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UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF LIPASE-PRODUCING THERMOPHILIC BACTERIA ISOLATED FROM HOT SPRINGS IN MALAYSIA

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By

AZURA LIANA BINTI KAMARUZAMAN

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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Specially for my loving parents,
Kamaruzaman Mohd Yatim and Rashidah Daud,
and siblings,
Azila Asfar and Azrul Azlan

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: **Associate Professor Nazamid Saari, PhD**

Faculty: **Food Science and Technology**

Lipase-producing thermophilic microorganisms were successfully isolated from water samples collected from six hot springs in the west coast of Peninsular Malaysia and were identified up to species level. Five hundred and forty-four microbial colonies were qualitatively screened using Rhodamine B-olive oil agar plate method and sixty-six were found to be positive as bacterial lipase producers after they formed orange fluorescent colour around the colonies when irradiated with UV light. Lipolytic activities were assayed using titrimetric method with olive oil emulsion as substrate. Comparative analysis among the positive bacterial lipase producers indicated that isolate ST 7 had the highest lipolytic activity of 4.58 U/ml, followed by isolate ST 6 with an activity of 3.51 U/ml. Eighteen isolates that showed high lipolytic activity were further examined for thermostability. Thermostability was determined by incubation of the crude lipase at temperature ranging from 40 to 80°C for 30 minutes. It was found that isolates ST 7 and

ST 8 produced the most thermostable lipase, which retained 86.70% and 87.52% of the original activity after incubation at temperature 80°C for 30 minutes, respectively.

Eighteen lipase-producing thermophilic bacteria were further characterized and identified using morphological characteristics, Biolog Microlog® Bacterial Identification System and conventional biochemical tests. Strains KW 6, KW 7, ST 1, ST 6 and ST 10 were identified as CDC Group IVc-2 (Alcaligenes-like) or *Ralstonia paucula*. Strains KW 8, KW 10 and DT 17 were identified as *Burkholderia cocovenenans*, *B. glumae* and *Photobacterium logei,* respectively. Biolog identified strains ST 7, ST 8, SY 7, KW 1, KW 9, KW 12 and TBN 3 were belonging to genus Bacilli and strains SY 9, DT 9 and DT 12 as *Staphylococcus* species. Four strains of the *Bacillus* genus with the highest thermostability, ST 7, ST 8, SY 7 and KW 12 were chosen for further identification using 16S rRNA gene sequence analysis.

The protocol of DNA extraction method applied in this study was Qiagen DNeasy Tissue Kit. Based on the quantification of extracted DNA and estimation of the purity by UV spectrophotometer, the Qiagen DNeasy Tissue Kit produced a high DNA yield of all samples. The primer pair used for specific-PCR generated the same expected PCR product for all the strains with molecular weight of 1500 bp. Sequences were determined using an automated sequencer; which was aligned with reference sequences of the closest related organisms in NCBI database. Based on 16S rRNA gene sequencing, the highest sequence similarity was found between strain ST 8 and *Bacillus subtilis* gene for 16S rRNA partial sequence (AB110598 with 100% similarity between nucleotides 1-

1532). Strains KW 12 and ST 7 were also showed a high sequence similarity at 99.0%; comparable to those of *Anoxybacillus kamchatkensis* 16S rRNA gene partial (accession number: AF510985) and *Anoxybacillus flavithermus* isolate AB05 16S rRNA gene partial sequence (accession number: AF001964), respectively. Strain SY 7 displayed 98.0% sequence similarity to *Bacillus cereus* strain J-1 16S rRNA gene partial sequence (accession number: AY305275). The 16S rRNA gene sequences for ST 8, ST 7, KW 12 and SY 7 have been deposited into Genbank Data Library and assigned the accession number DQ401073, DQ193516, DQ401072 and DQ401074, respectively.

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

PENGENALPASTIAN BAKTERIA TAHAN HABA PENGHASIL ENZIM LIPASE DIPENCILKAN DARI KOLAM AIR PANAS DI MALAYSIA

Oleh

AZURA LIANA BINTI KAMARUZAMAN

Januari 2008

Pengerusi: **Profesor Madya Nazamid Saari, PhD**

Fakulti: **Sains dan Teknologi Makanan**

Mikroorganisma tahan haba penghasil enzim lipase telah berjaya diasingkan dari sampel-sampel air yang diambil dari enam buah kolam air panas yang terletak di pantai barat Semenanjung Malaysia dan dikenalpasti hingga ke paras spesies. Lima ratus empat puluh empat koloni mikroorganisma telah disaring secara kualitatif menggunakan kaedah piring agar Rhodamine B-minyak zaitun dan enam puluh enam didapati positif sebagai penghasil enzim lipase setelah membentuk lingkaran cahaya berwarna jingga mengelilingi koloni apabila disinari cahaya UV. Aktiviti lipolitik dicerakin menggunakan kaedah titrimetrik dengan emulsi minyak zaitun sebagai substrat. Analisis perbandingan di antara semua bakteria penghasil lipase yang positif menunjukkan pencilan ST 7 mempunyai aktiviti lipolitik yang tertinggi iaitu 4.58 U/ml, diikuti oleh pencilan ST 6 dengan aktiviti 3.51 U/ml. Lapan belas pencilan yang menunjukkan aktiviti lipolitik yang tinggi kemudiannya diperiksa untuk ujian thermostabiliti.

