UNIVERSITI PUTRA MALAYSIA

GENE CLONING AND RECOMBINANT PROTEIN EXPRESSION OF LIPASE FROM MARINE BACTERIUM

LAILA BINTI NOH

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MOLECULAR CLONING AND EXPRESSION OF LIPASE FROM SEAWATER BACTERIA

By

LAILA BINTI NOH

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GENE CLONING AND RECOMBINANT PROTEIN EXPRESSION OF LIPASE FROM MARINE BACTERIUM

By

LAILA NOH

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Chairman : Adam Leow Thean Chor, PhD
Faculty : Biotechnology and Biomolecular Sciences

Nowadays salt tolerant enzymes have gained attention since they have advantages in food processing industries and pharmaceutical field. Marine microbes represent a potential source of bioactive compounds. Many reported studies only showed detection of lipase enzymes in marine bacteria instead of cloning and characterize the enzymes. The aim of this study was to isolate, clone and express lipase from a marine bacterium. The halophilic bacteria were isolated from seawater sample which collected from Tanjung Pelepas, Johor. All of the twenty seven putative lipase producing bacteria were screened for lipase production quantitative and qualitatively. Isolate J15 was selected for further study on the basis of lipase activity and novelty of the strain. The isolate J15 is a Gram negative bacterium with the ability to produce 0.096 U/ml of lipase activity extracellularly. Analysis of 16S rDNA revealed that it shows 97% identity to type strain *Photobacterium jeanii*
.
Lipase gene from *Photobacterium* sp. strainJ15 strain was isolated by using PCR amplification method with the closest homologous lipase being M37 lipase of *Photobacterium lipolyticum* sp. nov. with 83 % identity. The G+C content between the isolate and *P. lipolyticum* are 42.72 and 41.48 %, respectively. Phylogenetic analysis indicated that J15 lipase contained GHSKG as conserved pentapeptide and it clustered with the lipolytic enzymes from class III lipase with catalytic triad (Ser, Asp, and His). J15 lipase show rare oxyanion hole, (RG) normally found in filamentous fungi.

Further analysis was carried out by cloning the J15 lipase gene into an expression vector, peT32b(+) plasmid and transformed into RosettaGamiB(DE3)pLysS. Under the strong T7 promoter, functioning recombinant J15 lipase was synthesized. The open reading frame of J15 lipase was 1023 bp in length encoding an open reading frame containing 341 amino acid residues with a predicted molecular weight of approximately 38 kDa and pI 6.5. Total size of J15 lipase including fusion protein was about 55 kDa. Optimum recombinant lipase expression (18.4 U/ml) was observed when induced the recombinant strain with 0.05 mM IPTG at 30 ^{o}C after 16 h of incubation.

Crude enzyme of recombinant J15 was purified through Nickel-sepharose affinity chromatography obtaining 22.97 U/mg specific lipase activity with 5.39 fold and 97.79 % of recovery. Characterization of recombinant J15 lipase showed highest substrate

specificity for long carbon chain of triglycerides, triolein (C18:1) and long carbon chain of natural oil, coconut oil (C12). In addition, the recombinant displayed activity at pH 5-9 and temperature 15-55 ^{o}C in ranges and stable in the presence of 11 % NaCl. The recombinant J15 lipase showed total inhibition with the presence of EDTA and partially affected by reducing agents (dithiothreitol (DTT) and β-mercaptoethanol).

J15 lipase was classified into *Photobacterium* sp. with mesophilic characteristics. The moderate halophile lipase enzyme was successfully isolated, cloned and expressed. The purification of recombinant J15 lipase produced functioning salt tolerant protein with high recovery in a single step purification system. Further analysis of this lipase could bring to better understanding on its structural and function.

