UNIVERSITI PUTRA MALAYSIA

PRODUCTION, CHARACTERIZATION AND EXPRESSION OF AN ORGANIC SOLVENT TOLERANT LIPASE FROM PSEUDOMONAS AERUGINOSA S5

SYARUL NATAQAIN BAHARUM.

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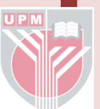

PRODUCTION, CHARACTERIZATION AND EXPRESSION OF AN ORGANIC SOLVENT TOLERANT LIPASE FROM *PSEUDOMONAS AERUGINOSA* S5

By

SYARUL NATAQAIN BAHARUM

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

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

PRODUCTION, CHARACTERIZATION AND EXPRESSION OF AN ORGANIC SOLVENT TOLERANT LIPASE FROM *PSEUDOMONAS AERUGINOSA* S5

By

SYARUL NATAQAIN BINTI BAHARUM

November 2005

Chairman : Professor Abu Bakar Salleh, PhD

Faculty : Biotechnology and Biomolecular Sciences

Lipolytic bacterium was screened from five pure bacteria cultures available in Enzyme and Microbial Technology laboratory in UPM. The stock cultures were tested for lipase production. Two isolates (S5 and 205W) showed the highest activity in tripticase soy broth and brain heart infusions. These isolates were further incubated in different basal media. Isolate S5 was shown to give higher activity (0.327 U/ml) than isolate 205W in media M1 and stable in various organic solvents tested. Therefore isolate S5 was chosen for further studies. Based on its morphological, biochemical characteristics and 16S rDNA sequence, strain S5 was identified as *Pseudomonas aeruginosa. P. aeruginosa* lipase exhibited the highest relative activity with n-hexane (410%) for 20 min reaction. Optimum lipase production was obtained at pH 7.0 and 37°C at static condition with peptone as the best nitrogen source and olive oil as the best carbon source. The best inoculum size was 6%. The surfactants, Tween 60 and Tween 80 were found to enhance for bacterial growth and lipase production by S5.

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The lipase was purified to homogeneity by affinity column chromatography and anion exchange column chromatography. The purified lipase was highly homogeneous as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular mass was estimated to be 60 kDa by SDS-PAGE and G-100 gel filtration column chromatography. The optimum temperature and pH of the purified enzyme was 45°C and pH 9.0, respectively. S5 lipase was stable at pH 6-9 for 30 min. The half-life of the S5 lipase at 45°C and 50°C was 2 h and 1 h, respectively. The lipase exhibited high stability in the presence of n-dodecane, 1-pentanol and toluene. As for metal ions, it was found that Ca^{2+} stimulated lipase activity in 15 min incubation time, while EDTA had no effect on lipase activity. However, the S5 lipase was strongly inhibited by the addition of 1 mM phenyl methyl sulfonyl fluoride (PMSF) (87% inhibition) and 1 mM of Pepstatin (76% inhibition) after 30 min incubation. The S5 lipase exhibited the highest activity in the presence of palm oil as a substrate and followed by coconut oil. S5 lipase was found to have the highest activity against triolein which possess longer carbon chain length. S5 lipase is a non-specific lipase as shown by triolein hydrolysis.

The gene encoding for the intracellular lipase of *P. aeruginosa* strain S5 was isolated via genomic DNA library and cloned into p*RSET*. The cloned sequence included two open reading frames (ORF) consisting of 1575 bp for the first ORF (ORF1) and 582 bp for the second ORF (ORF2). The ORF2 was located at the downstream and function as the *act* gene for ORF1. The conserved pentapeptide Gly- X- Ser- X-Gly was located in the ORF1. Catalytic triad resembling of that serine protease, consisting of serine, histidine,

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aspartic acid or glutamic acid residues was present in this lipase gene. Expression in *E.coli* resulted a 100-fold increase in enzyme activity after 9 h induction with 0.75 mM IPTG. The recombinant plasmid revealed a size of 60 kDa on SDS-PAGE. The Lip S5 gene was stable in the presence of 25% (v/v) n-dodecane and n-tetradecane after 2 h incubation at 37°C. Predicted 3D structure of S5 lipase revealed topological organization of α / β-hydrolase fold consisting of 10 α-Helices and 5 β-strands. The Ramachandran plot of S5 lipase showed that 85.8% (229) of residues lie in the most-favored region and only 2.2% (6) of residue lie in generously allowed regions and 1 residue lie in disallowed region.

