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CHARACTERIZATION OF A THERMOSTABLE LIPASE FROM *ANEURINIBACILLUS THERMOAEROPHILUS* STRAIN HZ

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By

MALIHE MASOMIAN

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

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Dedicated

To my dearly beloved family for their endless love, support, care and encouragement.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

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Chairman: Professor Raja Noor Zaliha Raja Abd Rahman, PhD

Faculty: Biotechnology and Biomolecular Sciences

Thermostable and organic solvent tolerant HZ lipase was an important enzyme which can withstand high temperature and presence of organic solvent for a long period of time. It is an extracellular enzyme secreted by *Aneurinibacillus thermoaerophilus* strain HZ, which isolated from hot spring in Sungai Kelah, Malaysia. Lipases are part of hydrolytic enzymes and widely use in industrial sectors. In addition, thermostable lipases are expected to play a significant role in industrial processing because running bioprocesses at elevated temperature lead to higher diffusion rate, increase solubility of polymeric substrates in water and reduced risk of contamination. To date, there are no local supplies of lipases even though the market is huge. Therefore, lipases derived from locally isolated microorganism are important in fulfilling the future industrial needs of enzymes. Meanwhile, to use any lipase for industrial application, it is important to purify and characterize the enzyme and study its properties.

Thermophilic lipolytic bacteria were screened from several samples collected from hot springs in Batang Kali, Selayang and Sungai Kelah, car service workshop in Port Dickson. The temperature of samples collected ranged from 45ºC to 90ºC. An

enrichment culture technique was used to isolate bacteria utilizing olive oil as substrate. Cultures were incubated at 55ºC for 3 days under shaking condition. From the comprehensive screening program for the isolation of thermophilic lipolytic bacteria, 90 positive isolates were obtained on Tributyrin, Rhodamine B, and Triolein agar plates. Twelve isolates demonstrated high lipase activity (0.05-0.2 U/mL). In order to select the best organic solvent tolerant lipase producer, all the twelve isolates were tested for their lipase stability in organic solvents. Four isolates that showed high stability in organic solvent were further investigated in different production media. Isolate A10 was observed to produce the highest level of lipase after 48h incubation and its crude enzyme was stable in the presence of dimethyl sulfoxide (DMSO), chloroform, octanol, dodecanol, and hexadecane. It was identified as *Aneurinibacillus thermoaerophilus* strain HZ based on its morphological study and 16S rRNA analysis.

Further optimization studies were conducted to determine the best lipase production condition. Inoculum size of 7% proved to be the best for lipase production with an optimum temperature of 60ºC when grown under shaking condition of 150 rpm. Among the various natural and synthetic triglycerides used, olive oil served as the best substrate for the production of extracellular lipase with peptone as the best nitrogen source. The cations, Mg^{2+}, Na^{+}, Ca^{2+} and K^{+} were found to enhance lipase production. In addition, lipase production was stimulated by Tween 85 as surfactant.

The enzyme was purified using two purification steps, anion exchange chromatography and gel filtration. HZ lipase was purified 15.6-fold with specific activity of 43.4U/mg. Purified lipase migrated as a single band with a molecular mass of ~50 KDa on SDS-PAGE. The purified lipase showed high activity at 65 ºC with

optimum pH at 7.0. The enzyme was stable from pH 4.0 to 10.0. It also showed high stability with half-life of 4 h 50 min at 60ºC, 3 h 10 min at 65ºC, and 1h 20 min at 70°C. Mg^{+} and Ca^{2+} at 28 and 39% respectively, gave an enhancement effect after 15 min of treatment. In addition, 46% increase in enzyme activity was observed after extended incubation (30 min), in the presence of Ca^{2+}. Heavy metal ions such as Cu^{2+}, Fe^{3+} and Zn^{2+} inhibited 45% of the HZ lipase activity. Dithiothreitol (DTT) and pepstatin had no effect on the lipase activity, while EDTA and PMSF showed slight inhibitory effect. The lipase exhibited high stability in the presence of dimethylsulfoxide (log *P* -1.3), methanol (log *P* -0.76) and n-tetradecane (log *P* 7.6). HZ lipase showed preference to natural oils as compared to triglycerides and it exhibited the highest activity in the presence of sun flower oil as substrate.