Thermostabiliti ditentukan dengan menginkubasi lipase mentah pada suhu 40 hingga 80°C selama 30 minit. Pencilan ST 7 dan ST 8 didapati menghasilkan enzim lipase yang paling tahan haba, iaitu mengekalkan 86.70% dan 87.52% daripada aktiviti asal setelah diinkubasi pada suhu 80°C selama 30 minit.

Pencirian dan pengenalpastian lapan belas bakteria tahan haba penghasil enzim lipase telah dilakukan menggunakan kaedah pencirian morfologi, sistem 'Biolog Microlog®' pengenalpastian bakteria dan ujian-ujian biokimia. Strain KW 6, KW 7, ST 1, ST 6 dan ST 10 telah dikenalpasti sebagai CDC Group IVc-2 (Alcaligenes-like) atau *Ralstonia paucula*. Bagi strain KW 8, KW 10 dan DT 17 pula, mereka telah dikenalpasti sebagai *Burkholderia cocovenenans*, *B. glumae,* dan *Photobacterium logei*. Hasil ujikaji Biolog mendapati strain ST 7, ST 8, SY 7, KW 1, KW 9, KW 12 dan TBN 3 tergolong sebagai genus Bacilli dan strain SY 9, DT 9 dan DT 12 sebagai spesies *Staphylococcus*. Empat strain daripada genus *Bacillus* dengan thermostabiliti yang tertinggi, ST 7, ST 8, SY 7 dan KW 12 telah dipilih untuk pengenalpastian selanjutnya menggunakan kaedah '16S rRNA Gene Sequence Analysis'.

Kaedah untuk pengekstrakan DNA yang digunakan di dalam kajian ini ialah Qiagen DNeasy Tissue Kit. Berdasarkan kepada keputusan kuantifikasi DNA ekstrak dan penganggaran ketulenannya menggunakan spektrofotometer UV, kaedah Qiagen DNeasy Tissue Kit memberikan kualiti DNA tertinggi untuk untuk semua strain. Pasangan primer yang digunakan untuk PCR-spesifik menghasilkan 'produk PCR' yang diharapkan untuk semua strain dengan berat molekul 1500 bp. Rangkaian jujukan dikenalpasti dengan menggunakan program rangkaian jujukan automatik; di mana

rangkaian jujukan akan dibandingkan dengan rujukan yang terhampir dengannya di dalam pangkalan maklumat NCBI. Keputusan dari analisis 'jujukan gen 16S rRNA' mendapati rangkaian jujukan ST 8 telah mendapat nilai persamaan tertinggi dengan *Bacillus subtilis* gene for 16S rRNA partial sequence (AB110598 dengan 100% persamaan antara nukleotida 1-1532). Strain KW 12 dan ST 7 juga menunjukkan nilai persamaan yang tinggi pada 99.0%; dibandingkan dengan *Anoxybacillus kamchatkensis* 16S rRNA gene partial (nombor kemasukan: AF510985) dan *Anoxybacillus flavithermus* isolate AB05 16S rRNA gene partial sequence (nombor kemasukan: AF001964). Strain SY 7 mempamerkan 98.0% persamaan rangkaian jujukan dengan *Bacillus cereus* strain J-1 16S rRNA gene partial sequence (nombor kemasukan: AY305275). Hasil dari ujikaji jujukan gen 16S rRNA untuk ST 8, ST 7, KW 12 dan SY 7 telah disimpan dalam 'Genbank Data Library' dan ditetapkan di bawah nombor kemasukan DQ401073, DQ193516, DQ401072 dan DQ401074.

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All of my hard work would have never been done properly without continuous support and encouragement especially from my dearest parent and my loving brother and sister. Because of their financial and spiritual support, I have been able to study at an advanced academic level.

I certify that an Examination Committee met on 22 January 2008 to conduct the final examination of Azura Liana Binti Kamaruzaman on her Master of Science thesis entitled "Identification of Lipase-Producing Thermophilic Bacteria Isolated from Hot Springs in Malaysia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of Science.

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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 declare that the thesis is 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 any other institution.

