(1996b)Errors in Protein structures. *Nature*, 381: 272.

Horchani, H., Aissa, I., Ouertani, S., Zarai, Z., Gargouri, Y., and Sayari, A. (2012). Staphylococcal lipases: Biotechnological applications. *Journal of Molecular Catalysis B: Enzymatic*, 76: 125–132.

Horchani, H., Mosbah, H., Ben Salem, N., Gargouri, Y., and Sayari, A.(2009). Biochemical and molecular characterisation of a thermoactive, alkaline and detergent-stable lipase from a newly isolated *Staphylococcus aureus* strain. *Journal of Molecular Catalysis B: Enzymatic, 56:* 237-245.

Hu, S., Huang, J., Mei, L., Yu, Q., Yao, S., and Jin, Z. (2010). Altering the regioselectivity of cytochrome P450 BM-3 by saturation mutagenesis for the biosynthesis of indirubin. *Journal of Molecular Catalysis B: Enzymatic*, 67:29-35.

Hyughues-Despointes, M.P.H., Thurlkill, R.L., Daily, M.D., Schell, D., Briggs, J.M., Antosiewicz, J.M., Pace, N., and Scholtz, M. (2003). pK Values of

Histidine residues in Ribonuclease Sa:Effect of salt and net charge. *Journal of Molecular Biology*, 325: 1093 -1105.

Jaeger, K.E., Dijkstra, B.W., and Reetz, M.T. (1999). Bacterial biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual review of Microbiology*, 53:315–351.

Jan, J. W., Tiesinga, Van Pouderoyen G., Ransac M.N.,S., and Dijkstra B.W. (2000). Structural Basis of Phospholipase Activity of *Staphylococcus hyicus* lipase. *Journal Molecular Biology*, 371: 447–456.

Jia, B., Liu, W.S., Yang, J.K., Ye, C.W., Xu, L., and Yan, Y.J., (2010). *Burkholderia cepacia* lipase gene modification and its constitutive and inducible expression in *Pichia pastoris*. *Acta Microbiologica Sinica*, 9: 76-89.

Jia, H., Li, Y., Liu, Y., Yan, Q., Yang, S., and Jiang, Z.(2012). Engineering a thermostable 1,3-1,4-glucanase from *Paecilomyces thermophila* to improve catalytic efficiency at acidic pH. *Journal of Biotechnology*, 159: 50– 55.

Joern, J.M., Meinhold, P. and Arnold, F.H.(2002). Analysis of Shuffled Gene Libraries. *Journal of Molecular Biology*, 316 : 643-656.

Johannes, T.W., Woodyer, R.D., Zhao, H. (2005). Directed evolution of a thermostable phosphite dehydrogenase for NAD(P)H regeneration. *Applied EnvironmentalMicrobiology*, 71: 5728-5734.

Jones, A., Lamsa, M., Frandsen, T.P., Spendler, T., Harris, P., Sloma, A., Xu, F., Nielsen, J.B., Cherry, J.R. (2008). Directed evolution of a maltogenic-amylase from Bacillus sp. TS-25. *Journal of Biotechnology*, 134: 325–333.

Jones, S., and Thornton, J.M. (2004).Searching for functional sites in protein structures. *Current Opinion in Chemical Biology*, 8: 3–7.

Joseph, B., Ramteke, P.W. and Thomas, G.(2008). Cold active microbial lipases: Some hot issues and recent developments. *Biotechnology Advances*, 26(5): 457-470.

Joshi, M.D., Sidhu, G., Pot, I., Brayer, G.D., Withers, S.D., and McIntosh, L.P. (2009).Hydrogen bonding and catalysis: a novel explanation for how a single amino acid substitution can change the ph optimum of a glycosidase. *Journal of Molecular Biology*, 299: 255-279.

Kampen, M.D.V. and Egmond, M.R. (2000). Directed evolution: From a Staphylococcal lipase to phospholipase. *European Journal of Lipid Science and Technology*, 102 (12): 717-726.

Kampen, M.D., Dekker, N., Egmond, M.R. and Verheij, H.M. (1998). Substrate Specificity of *Staphylococcus hyicus* Lipase and *Staphylococcus aureus* Lipase As Studied by *in vivo* chimeragenesis. *Biochemistry*, 37: 3459-3466.

Karkhane, A.S., Yakhchali, B., Jazii, F.R., and Bambai, B. (2009). The effect of substitution of Phe181 and Phe182 with Ala on activity, substrate specificity and stabilization of substrate at the active site of *Bacillus thermocatenulatus* lipase. *Journal of Molecular Catalysis B: Enzymatic*, 61: 162–167.