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

PENGKLONAN GEN DAN EKSPRESI REKOMBINAN PROTIN LIPASE DARI BAKTERIA MARIN

Oleh

LAILA NOH

Jun 2014

Pengerusi : Adam Leow Thean Chor, PhD
Fakulti : Bioteknologi dan Sains Biomolekular

Kini enzim yang toleran kepada garam mendapat perhatian dalam bidang pemprosesan makanan dan dalam bidang farmaseutikal. Mikrob laut merupakan sumber yang berpotensi untuk sebatian bioaktif. Banyak kajian yang telah didokumenkan hanya menunjukkan pengesanan enzim lipase dari bakteria marin daripada klon dan mengelaskannya. Jadi, kajian ini telah dijalankan untuk mengasing, mengklon, dan mengekspres lipase dari bakteria laut. Bakteria halofil telah diasingkan daripada sampel air laut yang diambil dari Tanjung Pelepas, Johor. Semua dua puluh tujuh bakteria yang dianggap dapat menghasilkan lipase telah disaring secara kuantitatif dan kualitatif. Bakteria J15 telah dipilih untuk kajian lebih lanjut berdasarkan aktiviti lipase yand ditunjukkan dan keunikannya. Bakteria J15 merupakan bakteria Gram negatif dengan keupayaan untuk menghasilkan 0.095 U/ml aktiviti lipase secara rembesan luar sel. Analisis 16S rDNA telah mendedahkan bahawa 97% identiti sama dengan strain *Photobacterium jeanii*'.

Gen lipase daripada strain J15 telah diasingkan dengan menggunakan kaedah amplifikasi PCR dengan lipase homolog yang terdekat adalah lipase M37 daripada*Photobacterium lipolyticum* sp. nov. dengan 83 % identiti. Komposisi G+C antara isolat J15 dan *P. lipolyticum* masing-masing adalah 42.72 dan 41.48%. Analisis filogenetik menunjukkan J15 lipase mempunyai GHSKG sebagai pentapeptide terpelihara dan ia tergolong dengan enzim lipolytik dari lipase kelas III dengan 'catalytic triad' (Ser, Asp, dan His). J15 menunjukkan lipase 'oxyanion hole' yang jarang berlaku, (RG) biasanya terdapat pada kulat filamen.

Analisis lanjut dilakukan oleh pengklonan lipase gen J15 ke dalam vektor ekspresi, peT32b(+) plasmid dan 'transform' ke dalam RosettaGamiB(DE3)pLysS. Di bawah promoter T7 yang kuat, rekombinan lipase J15 yang berfungsi telah disintesis. 'Open reading frame' bagi J15 lipase adalah sepanjang 1023 bp dan ia mengandungi 342 residu asid amino dengan berat molekul kira-kira 38 kDa dan pI 6.5. Pengekspresan lipase rekombinan yang optimum (18.4 U/ml) diperhatikan apabila strain rekombinan diinduksi dengan 0.05 mM IPTG pada 30 °C selepas 16 jam.

Enzim kasar bagi rekombinan J15 telah ditulenkan melalui 'Nickel-sepharose affinity chromatography'telah memperoleh aktiviti lipase tertentu 22.97 U/mg dengan pemulihan 97.79 %. Pencirian rekombinan lipase J15 menunjukkan pengkhususan

substrat tertinggi bagi rantai karbon yang trigliserida panjang, triolein (C18:1) dan rantai karbon yang panjang nagi minyak semula jadi, minyak kelapa (C12). Disamping itu, rekombinan yang memaparkan aktiviti pada pH 5-9 dan dalam julat suhu 15-55 °C dan stabil dengan kehadiran sehingga 11% NaCl. rekombinan J15 tehelah menunjukkan keterencatan secara keseluruhan dengan kehadiran EDTA dan keterencatan secara separuh dengan kehadiran agen penurunan, (dithiothreitol (DTT) danβ-mercaptoethanol).

Lipase J15 telah dikelaskan ke dalam *Photobacterium* sp. dengan ciri-ciri mesofil. Halophil enzim lipase yang sederhana telah berjaya diasingkan, diklon, dan diekspresikan. Penyulingan rekombinan lipase J15 telah menghasilkan protein yang toleran kepada garam dengan pulangan yang tinggi dalam satu langkah penyulingan. Analysis lanjut mengenai lipase ini boleh memberi kefahaman yang lebih baik berkaitan dengan struktur and fungsinya.