In conclusion, a new thermophilic lipolytic bacterium, *Aneurinibacillus thermoaerophilus* strain HZ, was successfully isolated as a lipase producer and so far no report was available on the isolation of lipase from *A. thermoaerophilus*. The nucleotide sequence of the bacterium 16S rRNA was deposited at GeneBank under the accession number DQ890194. Optimization studies have resulted in the production of crude enzyme to the level of 0.5 U/mL. HZ lipase was efficiently purified with 19.69% yield and characterization studies have shown its stability and activity at broad range of pH and elevated temperatures. In addition, HZ lipase showed selectivity towards long chain natural oils and stability in the presence of organic solvents. These unique properties will provide considerable potential for many biotechnological and industrial applications.

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

PENCIRIAN LIPASE THERMOSTABIL DARIPADA *ANEURINIBACILLUS THERMOAEROPHILUS* STRAIN HZ

Oleh

MALIHE MASOMIAN

Disember 2007

Pengerusi: **Profesor Raja Noor Zaliha Raja Abd Rahman, PhD**

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Lipase stabil haba dan stabil pelarut organik HZ merupakan enzim penting yang boleh bertahan dalam keadaan suhu tinggi berserta kehadiran pelarut organik untuk jangka masa yang lama. Enzim ini merupakan enzim luar sel yang dirembeskan oleh Aneurinibacilus thermaoaerophilus strain HZ, yang telah dipencilkan dari kolam air panas di Sungai Kelah, Malaysia. Lipase merupakan sebahagian daripada enzim hidrolitik dan banyak digunakan dalam sector industri. Selain itu, enzim stabil haba juga dijangka memainkan peranan penting dalam industri pemprosesan kerana pelaksanaan bioproses pada suhu tinggi membawa kepada kadar difusi yang lebi tinggi, meningkatkan keterlarutan substrat polimerik di dalam air dan mengurangkan risiko kontaminasi. Sehigga kini, didapati tiada pembekal lipase tempatan walaupun pasarannya amat luar. Oleh itu, lipase yang diperoleh daripada mikroorganisma yang dipencilkan di negara ini adalah penting bagi memenuhi keperluan bekalan enzim bagi industri masa depan. Dalam pada itu, penggunaan lipase untuk tujuan aplikasi industri adalah sangat penting untuk ditulenkan dan dicirikan.

Bakteria termofilik lipolitik telah disaring daripada beberapa sampel yang diperolehi daripada kolam mata air panas di Batang Kali dan Selayang, bengkel kereta di Port Dickson dan Pusat Rekreasi Air Panas, Sungai Kelah. Julat suhu semasa pemungutan sampel adalah antara 45°C hingga 90°C. Teknik pengkayaan kultur telah digunakan untuk memencilkan bakteria yang menggunakan minyak zaitun sebagai substrat. Kesemua kultur telah dieram pada suhu 55°C selama 3 hari sambil digoncang. Daripada penyaringan komprehensif yang dilakukan untuk mengasingkan bakteria termofilik lipolitik , 90 bakteria terpencil adalah positif terhadap agar Tributirin, Rhodamin B, dan Triolin. Dua belas pencilan menunjukkan akiviti yang tinggi (0.05-0.2 U/ml). Untuk memilih pengeluar lipase yang toleran terhadap pelarut organik untuk kajian selanjutnya, kesemua dua belas pencilan telah diuji untuk kestabilan dalam pelarut organik. Seterusnya, empat pencilan yang menunjukkan kestabilan yang tinggi dalam pelarut organik telah dieram dalam pelbagai media penghasilan. Pencilan A10 didapati telah menghasilkan jumlah lipase yang tertinggi selepas 48 jam inkubasi dijalankan dengan kehadiran dimetil sulfoksida (DMSO), klorofom, oktanol, dodekanol, dan heksadekana. Ia telah dikenalpasti sebagai *Aneurinibacillus thermoaerophilus* strain HZ berdasarkan kajian morfologi dan analisis 16s rRNA.

Kajian pengoptimuman lanjut telah dilakukan untuk menentukan keadaan optima untuk penghasilan lipase. Saiz inokulasi 7% telah dibuktikan sebagai keadaan optima untuk penghasilan lipase, dengan suhu optimum 60°C, apabila ditumbuhkan sambil digoncang pada kelajuan 150 rpm. Antara pelbagai triasilgliserida semulajadi dan sintetik yang digunakan, minyak zaitun adalah substrat terbaik untuk penghasilan lipase ekstraselular dan pepton sebagai sumber nitrogen terbaik. Ion logam Mg^{2+}, Na^{+},

Ca^{2+} dan K^{+} didapati telah meningkatkan penghasilan lipase. Selanjutnya, penghasilan lipase telah distimulasi oleh Tween 85 sebagai surfaktan.