Khurana, J., Singh, R.,and Kaur J.(2011). Engineering of Bacillus lipase by directed evolution for enhanced thermal stability: effect of isoleucine to threonine mutation at protein surface. *Molecular Biology Reports*, 38: 2919–2926.

Kim, E.K., Sung, M.H., Kim, H.M., and Oh, T.K. (1994). Occurrence of thermostable lipase in thermophilic *Bacillus sp.* strain 398. *Bioscience Biotechnology, and Biochemistry*, 58: 961–962.

Kim, H.K., Choi, H.J., Kim, M.H., Sohn, C.B. and Oh, T.K., (2002). Expression and characterization of Ca^{2+}- independent lipase from *Bacillus pumilus* B26. *Biochimica et Biophysica Acta*, 1583: 205-212.

Kim, M. H., Kim, H. K., Lee, J. K., Park, S. Y. and Oh, T. K. (2000). Thermostable Lipase of *Bacillus stearothermophilus*: High-level Production, Purification and Calcium-dependend thermostability. *Bioscience, Biotechnology and Biochemistry*, 64: 280-286.

Kojima, Y., Yokoe, M., and Mase, T. (1994). Purification and characterization of an alkaline lipase from *Pseudomonas fluorescens* AK 102. *Bioscience Biotechnology, and Biochemistry*, 58:1564–1568.

Krebber, A., Fleitz, F.J., Brands, J., Devine, P.N., Huisman, G.W., and Hughers, G.J. (2010) Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. *Science*, 329:305-309.

Kumar, A. (2006) *Protein Biotechnology*. Discovery Publishing House. 78-254.

Kumar, S., Kikon, K., Upadhyay, A., Kanwar, S.S. and Gupta, R. (2005). Production, purification, and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. *Protein Expression and Purification*, 41: 38–44.

Kumar, S., Tsai, C. and Nussinov, R. (2000). Factors enhancing protein thermostability. *Protein Engineering*, 13: 179-191.

Laemmli, U.K. (1970). Clevage of structural protein during the assembly of the head of Bacteriophage T4. *Nature*, 227: 680-685.

Laskowski, R.A., MacArthur, M.W., Moss, D.S. and Thornton, J.M. (1993). PROCHECK: a program to check the stereochemical quality of protein structure. *Journal of Applied Crystallography*, 26: 283-291.

Laskowski, R.A., Watson, J.D., and Thorton, M.(2005). Protein Function Prediction Using Local 3D Templates. *Journal of Molecular Biology*, 351: 614–626.

Laurents, D.V., Hyughues-Despointes, B.M.P., Bruix, M., Thurlkill, R.L., Schell, D., Newson, S., Grimsley, G.R., Shaw, K.L., Trevin, S., Rico, M., Briggs, J.M., Antosiewicz, J.M., Scholtz, M., and Pace, N.(2003). Charge–charge interactions are key determinants of the pK Values of ionizable groups in Ribonuclease Sa (pI53.5) and a basic variant (pI 510.2). *Journal of Molecular Biology*, 325: 1077–1092.

Lee, O.W., Koh, Y.S., Kim, K.J., Kim, B.C., Choi, H.J., Kim, D.S., Suhartono, M.T. and Pyun, Y.R. (1999). Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1.FEMS. *Microbiology Letter*, 179: 393–400.

Lehmann, M. and Wyss, M. (2001). Engineering proteins for thermostability: the use of sequence alignments versus rational design and directed evolution. *Current Opinion in Biotechnology*, 12: 371-375.

Lesuisse, E, Schanck, K, and Colson C. (1993). Purification andpreliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. *European Journal of Biochemistry*, 216:155–160.

Li, H. and Zhang, X. (2005) Characterization of thermostable lipase from thermophilic *Geobacillus* sp. *TW1*. *Protein Expression and Purification*, 42: 153–159.

Liang, J., Mundorff, E., Voladri, R., Jenne, S., Gilson, L., Conway, A., Krebber, A., Wong, J., Huisman, G., Truesdell, S. and Lalonde, J. (2010). Highly enantioselective reduction of a small heterocyclic ketone: Biocatalytic reduction of tetrahydrothiophene-3-one to the corresponding (R)-alcohol. *Organic process and Development*, 14:188-192.