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PERPUSTAKAAN SULTAN ABDUL SAMAD
UNIVERSITI PUTRA MALAYSIA

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

PENGHASILAN, PENCIRIAN, PENGKLONAN DAN PENGEKSPRESAN LIPASE DARIPADA *PSEUDOMONAS AERUGINOSA* S5 YANG TOLERAN TERHADAP PELARUT ORGANIK

Oleh

SYARUL NATAQAIN BINTI BAHARUM

November 2005

Pengerusi : Profesor Abu Bakar Salleh, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Bakteria yang menghasilkan enzim lipolitik telah disaring daripada lima kultur bakteria tulen yang terdapat di Makmal Enzim dan Teknologi Mikrob, UPM. Stok kultur ini telah diuji untuk penghasilan lipase. Dua pencilan (S5 dan 205w) menunjukkan aktiviti yang tertinggi di dalam media soya triptikas dan infusi hati dan otak. Kedua-dua pencilan ini seterusnya dieram di dalam basal media berlainan. Pencilan S5 dipilih untuk kajian selanjutnya kerana ia menunjukkan aktiviti yang tinggi (0.327 U/ml) berbanding pencilan 205w di dalam media M1 dan lebih stabil di dalam pelbagai pelarut organik yang telah diuji. Berdasarkan morfologi, pencirian biokimia dan jujukan 16S rDNA, strain S5 ini dikenalpasti sebagai *Pseudomonas aeruginosa*. Lipase *P. aeruginosa* menunjukkan aktiviti relatif tertinggi di dalam n-hexane (410%) di dalam tindak balas selama 20 minit. Kajian pengoptimuman terhadap penghasilan lipase oleh pencilan S5 menunjukkan penghasilan lipase adalah maksimum pada pH 7.0 dan 37ºC pada keadaan statik dengan pepton sebagai sumber nitrogen terbaik dan minyak zaitun sebagai sumber

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karbon terbaik. Saiz inokulum yang terbaik ialah 6%. Tween 60 dan Tween 80 merupakan sumber surfaktan terbaik bagi pertumbuhan bakteria dan penghasilan lipase bagi S5

Lipase ini telah ditulenkan sehingga homogeniti yang nyata menggunakan kromatografi turus afiniti dan penukaran cas anion. Lipase yang telah ditulenkan adalah berhomogenus tinggi berdasarkan poliakrilamid elektroforesis gel sodium dodecil sulfat (SDS-PAGE). Berat molekul lipase ini adalah 60 kDa berdasarkan SDS-PAGE dan gel penurasan kromatografi turus G-100. Suhu dan pH optimum bagi enzim tulen ini adalah 45°C dan pH 9.0, masing-masing. Lipase S5 adalah stabil pada pH 6-9 selama 30 minit. Separuh hayat bagi lipase S5 adalah 45°C dan 50°C masing- masing selama dua dan satu jam. Lipase ini menunjukkan kestabilan yang tinggi di dalam n-dodekana, 1-pentanol dan toluene. Ca^{2+} meransang lipase aktiviti dalam masa 15 min pengeraman, sementara EDTA tidak memberi kesan terhadap aktiviti lipase. Walau bagaimanapun, lipase S5 direncat dengan penambahan 1mM fenil metal sulfonil flourida (PMSF) (87% direncat) dan 1mM Pepstatin (76% direncat) setelah dieram selama 30 minit. Lipase S5 menunjukkan aktiviti yang tinggi dengan kehadiran minyak kelapa sawit dan minyak kelapa sebagai substrat. Lipase S5 didapati menunjukkan aktiviti yang tinggi terhadap triolein yang mempunyai rantaian karbon yang panjang. Lipase S5 adalah jenis tidak spesifik seperti ditunjukkan ke atas hidrolisis triolein.

