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EXPRESSION AND CHARACTERIZATION OF A COLD-ADAPTED LIPASE FROM AN ANTARCTIC PSEUDOMONAS SP.

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EXPRESSION AND CHARACTERIZATION OF A COLD-ADAPTED LIPASE FROM AN ANTARCTIC PSEUDOMONAS SP.

By

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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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EXPRESSION AND CHARACTERIZATION OF A COLD-ADAPTED LIPASE FROM AN ANTARCTIC *PSEUDOMONAS* SP.

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Eleven strains of unknown microbial isolates obtained from Antarctica were screened for extracellular lipolytic activity. Eight isolates showed positive results on Tributyrin, Rhodamine B and Triolein agar plates. A single isolate (designated as strain AMS8) that demonstrated the highest lipase activity (0.056 U/mL) was finally used in the current research. It was identified as Pseudomonas sp. based on its morphological study and 16S rDNA analysis.

A lipase gene (*lipAMS8*) was amplified from strain AMS8 via polymerase chain reaction (PCR) amplification. The open reading frame (ORF) of LipAMS8 is 1431 bp in length coding for 476 amino acids. LipAMS8 lacks an N-terminal signal peptide and contains a glycine- and aspartate-rich nanopeptide sequence at the C-terminus. The catalytic triad of LipAMS8 was predicted as Ser-207, Asp-255 and His-313, based on multiple sequence alignment.

Both soluble and insoluble protein of LipAMS8 was expressed in *Escherichia coli*. The ORF of LipAMS8 was expressed using pTrcHis TOPO TA, pET-32b(+) and pGEX-4T1, which are under the control of trc, T7lac and tac promoters, respectively. An optimum expression level for pTrcHis/*lipAMS8*, pET32b/*lipAMS8* and pGEX/*lipAMS8* under constant expression conditions was 0.346 U/mL, 6.066 U/mL and 1.533 U/mL, respectively. An improved lipase expression (9.493 U/mL) was attained using pET-32b(+) vector in *E. coli* BL21(DE3) expressed at 15°C for 8 hours, induced with 0.05 mM isopropyl β-D thiogalactoside (IPTG) at *E. coli* growth optimal density of 0.5.

Only a small amount of lipAMS8 was expressed in soluble form. A huge amount of expressed proteins were in the form of inclusion bodies or insoluble proteins. The inclusion bodies were solubilized by means of urea, a strong denaturating agent and are then refolded via single step dilution. The level of expression obtained from insoluble

protein (41.84 U/mL) was almost four times higher compared to the soluble protein (9.493 U/mL).

Crude enzyme obtained from intracellular inclusion bodies expression was then purified. The His-tagged recombinant LipAMS8 was purified with 23.0% total recovery and an average purification factor of 9.7. The purified LipAMS8 migrated as a single band with a molecular weight approximately 65 kDa during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

LipAMS8 was highly active at 30°C and pH 10. It retained almost 19 and 68% of its relative activity at 0 and 10°C, respectively. The half-life of LipAMS8 was 4 and 2 hours at 30 and 40°C, respectively. The lipase was stable over a broad range of pH (pH 6 to 12). LipAMS8 showed enhancement effect in its relative activity under the presence of Li^{+}, Na^{+}, K^{+}, Rb^{+} and Cs^{+} after 30 minutes treatment. Nonetheless, the enhancement effect decreases as the metallic character increases from Li+ to Cs^{+}. As for divalent metals, a lower concentration (1 mM) of Mg^{2+} and Ca^{2+} gave an enhancement effect to the LipAMS8 activity after 30 minutes treatment. Heavy metal ions such as Cu^{2+}, Fe^{3+} and Zn^{2+} inhibited LipAMS8 activity. As for the organic solvent, methanol, ethanol and xylene had almost no effect on lipase activity, whereas β-mercaptoethanol, pyridine, 1-butanol, iso-amylalcohol, propylacetate and 1-propanol exhibited an inhibitory effect. The LipAMS8 demonstrated high stability in the presence of dimethylsulfoxide, isooctane, octane, n-decane, n-tridecane, n-tetradecane and n-hexadecane.

In conclusion, a new cold-adapted lipase was successfully isolated and its nucleotide sequence was deposited at gene bank under the accession number HQ162821. It exhibited stability and activity at broad range of pH, elevated temperatures and also in the presence of certain metal ions and organic solvents. These unique properties of LipAMS8 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

PENGEKSPRESAN DAN PENCIRIAN LIPASE DARIPADA *PSEUDOMONAS* SP. ANTARTIKA

Oleh

MENEGA GANASEN

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Pengerusi: Dr Mohd Shukuri Mohamad Ali, PhD

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Beberapa strain mikrob yang tidak diketahui, dan diperolehi daripada Antartika telah disaring untuk aktiviti enzim lipase. Semua mikrob menunjukkan pemerhatian yang positif untuk analisis atas tributirin, Rodamin B dan triolin agar. Satu mikrob (dinamakan sebagai strain AMS8) yang menunjukkan aktiviti lipase yang paling tinggi (0.056 U/mL) telah digunakan dalam kajiam semasa. Ia telah dikenal pasti sebagai *Pseudomonsa* sp. berdasarkan kajian morfologi dan analisis 16S rDNA.