Lithauer D, Ginster A, Skein E (2002). Pseudomonas luteola lipase: a new member of the 320 – residue Pseudomonas lipase family. *Enzyme Microbiology Technology*, 30: 209- 215.

Lin, S.F., Chiou, C.M., Yeh, C.M., and Tsai, Y.C. (1996). Purification and partial characterization of an alkaline lipase from *Pseudomonas pseudoalcaligenes* F-111. *Applied and Environmental Microbiology*, 62:1093–1095.

Liu, L., Murphy, P., Baker, D., and Lutz, S. (2010). Computational design of orthogonal nucleoside kinase. *Chemical Communications*, 46: 8803-8805.

Lotti, M. and Alberghina, L. (2000). *C. rugosa* lipases: from molecular evolution analysis to the design of a synthetic gene. In: Protein Engineering in Industrial Biotechnology, Harwood Academic Publishers, New Jersey. 63-74.

Ma, S.K., Gruber, J., Davis, C., Newman, L., Gray, D., Wang, A., Grate, J., Huisman, G.W.,and Sheldon, R.A.(2010). A green-by-design biocatalytic process for atorvastatin intermediate. *Green Chemistry*, 12:81-86.

Macrae, A.R. (1983). Extracellular microbial lipases. In *Microbial Enzymes and Biotechnology*. Applied Science Publishers Ltd, London. 225-250.

Mahadik, N.D., Puntambekar, U.S., Bastawde, K.B., Khire, J.M. and Gokhale, D.V. (2002). Production of acidic lipase by Aspergillus niger in solid state fermentation. *Process Biochemistry*, 38(5): 5715-721.

Malakar, P. and Venkatesh, K.V. (2012). Effect of substrate and IPTG concentrations on the burden to growth of *Escherichia coli* on glycerol due to the expression of Lac proteins. *Applied Microbiology Biotechnology*, 93: 2543–2549.

Michaux, C., Pouyez, J., Mayard, A., Vandurm, P., Housen, I., and Wouters, J. (2010). Structural insights into the acidophilic pH adaptation of a novel endo-1,4-b-xylanase from *Scytalidium acidophilum. Biochimie*, 92: 1407-1415.

Mosbah, H., Sayari, A., Bezzine, S.,and gargouri, Y. (2006). Expression, purification and characterization of His-tagged *Staphylococcus xylosus* lipase wild-type and its mutant Asp 290 Ala. *Protein Expression and Purification*, 47: 516–523.

Mosbah, H., Sayari, A., Horchani H., and gargouri, Y. (2007). Involvement of Gly 311 residue on substrate discrimination, pH, and temperature dependency of recombinant *Staphylococcus xylosus* lipase: A study with emulsified substrate. *Protein Expression and Purification*, 55: 31–39.

Muriel, D., Van Kampen, Maarten, R., and Egmond. (2000). Directed evolution: From a Staphylococcal lipase to a phospholipase. *European Journal of Lipid Science and Technology*, 102: 717-726.

Muraoka, T., Ando, T. and Okuda, H. (1982). Purification and properties of a novel lipase from *Staphylococcus aureus* 226. *Journal of Biochemistry*. 92(6): 933-939.

Nayek, A., Gupta, P.S.S., Banerjee, S., Mondal, B., Amal, K., and Bandyopadhyay (2014). Salt Bridge Energetics in halophilic Proteins. *Public Library of Science*, 9: 1-11.

Nawani, N. and J. Kaur, (2000). Purification, characterization and thermostability of lipase from a thermophilic *Bacillus* sp. J33. *Molecular Cellular Biochemistry*, 206: 91-96.

Nielsen, J.E., V.Borchert, T. And Vriend, G. (2001). The determinants of α-amylase pH activity profiles. *Protein Engineering*, 14: 505-512.

Nielsen, J.E. and McCammon, J.A. (2003). Calculating pKa values in enzyme active sites. *Protein Science*. 121: 894–1901.

Niu, W.N., Li, Z.P., Zhang,D.W.,Yu, M.R., and Tan, T.W. (2006). Improved thermostability and the optimum temperature of *Rhizopus arrhizus* lipase by directed evolution. *Journal of Molecular Catalysis B: Enzymatic*, 43: 33–39.

Oh, B.C., Kim, H.K., Lee, J.K., Kang S.C., and Oh, T.K. (1999). Staphylococcus haemolyticus lipase: biochemical properties, substrate specificity and gene cloning. *Federation ofMicrobiological Societies: Microbiology Letter*, 179: 385–392.