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AZURA LIANA BINTI KAMARUZAMAN

Date:

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rpm	revolution per minute
v/v	volume per volume
min	minute
h	hour
g	gram
ml	millileter
mg	milligram
µm	micrometer
nm	nano meter
mM	milliMolar
µmol	micromole
ng	nanogram
µl	microliter
M	Molar
N	Normality
Gly	Glycine
Ser	Serine
kDa	kilo Dalton
ATCC	American Type Culture Collection
pI	isoelectric point
U/ml	unit per millileter
U/g	unit per gram

w/v	weight per volume
UV	ultraviolet
DNA	deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
bp	base pair
kb	kilo basepair
C18	stearate/ stearic acid
C18:1	oleate/ oleic acid
BUG	Biolog Universal Growth medium
GN	Gram-negative
GP	Gram-positive
IF	Inoculation fluid

CHAPTER 1

INTRODUCTION

Nowadays, lipases arise amongst the most important biocatalysts carrying out novel reactions in both aqueous and non-aqueous media. This is mainly due to their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents, and show chemo-, regio- and enantioselectivity. In recent times, the determination of their three-dimensional structures has thrown light into their unique structure–function relationships.

Lipase or glycerol ester hydrolases (EC 3.1.1.3) is defined by the Enzyme Commission of International Union of Biochemistry and Molecular Biology as the enzyme that catalyzes hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved, under a natural conditions. Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol. The catalytic action that occurs is as follows:

$$\text{TRIACYLGLYCERIDE} + H_2O \leftrightarrows \text{GLYCEROL} + \text{FREE FATTY ACIDS}$$

(Ester) (Alcohol) (Acid)

In nature, lipases are ubiquitous and produced from various sources including animals, plants and microorganisms. However, lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reaction including alcoholysis, aminolysis, hydrolysis, esterification and interesterification (Pandey *et al.*, 1999). Besides, microbes can be easily cultivated and the lipases produced are mostly extracellular. Several *Bacillus* sp. have been reported to be the main source of lipolytic enzymes (Schmidt *et al.*, 1994).

The isolation of a large number of beneficial thermophilic microorganisms from different exotic ecological zones of the earth and followed by the extraction of thermostable lipases shows that the advances of this area have been possible (Gowland *et al.*, 1987; Dong-Woo *et al.*, 1999; Kambourova *et al.*, 2003). Thermostable lipases, which are commercially available, are mostly produced from mesophilic bacteria and fungi. Although many lipases from mesophilic group are stable at elevated temperatures, lipases from thermophilic have become the subject of special interest for structural investigation and biotechnological applications (Herbert, 1992). Thermostable lipases, which have been isolated from thermophilic organisms, play an important role in commercial applications because of their overall inherent stability (Demirjian *et al.*, 2001). They are also stable and active at temperatures which are higher than optimum temperatures for the growth of the microorganisms. This reduces 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 *et al.*, 1997).

With the rapid development of enzyme technology, many novel biotechnology applications for lipases have been identified. The esters produced play a relevant role in the food industry as flavour and aroma constituents (Gandhi, 1997). Other applications include the removal of the pitch from pulp in the paper manufacturing industry, drug formulations in the pharmaceuticals industry and the removal of subcutaneous fat in the leather industry (Pandey *et al*., 1999). Lipases are extensively used in dairy industry for flavour development in cheese, butter and margarine and hydrolysis of milk fat (Paiva *et al*., 2000). Other applications are in the fats and oil industries including production of modified triacylglycerides and upgrading of low-value oils and fats.

As industrial application increase, the requirements for large amounts of lipases become a limiting factor. Therefore, several workers have tried to obtain thermostable lipases from thermophilic or extreme thermophilic strains such as *Pseudomonas fluorescens* SIK W1 (minimal activity at 60ºC) (Andersson *et al*., 1979) or *Bacillus* sp. (maximal activity at 60ºC) (Emanuilova *et al.*, 1993). The alkalophilic and thermophilic *Bacillus* sp. has been the focus of a number of investigations as the source of lipases that are stable and function optimally at alkaline pH values and high temperature

This research was done with the ultimate goal of isolating lipase-producing thermophilic bacteria from hot springs in Malaysia. The strain that produced a high thermostable lipase was chosen for further studies. Identification and characterization of the thermophilic bacteria producing lipase was carried out by using three methods;

Biolog Micrologg® Bacterial Identification System, conventional biochemical test and 16S rRNA gene sequencing.

1.1 General Objectives of the Study

The study was undertaken with the following general objectives:

a) To isolate lipase-producing thermophilic bacteria from hot springs in Malaysia

b) To identify and characterize the thermophilic bacteria producing lipase using three different methods

   i) Biolog Micrologg® Bacterial Identification System

   ii) Conventional biochemical tests

   iii) 16S rRNA gene sequencing