Gen yang mengkodkan intrasel lipase dari *P. aeruginosa* S5 dipencilkan menerusi perpustakaan genomik DNA dan diklonkan ke dalam p*RSET*. Klon ini mengandungi dua rangka bacaan terbuka (ORF) yang mengandungi 1575 bp untuk ORF pertama (ORF1)

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dan 582 bp untuk ORF kedua (ORF2). ORF2 ini yang terletak di aliran ke bawah yang berperanan sebagai gen bertindak (*act gene*) untuk ORF1. Pentapeptida terpelihara Gly-X- Ser- X- Gly terletak di ORF1. Triad pemangkin yang seperti didapati dalam protease serina yang mengandungi serina, histidina, asid aspartik atau asid glutamik terdapat di dalam gen lipase ini. Ekspresi di dalam *E.coli* menghasilkan peningkatan aktiviti enzim sebanyak 100 kali ganda selepas 9 jam induksi dengan 0.75 mM IPTG. Plasmid rekombinan di atas SDS-PAGE menunjukkan saiz 60 kDa. Lip S5 adalah stabil dengan kehadiran 25% (v/v) n-dodekana dan n-tetradekana selepas 2 jam pada 37ºC. Ramalan struktur tiga dimensi (3D) S5 lipase menunjukkan organisasi topologi lipatan $\alpha/\ \beta$- hidrolase mengandungi 10 α-Heliks and 5 β-bebenang. Plot Ramachandran menunjukkan 85.8% (229) residu terletak di dalam kawasan yang diyakini betul, sementara 11.6% (31) terletak di dalam kawasan yang betul. Hanya 2.2% (6) residu terletak di dalam kawasan yang diragui betul dan satu residu terletak di dalam kawasan yang tidak betul.

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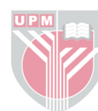

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LIST OF ABBREVIATIONS

APS	ammonium persulfate
BHI	Brain heart infusion
bp	base pair
BTEX	Benzene, Toluene, Ethyl-benzene, p-xylene
cm	centimeter
CM	carboxymethyl
CMC	critical micellar concentration
ConA	Concanavalin A
Da	Dalton
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
DMF	n-n-dimethylformamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acid
FPLC	fast protein liquid chromatography
g	gram
g/L	gram per liter
h	hour
IPTG	isopropyl β-D thiogalactoside
Kb	kilobase
kDa	kiloDalton

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L	liter
LB/amp	Luria-Bertani containing ampicilin
M	molar
mA	milliampear
mM	millimolar
mg	milligram
min	minute
NB	nutrient broth
nm	nanometer
ORF	open reading frame
PAH	polyaromatic hydrocarbon
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel elecrophoresis
TEMED	N, N, N, N- Tetramethylenediamide
TSB	trypticase soy broth
μg	Microgram
μl	microliter
μm	micrometer
U/ml	Unit per milliliter
U/mg	Unit per milligram
v/v	volume per volume
w/v	weight per volume

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CHAPTER 1

INTRODUCTION

The estimated world-wide sales volume for industrial enzymes in 1995 is US$ 1 billion and this volume is foreseen to double by 2005 (Godfrey and West, 1996). Following proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volume. The demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes. It is in the last decade that lipases have gained importance to a certain extent over proteases and amylases, especially in the area of organic synthesis (Saxena *et al.*, 2005). The interest of lipases stems primarily from their ability to preferentially hydrolyze long/short or saturated/unsaturated fatty acyl residues, but they also exhibit a positional specificity for either the 1 (3) or 1,2 (2,3) positions of a triacylglycerol molecule.

Lipases (E.C. 3.1.1.3) are glycerol ester hydrolases that catalyze the hydrolysis of triaclyglycerols into fatty acid, partial acylglycerols and glycerol. Lipases are unique in catalyzing the hydrolysis of fats into fatty acids and glycerol at the water-lipid interface and reversing the reaction in non-aqueous media. The enzyme conformation changes when it contacts with a water-insoluble substrate. This phenomenon is becoming more interesting in understanding the structure-function of enzymes.

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