Pace, C.N., Huyghues-Despointes, B.M.P., Briggs J.M., Grimsley, G.R., and Scholtz, J.M. (2002) Charge–charge interactions are the primary determinants of the pK values of the ionizable groups in Ribonuclease T1 *Biophysical Chemistry*, 101: 211–219.

Paiva, A.L., Balcão, V.M., and Malacta, F.X. (2000). Kinetics and mechanisms of reactions catalyzed by immobilized lipases. *Enzyme and Microbial biotechnology*, 27:187–204.

Patel, R.N. (2002). Microbial/enzymatic synthesis of chiral intermediates for pharmaceuticals. *Enzyme and Microbial Technology*, 31(6): 804-826.

Patrick, W.M., Firth, A.E. and Blackburn, J.M. (2003). User-friendly algorithms for estimating completeness and diversity in randomized protein-encoding libraries. *Protein Engineering*, 16: 451-457.

Petsko, G.A. and Ringe, D. (2004). From sequence to structure. In Protein Structure and Function. New Science Press Ltd, London. 2-47.

Rashid, N., Shimada, Y., Ezaki, S., Atomi, H., and Imanaka, T. (2001). Low temperature lipase from psychrotrophic Pseudomonas sp. strain KB700A. *Applied and Environmental Microbiology*, 67:4064–4069.

Richardson, T.H., Tan, X., Frey, G., Callen, W., Cabell, M., Lam, D., Macombe, J., Short, J.M., Robertson, D.E., and Miller, C. (2002). A novel, high performance enzyme for starch liquefaction. Discovery and optimization of alow pH, thermostable alpha-amylase. *The Journal of Biological Chemistry*, 277: 26501–26507.

Rosenstein, R. and Gotz, F. (2000). Staphyloccocal lipases: biochemical and molecular characterization. *Biochimie*, 82(11): 1005-14.

Rosic, N.N.,Huang, W., Johnston, W.A., DeVoss, J.J., Elizabeth, M.J., and Gillam (2007). Extending the diversity of cytochrome P450 enzymes by DNA family shuffling. *Gene*, 395: 40–48.

Rostkowski, M., Olsson, M.H.M., Sondergaard, C.R. and Jensen, J.H. (2011). Graphical analysis of pH-dependent properties of proteins predicted using PROPKA. *BMC Structural Biology*, 11: 6.

Rothlisberger, D., Khersonsky, O., Wollacott, A.M., Jiang, L., DeChancie, J., Betker, J., Gallaher, J.L., Althoff, E.A., Zanghellini, A., Dym, O., Albecks, S., Houk, K.N., Tawfik, D.S., and Baker D. (2008). Kemp elimination catalysts by computational enzyme design. *Nature*, 453:190-195.

Sammour, R.H. (2005). Purification and Partial Characterisation of an Acid Lipase in Germinating Lipid body Linseedlings. *Turky Journal of Botanical*, 29 :177-184.

Sarkar, P., Yamasaki, S., Basak, S., Bera, A., and Bag, P.K. (2012). Purification and characterization of a new alkali-thermostable lipase from *Staphylococcus aureus* isolated from *Arachis hypogaea* rhizosphere. *Process Biochemistry*, 47: 858–866.

Savile, C.K, Janey, J.M., Mundorff, E.C., Moore, J.C., Tam, S., Jarvis, W.R., Colbeck, J.C., Krebber, A., Fleitz, F.J., Brands, J., Devine, P.N., Huisman, G.W. and Hughes, G.J.(2010). Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. *Science*, 329(5989):305-9.

Saxena, R.K., Sheoran, A., Giri, B. and Davidson, W.S. (2003). Review: Purification strategies for microbial lipases. *Journal of Microbiological Methods*, 52: 1 – 18.

Sayari, A., Agrebi, N., Jaoua, S. and Gargouri, Y. (2001). Biochemical and molecular characterization of *Staphylococcus simulans* lipase. *Biochemistry*, 83: 863-871.

Schmitt, J., Brocca, S., D.Schmid, R. and Pleiss, J. (2002). Blocking the tunnel: engineering of *Candida rugosa* lipase mutants with short chain length specificity. *Protein Engineering*, 15: 595-601.

Secundo, F., Carrea, G., Tarabiono, C., Gatti-Lafranconi, P., Brocca, S., Lotti, M. (2006). The lid is a structural and functional determinant of lipase activity and selectivity. *Journal of Molecular Catalysis B: Enzymatic*, 39: 166–170.