Lipase HZ telah ditulenkan menjadi homogenus menggunakan dua langkah penulenan, kromatografi penukaran anion dan penapisan gel. Lipase HZ telah ditulenkan sebanyak 15.6 kali ganda dengan aktiviti spesifik sebanyak 43.4 U/mg. Lipase tulen bergerak sebagai satu garisan dengan jisim molekul sebanyak ~50kDa dalam SDS-PAGE. Lipase tulen menunjukkan aktiviti yang tinggi pada 65°C dengan pH optimum pada pH7. Enzim tersebut stabil dalam julat pH yang besar daripada 4 hingga 10. ia juga menunjukkan kestabilan yang tinggi dengan separuh hayat 4 jam 50 minit pada suhu 60°C, 3 jam 10 minit pada suhu 65°C dan 1 jam 20 minit pada suhu 70°C. Mg^{+} dan Ca^{2+} memberikan kesan peningkatan selepas didedahkan selama 15 minit dengan peningkatan yang agak tinggi sebanyak 28 dan 39 peratus, masing-masing. Sebanyak 46% peningkatan dalam aktiviti enzim telah diperhatikan selepas pengeraman berpanjangan (30 minit), dengan kehadiran Ca^{2+}. Ion logam berat seperti Cu^{2+}, Fe^{3+} dan Zn^{2+} mempengaruhi aktiviti lipase HZ dengan menyebabkan penindasan lebih daripada lebih daripada 45% aktiviti selepas rawatan. DDT dan pepstatin tidak mempunyai kesan terhadap aktiviti lipase, sementara EDTA dan PMSF menunjukkan sedikit kesan penindasan. Lipase tersebut menunjukkan kestabilan yang tinggi dalam kehadiran dimetilsulfoksid (log *P* -1.3), methanol (log *P* -0.76) dan n-tetradekana (log *P* 7.6). lipase HZ menunjukkan keutamaan terhadap minyak semulajadi berbanding trigliserida dan ia menunjukkan aktiviti tertinggi di dalam kehadiran minyak bunga matahari sebagai substrat.

Kesimpulannya, bakteria lipolitik termofilik baru, *Aneurinibacillus thermoaerophilus* strain HZ telah berjaya dipencilkan sebagai penghasil lipase yang setakat ini tidak pernah dilaporkan sehingga kini. Analisis jujukan nukleotida 16sRNA telah ditempatkan di GenBank dan diberikan nombor rujukan DQ890194. Kajian pengoptimuman menunjukkan penghasilan enzim (yang belum ditulenkan) menghasilkan aktiviti sebanyak 0.5 U\ml. Lipase HZ telah ditulenkan secara efisien dengan 19.69% pulangan aktiviti. Kajian pencirian menunjukkan kestabilan serta aktiviti enzim pada julat pH yang besar serta suhu yang tinggi. Tambahan pula, lipase HZ telah menunjukkan kecenderungan ke arah minyak asli berantai panjang dan kestabilan dalam pelarut organik. Ciri-ciri unik ini adalah penting bagi meluaskan potensi dalam bidang bioteknologi aplikasi 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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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at other institution.

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MALIHE MASOMIAN

Date:

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LIST OF ABBREVIATIONS

APS	ammonium persulfate
bp	Base pair
cm	centimetre
Con A	Concanavalin A
Da	Dalton
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acid
g	gram
g/L	gram per liter
h	hour
Kb	kilobase
kDa	kiloDalton
L	liter
M	molar
mA	milliampere
mM	millimolar
mg	milligram
min	minute
NB	nutrient broth
nm	nanometer
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TSB	trypticase soy broth

µg	microgram
µL	microliter
U/mL	unit per milliliter
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Lipases (EC 3.1.1.3) classified as hydrolyses (EC 3.4), are lipolytic enzyme that catalyse both the hydrolysis and the synthesis of esters. Lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reactions (Vulfson, 1994). These include hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis (Jaeger *et al.,* 1994; Pandey *et al.,* 1999; Nagao *et al.,* 2001; Kim *et al*., 2002a, b). Their unique characteristics include substrate specificity, stereospecificity, regioselectivity and ability to catalyze heterogeneous reactions at the interface of water soluble and water insoluble systems (Borgstorm and Brockman, 1994; Jaeger and Reetz, 1998).

