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PRODUCTION AND CHARACTERISATION OF A RECOMBINANT COLD ADAPTED LIPASE FROM *Pseudomonas* sp. LSK25 ISOLATED FROM SIGNY STATION, ANTARCTICA

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman : Associate Professor Mohd Shukuri Mohamad Ali, PhD
Faculty : Biotechnology and Biomolecular Sciences

Enzyme-mediated catalysis has increasingly become a preferred approach in numerous industries, facilitating reactions with fewer by-products, consuming less energy and adding value to products. Increasing interest has been taken in enzymes obtained from organisms living in extreme environments, for instance from Antarctica, based on their ability to function under harsh conditions. Cold-adapted enzymes, especially lipases, have gained importance in commercial applications. These enzymes are favoured for their high catalytic activity at low temperatures, reducing energy costs and improving the cost-effectiveness of industrial production. Therefore, the search for new cold-adapted lipases is an ongoing effort. Lack of indepth study on the production and characterisation of cold adapted lipases hinder our understanding of the potential of these enzymes. In order to better understand the biocatalytic potential of these unique cold-adapted lipases, in-depth studies of their production, biochemical characterisation and structural analysis are pivotal. The current study set out to identify, isolate, production, express, characterise and predict the structure via *in silico* simulation, of a new cold-adapted lipase produced by a soil bacterium originally isolated from Signy Island, Antarctica. The strain *Pseudomonas* sp. LSK25 was isolated and its lipase gene expression was quantified. Lipase production of strain LSK25 was investigated via optimised physical and nutritional factors. A recombinant lipase gene (LSK25 lipase), consisting of 1432 nucleotides encoding 476 amino acids for a protein of predicted molecular mass of 65kDa, was successfully expressed at optimal conditions of 25°C, 0.1 mM IPTG (inducer) and 16 h post-induction time. The enzyme was expressed in the form of an inclusion body and was purified via one step Ni-Sepharose affinity chromatography. Biochemical characterisation of LSK25 lipase showed an optimal and stable temperature profile around 25-30 °C with high lipolytic activity retained at pH 6. Elevated lipolytic activity was also observed in the presence of the Ca^{2+} ion. The enzyme was able to hydrolyse long chain lipid substrates. An added advantage of LSK25 lipase is its

ability to tolerate a wide range of organic solvents. The *in silico* study of the predicted structure of the cold-adapted LSK25 lipase further improved the understanding of the stability and molecular flexibility of this enzyme over a broad range of temperature.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN DAN PENCIRIAN LIPASE REKOMBINAN TAHAN SEJUK *Pseudomonas* sp.LSK25 YANG DIPENCILKAN DARIPADA STESEN SIGNY, ANTARTIKA

Oleh

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Pengerusi : Profesor Madya Mohd Shukuri Mohamad Ali, PhD
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Enzim-pengantara pemangkinan semakin menjadi pilihan dalam pelbagai industri. Penggunaan enzim dalam industri dapat memudahkan tindak balas dengan mengurangkan sisa keluaran sampingan, menggunakan kurang tenaga dan menambah nilai kepada produk. Enzim yang diperolehi daripada kawasan ekstrem seperti Antartika menarik minat yang semakin mendalam berdasarkan keupayaan mereka untuk berfungsi dalam keadaan cuaca kutub yang melampau. Enzim tahan sejuk terutama lipase, telah mendapat kepentingan dalam aplikasi komersial. Enzim tahan sejuk dipilih kerana mampu menjalankan aktiviti pemangkin yang tinggi pada suhu rendah, mampu mengurangkan kos tenaga dan meningkatkan keberkesanan kos pengeluaran perindustrian. Walau bagaimanapun, penggunaan enzim psikrofilik adalah terbantut oleh kerana jumlah enzim tahan sejuk yang terhad. Oleh yang demikian, usaha untuk mencari lipase tahan sejuk baru adalah satu usaha yang berterusan. Dalam usaha untuk lebih memahami potensi biopemangkin lipase tahan sejuk, pencarian mendalam mengenai pengeluaran enzim, pencirian biokimia dan analisis struktur adalah penting. Kajian yang dijalankan merangkumi proses mengenal pasti, mengasingkan, pengeluaran, mencirikan dan meramalkan struktur melalui simulasi *in silico*, daripada lipase tahan sejuk baru yang dihasilkan oleh bakteria berasal dari Pulau Signy, Antartika. *Pseudomonas* sp. LSK25 telah diasingkan dan expresi gen lipase dikenalpasti. Pengeluaran lipase LSK25 dikaji melalui faktor-faktor fizikal dan nutrisi yang optimum. Gen rekombinan lipase (LSK25 lipase), yang terdiri daripada 1432 nukleotida pengekodan 476 asid amino meramalkan jisim molekul protein sebanyak 65kDa telah berjaya diekspres dalam keadaan optimum 25°C, 0.1 mM IPTG (pencetus) dan 16 jam selepas- masa induksi. Enzim dinyatakan dalam bentuk jasad perangkuman dan telah ditulenkan melalui satu langkah Ni-Sepharose afiniti kromatografi. Pencirian biokimia LSK25 lipase menunjukkan profil suhu optimum dan stabil sekitar 25-30°C dengan aktiviti lipolitik tinggi dikekalkan pada pH 6. Kadar aktiviti enzim yang tinggi juga diperhatikan dengan kehadiran ion