Sharma, D., Sharma, B and Shukla, A.K.(2011).Biotechnological approach of Microbial Lipase:A Review. *Biotechnology*, 10(1) : 23-40

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

Shivange, A.V., Denniga, A., and Schwaneberg, U. (2014). Multi-site saturation by OmniChange yields a pH- and thermally improved phytase. *Journal of Biotechnology*, 170: 68– 72.

Simons, J.F.A., Kampen, M.D., Riel, S., Götz, F., Egmond, M.R. and Verheij, H.M. (1998). Cloning, purification and characterisation of the lipase from Staphylococcus epidermidis: Comparison of the substrate selectivity with those of other microbial lipases. *Europeon Journal of Biochemistry*, 253: 675-683.

Simons, J.W.F.A., Adams, H., Cox, R.C., Dekker, N., Gotz, F., Slotboom, A.J. and Verheij, H.M. (1996). The lipase from Staphylococcus aureus: expression in Escherichia coli, large-scale purification and comparison of substrate specificity to *Staphylococcus hyicus* lipase. *Europen Journal of Biochemistry*, 242:760–769.

Snellman, E.A., Sullivan, E.R. and Colwell, R.R. (2002). Purification and properties of the extracellular lipase, Lip A, of Acinetobacter sp. RAG-1. *European Journal of Biochemistry*, 269: 5771–5779.

Stein, S. (ed.) (1990) Fundamental of Protein Biotechnology. Marcel Dekker Inc, New York. 9- 55.

Stemmer, W.P.C. (1994). Rapid evolution of a protein in vitro by DNA shuffling. *Nature*, 70: 389-391.

Suen, W.C., Zhang, N.Y., Xiao, L., Madison, V., and Zaks, A. (2004). Improved activity and thermostability of Candida antarctica lipase B by DNA family shuffling. *ProteinEngineering design and Selection*, 17: 133-140.

Sumita, T.S. (2012). Lipases, its sources, Properties and Applications. *International Journal of Scientific & Engineering Research*, 3: 1-29.

Surinenaite, B., Bendikiene, V., Juodka, B., Bachmatova, I., and Marcinkevichiene, L. (2002). Characterization and physicochemical properties of a lipase from *Pseudomonas mendocina*3121-1.*Biotechnology and Applied Biochemistry*, 36:47–55.

Talon, Dubit, R.N., Montel, C.M., and Cantonnet, M. (1995). Purification and characterization of extracellular *Staphylococcus warneri* lipase. *Microbiology*, 30: 11-16.

Thomson, C. A., Delaquis P.J., and Mazza, G. (1999). Detection and Measurement of Microbial Lipase Activity: A Review, *Critical Reviews in Food Science and Nutrition*, 39 (2): 165- 187.

Tiesinga, J.J.W., Pouderoyen, G.V., Ransac, M.N.S. and Dijkstra, B.W. (2007). Structural Basis of Phospholipase Activity of *Staphylococcus hyicus* lipase. *Journal of Molecular Biology*,371: 447-456.

Tishkov, V.I., Gusakov, A.V., Cherkashina, A.S., and Sinitsyn, A.P. (2013). Engineering the pH-optimum of activity of the GH12 family endoglucanase by site-directed mutagenesis. *Biochimie*, 95: 1704-1710.

Tursi, J.M., Phair, P.G. and Barnes, G.L. (1994). Plant sources of acid stable lipases: potential therapy for cystic fibrosis. *Journal of Pediatric Child Health*, 30(6):539-543.

Tynan-Connolly, B. and Nielsen, J.E. (2006). pKD: Re-designing protein pKa values. *Nucleic Acids Resolution*, 34**:** 48-51.

Van Kampen, M.D., Rosenstein, R., Götz, F., Egmond, M.R. (2001). Cloning, purification and characterization of *Staphylococcus warneri* lipase 2. *Biochimica et Biophysica Acta* , 1544:229–241.

Vazquez-Figueroa, E., Chaparro-Riggers, J., and Bommarius, A.S.(2007). Development of a thermostable glucose dehydrogenase by a structure-guided consensus concept. *Chem Bio Chem*, 8: 2295-2301.

Venselaar, H., Joonsten, R. P., Vroling, B., Baakman, C. A. B., Hekkelman, M. L., Krieger, E. and Vriend, G. (2010). Homology modelling and spectroscopy, a never-ending love story. *European Biophysics Journal*, 39: 551-563.