Satu gen lipase (*lipAMS8*) telah diperoleh dari mikrob AMS8 melalui kaedah Reaksi Berantai Polimer (PCR). Rangka bacaan terbuka (ORF) bagi LipAMS8 adalah 1431 bp atau 476 asid amino. LipAMS8 kekurangan satu peptida isyarat N-terminal dan mengandungi ulangan nanopeptida glisin dan aspartat di terminal C. Residu-residu katalitik telah dikenal pasti sebagai Ser-207, Asp-255 dan His-313, berdasarkan pelarasan dengan lipase yang lain.

Kedua-dua protein larut dan tidak larut LipAMS8 telah diekspreskan dalam *Escherichia coli*. LipAMS8 telah diekspres dalam pTrcHis TOPO TA, pET-32b(+) dan pGEX-4T1 vektor, yang berada di bawah kawalan *trc*, T7*lac* dan promoter *tac*, masing-masing. Tahap pengekspresan optimum untuk pTrcHis/*lipAMS8*, pET32b/*lipAMS8* dan pGEX/*lipAMS8* bawah keadaan pengekspresan yang sama adalah 0.346 U/mL, 6.066 U/mL dan 1.533 U/mL, masing-masing. Pengekspresan lipase yang lebih baik (9.493 U/mL) telah dicapai menggunakan pET-32b(+) vektor dalam *E. coli* BL21(DE3), diekspreskan pada 15˚C selama 8 jam, dipercepatkan oleh 0.05 mM isopropil β-D tiogalaktosida (IPTG) pada kepadatan optimum pertumbuhan *E. coli* (0.5).

Hanya sejumlah kecil LipAMS8 telah diekspres dalam bentuk larut. Jumlah besar protein diekspres berada dalam bentuk protein tidak larut. Protein ini telah dilarutkan semula menggunakan urea, ejen pelarut yang kuat dan kemudian, protein itu dilipatkan balik ke bentuk asal melalui langkah pencairan tunggal. Tahap pengekspresan yang

diperolehi daripada protein tidak larut (41.84 U/mL) adalah hamper empat kali ganda lebih tinggi berbanding dengan protein yang larut (9.493 U/mL).

Enzim mentah yang diperolehi daripada intrasel (protein tidak larut) kemudian ditulenkan. LipAMS8 yang mengandungi His-tag telah ditulenkan dengan jumlah pemulihan sebanyak 23.0%. LipAMS8 yang ditulenkan mengandungi berat molekul lebih kurang 65 kDa, dianalisiskan atas natrium sulfat dodecyl gel elektroforesis (SDS-PAGE).

LipAMS8 adalah sangat aktif pada 30°C dan pH 10. Ia mengekalkan hampir 19 dan 68% daripada aktiviti relatif pada 0 dan 10°C, masing-masing. Separuh hayat untuk LipAMS8 adalah 4 dan 2 jam pada 30 dan 40°C, masing-masing . Lipase itu stabil pada pelbagai pH (pH 6 hingga 12). LipAMS8 menunjukkan kesan peningkatan dalam aktiviti relatif di bawah kehadiran Li^+, Na^+, K^+, Rb^+ dan Cs^+ selepas rawatan 30 minit. Walaubagaimanapun, kesan peningkatan berkurang dari Li^+ ke Cs^+. Bagi logam divalent, konsentrasi yang lebih rendah (1 mM) daripada Mg^{2+} dan Ca^{2+} memberikan kesan peningkatan kepada aktiviti LipAMS8 selepas rawatan 30 minit. Ion logam berat Cu^{2+}, Fe^{3+} dan Zn^{2+} merencat aktiviti LipAMS8. Bagi pelarut organik seperti metanol, etanol dan xilena, mereka hamper tiada kesan ke atas aktiviti lipase. Manakala, β-mercaptoetanol, piridin, 1-butanol, iso-amilalkohol, propilasetat dan 1-propanol merencat aktiviti LipAMS8 menunjukkan kestabilan yang tinggi di dalam dimetilsulfoksida, isooktana, oktana, *n*-dekana, *n*-tridekana, *n*-tetradekana dan *n*-hexadekana.