Ca^{2+}. Enzim lipase dapat menghidrolisiskan substrat lipid berantai panjang. Satu kelebihan tambahan LSK25 lipase mencatatkan peningkatan yang tinggi dalam pelbagai pelarut organik. Dalam kajian *in silico* struktur yang diramalkan LSK25 lipase tahan sejuk meningkatkan pemahaman kestabilan dan fleksibiliti molekul enzim dalam pelbagai suhu.

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Work hard in silence; let your success be your noise- Frank Ocean

Don’t limit your challenges, challenge your limit- Oprah Winfrey

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%	percentage
α	alpha
Å	Angstrom
A_{600}	Absorbance
approx	approximately
β	beta
µg	microgram
µl	microlitre
Amp	ampicillin
APS	Ammonium Persulfate Solution
*Bam*HI	*Bacillus amyloliquefaciens*
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	Bovine Serum Albumin
cDNA	complementary DNA
cm	centimeter
dH_2O	distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FFA	Free fatty acid
g	gram
h	hour
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
K	Kelvin
kb	kilo base
kDa	kilo Dalton
l	litre

LB	Luria-bertani
M	Molar
mg/mL	milligram per mililiter
min	minute(s)
mL	mililiter
mM	millimolar
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information,
No.	Number
ºC	degree Celsius
OD	optical density
PCR	Polymerase Chain Reaction
ps	picosecond
RE	restriction enzyme
rpm	revolution per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-EDTA-buffer
TEMED	N,N,N',N' –tetramethylenediamine
U	enzyme unit
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside
*Xho*I	*Xanthomonas vasicola*

CHAPTER 1

INTRODUCTION

Extreme environments such as those of the polar regions, have been successfully colonised by an abundance of microorganisms, and the biodiversity of these microorganisms is becoming increasingly well documented (Flocco et al.,2019; Giudice et al.,2019; Kochkina et al.,2019). Microorganisms which typically inhabit cold polar environments are commonly categorised as psychrophilic or psychrotolerant. These microorganisms have developed various adaptations enabling them to survive the harsh effects of such environments (Gerday et al.,2000; Cavicchioli et al.,2011). Psychrophiles are defined as microorganisms with an optimal growth temperature below 15ºC and unable to grow above 20°C whereas psychrotolerant's have their optimal temperature for growth above 20°C (Verma et al.,2015). It is crucial that all components of the cell, including metabolism and protein synthesis, are well adapted to function at low temperature (Feller & Gerday, 2003).

Adaptations of these psychrophiles and psychrotolerant microbes includes crucial features such as the maintenance of functional membranes, the evolution of cold-adapted enzymes, high catalytic efficiency at low temperatures and the inclusion of a range of structural features which endow a high level of flexibility in protein structure especially around the active site (Thomas & Diekmann, 2002; Siddiqui et al.,2013). Cold-adapted enzymes are increasingly reported as an essential component in these adaptations, also receiving attention owing to their potential application in biotechnological industries (Kavitha & Shanthi, 2013; Maiangwa et al.,2015; Miao et al., 2016).

Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) are able to catalyse the hydrolysis of triacyglycerols to glycerols and fatty acids at oil-water interfaces (Jaeger & Eggert, 2002; Verma et al., 2012). Cold-adapted lipases have emerged as advantageous in temperature sensitive applications, such as in detergent additives, permitting effective washing in cooler water and thereby improving energy efficiency. As additives in the food industry, cold-adapted lipases aid in improving cold storage through reducing contamination and food spoilage, and they can also be used as efficient bioremediation agents in waste water treatment and in the *in situ* bioremediation of fat-contaminated environments (Maiangwa et al., 2015; Salihu & Alam, 2015). Cold-adapted lipases with the specific features of being organic solvent tolerant also have high potential value for use in enzyme-catalyzed transesterification reactions such as are employed in biodiesel production (Kamarudin et al.,2014). Cold regions, and in particular the Arctic and Antarctic, provide ideal potential sources for the isolation of novel cold-adapted lipases (Gerday et al.,2000; Margesin et al.,2003; Cavicchioli et al., 2011).