Verma, N. ,Thakur, S., and Bhatt, A.K.(2012). Microbial Lipases: Industrial Applications and Properties. *International Research Journal of Biological Sciences*, 1: 88-92.

Vita, C., Drakopoulou, E., Vizzavona, J., Rochette, S., Martin, L., Ménez, A., Roumestand, C., Yang, Y.S., Ylisastigui, L., Benjouad, A., and Gluckman, J.C. (1999). Rational engineering of a miniprotein that reproduces the core of the CD4 site interacting with HIV-1 envelope glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America*, 96: 13091–13096.

Walsh, G. (2005). Proteins: Biochemistry and Biotechnology. John Wiley & Sons Ltd, New York. 1-47.

Wang, D., Wang, J., Wang, Bo., and Yu, H. (2012). A new and efficient colorimetric high throughput screening method for triacylglycerol lipase directed evolution. *Journal ofMolecular Catalysis B: Enzymatic*, 82: 18– 23.

Wang, H.K., Liu, R.J., Lu, F.P., Qi, W., Shao, J.,and Ma, H.J. (2009). A novel alkaline and low temperature lipase of *Burkholderia cepacia* isolated

from Bohai in China for detergent formulation. *Annals of Microbiology*, 59: 105-110.

Wang, T., Liu, X., Yu, Q., Zhang, X. Qu, Y., Gao, P., and Wang, T. (2005). Directed evolution for engineering pH profile of endoglucanase III from *Trichoderma reesei*. *Biomolecular Engineering*, 22: 89–94.

Wang, Y., Feng, S., Zhan, T., Huang, Z., Wu, G., and Liu, Z. (2013). Improving catalytic efficiency of endo-β-1,4-xylanase from *Geobacillus stearothermophilus* by directed evolution and H179 saturation mutagenesis. *Journal of Biotechnology*, 168: 341– 347.

Wen, F., Mclachlan, M., and Zhao, H.(2008). Directed evolution: Novel and improved enzymes. Wiley encyclopedia of chemical biology. John Wiley & Sons, Inc. 1-10.

Wena, S., Tan, T., and Zhao, H.(2012) .Improving the thermostability of lipase Lip2 from *Yarrowia lipolytica*. *Journal of Biotechnology*, 164: 248–253.

Wieser, M., and Busse, H. J. (2000). Rapid identification of Staphylococcus epidermidis. *International journal of systematic and Evolutionary*, 50: 1087–1093.

Williams, G.J., Nelson, A.S. and Berrya, A. (2004). Review: Directed evolution of enzymes for biocatalysis and the life science. *Cell and Molecular Life Science*, 61: 3034-3046.

Xiao-Lu, L., Wen-Hui, Z., Ying-Dong, W., Yu-Jie, D., Hui-Tu, Z., Yue, W., Hai-Kuan, W., and Fu-Ping, L. (2014). A high-detergent-performance, cold-adapted lipase from *Pseudomonas stutzeri* PS59 suitable for detergent formulation. *Journal of Molecular Catalysis B: Enzymatic*, 102: 16–24.

Yang, J., Koga, Y., Nakano, H. and Yamane, T. (2002). Modifying the chain-length selectivity of the lipase from *Burkholderia cepacia* KWI-56 through in vitro combinatorial mutagenesis in the substrate-binding site. *Protein Engineering*, 15 (2): 147-52.

Yu, S., Yu, S., Han, W., Wang, H., Zheng, B., and Feng, Y. (2010). A novel thermophilic lipase from *Fervidobacterium nodosum* Rt17-B1 representing a new subfamily of bacterial lipases. *Journal of Molecular Catalysis B: Enzymatic*, 66: 81–89.

Zhang, H., Huang, X., Fukamizo, T., Muthukrishnan, S., and Kramer, K.J. (2002). Site-directed mutagenesis and functional analysis of an active site tryptophan of insect chitinase Insect. *Biochemistry and Molecular Biology*, 32: 1477–1488.

Zhang, N., Suen, W., Windsor, W., Xiao, L., Madison, V. and Zaks, A. (2003). Improving tolerance of Candida antarctica lipase B towards irreversible

thermal inactivation through directed evolution. *Protein Engineering*, 16(8): 599-605.

Zuo, Z.Y., Zheng, Z.L., Liu, Z.G., Yi, Q.M., and Zou, G.L. (2007). Cloning, DNA shuffling and expression of serine hydroxymethyltransferase gene from *Escherichia coli* strainAB90054. *Enzyme and Microbial Technology*, 40: 569–577.