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Dedicated to my late father, Noh bin Idris.

I certify that a Thesis Examination Committee has met on 27 June 2014 to conduct the final examination of Laila binti Noh on her thesis entitled "Gene Cloning and Recombinant Protein Expression of Lipase from Marine Bacterium" 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.

Members of the Thesis Examination Committee were as follows:

Muhajir bin Hamid, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Janna Ong binti Abdullah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Sieo Chin Chin, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Roohaidah Othman, PhD
Associate Professor
Universiti Kebangsaan Malaysia
Malaysia
(External Examiner)

NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 19 September 2014

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Adam Leow Thean Chor, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raja Noor Zaliha Raja Abd Rahman, D. Eng
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Mohd Shukuri Mohamad Ali, PhD
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

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Signature: _____
Name of
Chairman of
Supervisory
Committee: Adam Leow Thean Chor, PhD

Signature: _____
Name of
Member of
Supervisory
Committee: Raja Noor Zaliha Raja Abd Rahman, D. Eng

Signature: _____
Name of
Member of
Supervisory
Committee: Mohd Shukuri Mohamad Ali, PhD

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LIST OF ABBREVIATIONS

C	Cytosine
cm	Centimeter
Da	Dalton
dH_2O	Distilled water
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
G	Guanine base nucleotide
g	Gram
g/L	Gram per liter
GST	Glutathione-S-Transferase
h	Hour
IPTG	Isopropyl β-D Thiogalactoside
kDa	Kilo Dalton
L	Liter
M	Molar
mM	Millimolar
mg	Milligram
ml	Milliliter
min	Minute
nm	Nanometer
ORF	Open reading frame
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
RBS	Ribosome binding site
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N, N-Tetramethyllenediamide
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

CHAPTER 1

INTRODUCTION

Lipases (triacylglycerol hydrolases, E.C.3.1.1.3) are enzymes that catalyze the hydrolysis of triglyceride to glycerol and fatty acids. Lipase has become one of the valuable enzymes due to its broad application in various industries detergents, pharmaceuticals, dairy, oleochemicals, and cosmetics. These enzymes are important industrial productions due to their characteristics, such as broad substrate specificity, stability in organic solvents, region specificity and stereoselectivity (Roh and Villatte, 2008). Lipases can be found ubiquitously in animals, plants, fungi and bacteria (Hasan *et al.,* 2006; Bornscheuer, 2002).

Microbial lipases are potential industrial enzyme due to minimum cost of production and easy handling. Different sources of lipases contribute to diversity of lipase genes. Since 1970's psychrotolerant lipases and thermostable lipases have received attracted attention in various research fields to produce salt tolerant and thermostable lipases which are able to function in extreme high salinity and temperature, respectively (Joseph *et al.,* 2008).

Marine microbes play an important role in cycling the global compounds such as carbon, oxygen, nitrogen, phosphorus, iron and trace elements (Lennon and Pfaff, 2005). Marine environments are considered to be a potential source for the isolation of salt tolerant enzyme producing bacteria (Yang *et al*., 2008). Halophiles are microorganisms adapted to diverse extreme saline environments. They are high potential biotechnology source of hydrolases with various biotechnological applications.

Marine enzyme isolation leads to great discovery of a novel gene, led the identification of a new family (Ryu *et al.*, 2006). This finding shows that there are much more novel enzymes and novel microbes from open sea since over two thirds of our earth surface are covered by the ocean.

Advance in molecular cloning, expression and purification techniques had led to rapid discovery of new isolates with unique properties. Expression in heterologous host helps in overcomes the expression level of gene of interest. Seawater is a high potential source of new bacterium with novel lipase characteristics. Therefore, the research project entails bacterial isolation and identification, molecular expression, purification and characteristic of a marine lipase. The objectives of the study are;

1. To screen and identify lipase producing bacteria from seawater
2. To clone and express the salt tolerant lipase gene
3. To purify and characterize recombinant lipase from marine bacterium

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