To date, however, most studies have focused on the production of lipase enzymes from thermophiles and mesophiles (El-moniem et al.,2008; Rahman et al.,2009; Masomian et al.,2010; Babu et al.,2015). Little attention also has been paid to the potential for the production and characterisation of cold-adapted lipases sourced from the microbiota of more cold environments The rising interest in the biotechnological applications of cold-adapted lipases has led to the development of recombinant lipases, whereby the lipase genes are integrated into heterologous expression host systems in order to increase the efficiency and scale of production of the desired enzyme (Thanassi et al.,2000). With the ever growing demand for cold-adapted lipases, there is continual activity to isolate, express and characterise new cold-adapted lipases. Such work also contributes to increasing the still limited understanding of the structural and functional adaption of these enzymes to the challenges of extreme environments. The principle objective of this study was to isolate a cold-adapted lipase gene from an Antarctic bacterium, and to study the production and feature of the cold-adapted recombinant lipase enzyme *via* biochemical and biophysical analyses. The specific objectives of this study were as follows:

1. To screen and isolate a cold-adapted lipase from an identified Antarctic bacterium
2. To examine the potential of various physical and nutritional factors to enhance lipase production from selected wildtype strain
3. To optimise recombinant lipase expression *via* molecular approaches
4. To purify and characterise the recombinant cold adapted lipase *via* biochemical and biophysical approaches
5. To predict the 3D structure and perform molecular dynamic simulation of the cold adapted lipase

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BIODATA OF STUDENT

Leelatulasi Salwoom , the eldest of 3 girls was born on the 25th of January 1980 in Kuala Lumpur Malaysia. She received her education at Sekolah Kebangsaan Ampang Campuran and continued her secondary in Convent Bukit Nanas , Kuala Lumpur. Upon completion of Sijil Tertinggi Persekolahan Malaysia (STPM) she was offered to pursue her degree in Medical Microbiology, Faculty of Medicine University Malaya Kuala Lumpur and continued her masters in the field of Antarctic Microbiology under the National Antarctic Research Centre, Institute of Postgraduate Studies University Malaya. The author has also conducted her own sampling from the Antarctic region for the completion of her MSc project. The author joined Universiti Putra Malaysia (UPM) , Faculty of Biotechnology and Biomolecular Sciences under the supervision of Assoc. Prof. Dr. Mohd Shukuri Mohamad Ali for PhD programme with dissertation.. Enzymology was the chosen field of study specialising in cold adapted lipases from the Antarctic region. Author is also the recipient of the Sultan Mizan Antarctic Research Fellowship from the Academy of Sciences Malaysia.

LIST OF PUBLICATIONS

Published research article

Salwoom, L.; Rahman, R.N.Z.R.; Salleh, A.B.; Shariff, F.M.; Convey, P.; Pearce, D.; Ali, M.S.M. (2019). Isolation, Characterisation, and Lipase Production of a Cold-Adapted Bacterial Strain *Pseudomonas* sp. LSK25 Isolated from Signy Island, Antarctica. *Molecules, 24*(4), 715. (Impact factor: 3.098, Q2)

Salwoom, L.; Rahman, R.N.Z.R.; Salleh, A.B.; Shariff, F.M.; Convey, P.; Ali, M.S.M. (2019). New Recombinant Cold-Adapted and Organic Solvent Tolerant Lipase from psychrophilic *Pseudomonas* sp. LSK25, Isolated from Signy Island Antarctica. *International Journal of Molecular Sciences, 20(6),* 1264.(Impact factor: 4.183, Q2)

Pending submissions

1. Unravelling protein structure of a new recombinant cold adapted lipase from psychrophilic *Pseudomonas* sp. LSK25 via insilico approaches

2. Effect non polar solvents on the interfacial activation of organic solvent tolerant lipase *Pseudomonas* sp. LSK25

3. A comprehensive review of cold adapted lipases from *Pseudomonas* sp. isolated from the Antarctic region

Conferences

1. Scientific Committee on Antarctic Research's (SCAR) Biology Conferences. Barcelona Spain . 2-10th August 2013. Poster presenter

2. Scientific Committee on Antarctic Research's (SCAR) Biology Conferences. Kuala Lumpur Malaysia. 25-30th August 2016. Poster presenter

3. Malaysian International Antarctic Seminar, Terengganu Malaysia 10-15th August 2017. Oral presenter

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