Although lipases are produced by animals, plants, and microorganisms, the majority of lipases used for biotechnological purposes have been isolated from bacteria and fungi (Saxena *et al.,* 1999). Lipases of microbial origin are divided into three groups: 1) extracellular enzymes; 2) intracellular enzymes; and 3) cell-bound enzymes. Of the three groups, the extracellular enzymes have been extensively investigated in application to detergents, and certain lipases have been utilized as detergent additives. The reasons for the enormous biotechnological potential of microbial lipases include the fact that they are stable in organic solvents (Niehaus *et al.,* 1999; Pennisi, 1997), do not require cofactors (Rubin *et al.,* 1997), possess a broad substrate specificity (Rubin *et al.,* 1997) and exhibit a high enantioselectivity (Kazlauskas *et al.,* 1998).

Lipases, which display maximum activity toward water-insoluble long-chain acylglycerides (Bornscheuer, 2002), can catalyse a number of different reactions. They are most interesting because of their potential applications in various industries such as food, dairy, pharmaceutical, detergents, textile, and biodiesel, cosmetic industries, in synthesis of fine chemicals, agrochemicals, and new polymeric materials (Saxena *et al.,* 1999; Jaeger *et al.,* 2002). Each application requires unique properties with respect to specificity, stability, temperature, and pH dependence, and/ or ability to catalyze synthetic ester reactions in organic solvents. Therefore, screening of microorganisms with lipolytic activities could facilitate the discovery of novel lipases. Thermostable enzymes are particularly attractive for industrial applications because of their high activities at the elevated temperatures and stabilities in organic solvents (Niehaus *et al.,* 1999; Pennisi, 1997).

Microbial lipases have been studied in a wide variety of microorganisms which include bacteria, yeast and fungi. Within the bacteria, lipase production in various species have been investigated, which include *Geobacillus* sp.*TW1* (Li and Zhang, 2005), *Thermus themophilus* HB27 (Dominguez *et al.,* 2005), *Bacillus stearothermophilus* MC7 (Kambourova *et al.,* 2003), *Lactobacillus plantarum* (Lopes *et al.,* 2001), *Pseudomonas tolaasii* (Baral and Fox, 1997) , *Pseudomonas fluorescens* (Kim *et al.,* 2005), *Pseudomonas aeruginosa* LST-03 (Ogino *et al.,* 2000; Ito *et al.,* 2001), *Bacillus* sp.RSJ-1 (Sharma *et al.,* 2002), *Bacillus coagulans* BTS-3 (Kumar *et al.,* 2005), *Bacillus thermoleovorans* ID-1 (Lee *et al.,* 1999). Many species of yeast and fungi have shown lipase production such as *Aspergillus carneus* (Saxena *et al.,* 2003), *Aspergillus niger* (Ellaiah *et al.,* 2004; Gandhi, 1997), *Aspergillus oryzae* (Tsuchiya *et al.,* 1996), *Antrodia cinnamomea* (Lin and Ko, 2005), *Rhizopus oryzae*

(Minning *et al.*, 2001), *Yarrowia lipolytica* 681 (Corzo and Revah, 1999), *Rhizopus delemar*, *Geotrichum candidum*, and *Candida rugosa* (Gandhi, 1997).

Enzymes from thermophile and hyperthermophile microorganisms, however, have been shown to be inherently more resistant to a variety of enzyme denaturants and, thus, represent promising alternatives for the development of industrial biocatalytic processes (Niehaus *et al.,* 1999). One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Allowing a higher operation temperature has also a significant influence on the bioavailability and solubility of organic compounds and thereby provides efficient bioremediation (Becker, 1997). Other values of elevated process temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates and higher process yield due to increased solubility of substrates and products and favorable equilibrium displacement in endothermic reactions (Mozhaev, 1993; Krahe *et al.,* 1996; Kumar and Swati, 2001). Such enzymes can also be used as models for the understanding of thermostability and thermo-activity, which is useful for protein engineering. Therefore thermophilic microorganisms have been the focus of a number of investigations into the sources of lipases that are stable and function optimally at high temperature, then the search for new microorganisms producing new and novel lipase for industrial purposes should be continuously pursued. This research was undertaken with the following objectives:

1) to screen and isolate a thermophilic lipolytic bacterium.
2) to identify the bacterium.
3) to optimize the production of lipase .