Kesimpulannya, lipase tahan sejuk baru telah berjaya diperoleh dan urutan nukleotida telah didepositkan di Gene Bank di bawah nombor kesertaan HQ162821. LipAMS8 menunjukkan kestabilan dan aktivitinya pada pelbagai pH, pelbagai suhu, dalam kehadiran ion logam dan pelarut organic tertentu. Ciri-ciri unik LipAMS8 menyediakan potensi besar untuk banyak aplikasi bioteknologi dan industri.

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I certify that a Thesis Examination Committee has met on 13 June 2014 to conduct the final examination of Menega Ganasen on her thesis entitled "Expression and Characterization of a Cold-adapted Lipase from an Antarctic *Pseudomonas* sp." In accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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A	Adenine
APS	Ammonium persulfate
Bp	Base pair
BRP	Bacteriocin release protein
C	Cytosine
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FFA	Free fatty acid
G	Guanine
g	Gram
h	Hour
IBs	Inclusion bodies
IPTG	Isopropyl β-D thiogalactosidase
kbp	Kilo base pair
kDa	Kilo Dalton
L	Liter
M	Molar
mM	Milimolar
mg	Miligram
min	Minute
nm	Nanometer
ORF	Open reading frame
PCR	Polymerase chain reaction
RBS	Ribosomal binding site
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T	Thymine
U/mL	Unit per milliliter
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Earth's biosphere is dominated by low temperature environments and has been successfully colonized by largely diversified organisms, in particular bacteria, yeasts, unicellular algae and fungi. These unique microorganisms have lately attracted attention due to their ability to permanently cope to cold environments, unlike microorganisms that may experience only transient period of cold (Cavicchioli et al., 2002; Parra et al., 2007). While the cytoplasmic membranes and enzymes of most cells tend to become rigid and inactive when the temperature drops, these organisms have successfully overcome those barriers existing at low temperatures by maintaining membrane fluidity and optimal enzymatic activity (Amico *et al*., 2006).

Owing to the current issue of energy crisis and global warming these decades, the development of energy saving products has grabbed a huge interest in present industry. For instance, detergent manufacturers are involved in development of detergent products, which are efficient at low temperatures (Lu *et al*., 2010). Chemical processes like biomass conversion, bioremediation and fermentation are highly desired to be run at lower temperature because it reduces the risk of contamination by mesophiles as well as saves energy consumption (Cotarlet and Bahrim, 2011). In conjunction to those, studies on cold adapted enzymes have gained an increasing interest. Due to the requirement of low activation energy and its capability to possess high activity at low temperature, these enzymes have great prospect in the manufacture of energy saving products.

Psychrophiles produce cold active enzymes, which are more active at low and moderate temperatures compared to homologous mesophilic enzymes. These enzymes are capable of maintaining the flexibility and dynamics of their active site at low temperature. They are highly useful in biotechnological application as they save energy cost, prevent undesired chemical transformation, prevent the loss of volatile compounds and do not require expensive heating or cooling system (Margesin *et al*., 2003).

At present, proteases, lipases and amylases are the pioneers in enzyme studies as they hold a huge role in industrial applications. Although lipases share only 5% of the industrial enzyme market, they have gained focus as biotechnologically valuable enzyme (Sangeetha *et al*., 2011). In general, lipases are long chain fatty acid ester hydrolases, and are currently attracting enormous attention because of their biotechnological potential. Cold-adapted lipases are largely used in the detergent industry, where cold washing reduces both energy consumption and the wear and tear of textile fibers. They are also preferred in the food industry, as these enzymes can be inactivated at reasonably low temperatures, and by that conserving the nutritional quality of the food. Additionally, they are used in environmental applications such as wastewater treatment and bioremediation of fat-contaminated cold environments (Rashid *et al*., 1999; Joseph *et al*., 2008; Lu *et al*., 2010). Due to the above-mentioned criteria, attempts have been made to isolate, express and characterize a cold adapted lipase from a psychrophilic microorganism, which would have advantages over heat-stable lipases to fulfil industrial applications in future.

Although psychrophiles and their enzymes have potential applications, there are drawbacks on the large-scale fermentation of psychrophilic microorganisms. This is due to low production level of the native strains and the excessive cost of growing wild strains at low temperatures. These accentuate the need for the heterologous expression systems, where the gene encoding for a psychrophilic protein is overexpressed in a mesophilic host. Besides assisting in the large-scale fermentation, the use of recombinant bacterial lipases also minimize the hitch in further research exploration as it simplify the downstream purification (Feller *et al*., 1997).

To date, there is a lack of understanding and limited information on cold-adapted enzymes. In accordance to this, the principle objectives of this study are to isolate and express a cold-adapted lipase via molecular approach, and to analyze the lipase in terms of its physical and biochemical characteristics.

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