UNIVERSITI PUTRA MALAYSIA

REVERSE MICELLAR EXTRACTION OF A RECOMBINANT COLD-ADAPTED AMS8 LIPASE FROM THE ANTARCTIC *Pseudomonas fluorescens*

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FBSB 2019 9

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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

April 2019

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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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April 2019

Chairman : Mohd. Shukuri Mohamad Ali, PhD
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A moderate yield of a purified enzyme can be achieved by using simple technique of reverse micellar extraction (RME). RME is a liquid-liquid extraction method that uses a surfactant and an organic solvent to extract biomolecules. However, there is a lack of study of` RME in extracting cold-adapted enzyme. Instead of traditional chromatographic purification methods, which are tedious and expensive, RME using the non-ionic surfactant Triton X-100 and toluene is used as an alternative purification technique to purify a recombinant cold-adapted lipase, AMS8. AMS8 lipase was isolated from soil samples of Casey Station, Antarctica and was recombinant expressed in *Escherichia coli* strain BL21 (DE3) (pET32b/AMS8).Various process parameters were optimized to maximize the activity recovery of AMS8 lipase. The optimal conditions were found to be 50 mM sodium phosphate buffer, pH 7, 0.125 M NaCl, 0.07 M Triton X-100 in toluene at 10°C with the 90 kU lipase unit loaded. Approximately 86% of lipase activity was successfully recovered. Structural analysis of the lipase in a reverse micelle (RM) was performed using an *in silico* approach. The predicted model of AMS8 lipase was simulated in the Triton-X-100/toluene reverse micelles from 5 to 40°C. The lid 2 covering the active site was slightly opened at 10°C. The secondary structure of AMS8 lipase was most affected in the non-catalytic domain compared to the catalytic domain, with an increased coil conformation. Solvent-accessible surface area and radius of gyration supported the evidence of opening lid AMS8 lipase. Then, to further investigate characteristic of AMS8 lipase in reverse micelles, it was simulated in the different Triton X-100 molecules under optimum conditions. AMS8 lipase in 100 molecules of Triton X-100 reverse micelle gives the most stable form. These results suggest that an AMS8 lipase can be extracted using Triton X-100/toluene micelles at low temperature. This RME approach will be an important tool for the recombinant cold-adapted lipases.

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

PENGEKSTRAKAN MISEL TERBALIK REKOMBINAN AMS8 LIPASE TAHAN SEJUK DARI *Pseudomonas fluorescens* ANTARKTIKA

Oleh

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Pengerusi : Mohd. Shukuri Mohamad Ali, PhD
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Penulinan enzim dalam kuantiti sederhana dapat dilakukan dengan menggunakan teknik pengekstrakan misel terbalik (RME). RME adalah kaedah pengekstrakan cecair yang menggunakan surfaktan dan pelarut organik untuk mengekstrak biomolekul. Namun begitu, tidak banyak kajian penggunaan RME dalam pengestrakan enzim tahan sejuk. Jika dibandingkan dengan teknik penulinan tradisional yang lama dan mahal, RME menggunakan surfaktan non-ionik iaitu Triton X-100 dan toluena sebagai alternatif untuk penulinan AMS8 lipase yang merupakan enzim rekombinan tahan sejuk. AMS8 lipase berasal daripada sampel tanah Stesyen Casey, Antarktika dan diekspreskan di dalam Escherichia coli strain BL21 (DE3) (pET32b/AMS8). Berbagai parameter telah dioptimumkan untuk pemulihan aktiviti AMS8 lipase yang paling maksimum. Penimbal 50 mM natrium fosfat, pH 7, 0.125 M NaCl, 0.07 M Triton X-100 di dalam toluena pada suhu 10°C merupakan kondisi terbaik untuk pemulihan 90 kU AMS8 lipase. Anggaran 86% aktiviti lipase berjaya dipulihkan. Analisis struktur lipase di dalam keadaan misel terbalik juga telah dilakukan dengan menggunakan teknik in siliko. Model AMS8 lipase telah dijangka menggunakan teknik in siliko. Model kemudian disimulasi di dalam misel terbalik Triton X-100/toluena pada suhu 5°C ke 40°C. Penutup kedua yang melindungi kawasan pemangkinan AMS8 lipase kelihatan terbuka pada suhu 10°C. Struktur sekunder AMS8 lipase pada kawasan bukan pemangkin adalah yang paling terkesan pada perbezaan suhu berbanding kawasan pemangkin berdasarkan peningkatan struktur gegelung pada struktur sekunder kawasan tersebut. Kajian pada permukaan yang terdedah untuk pelarut dan radius gyration juga menyokong pembukaan penutup kedua pada AMS8 lipase. Untuk mengkaji ciri-ciri AMS8 lipase di dalam keadaan misel terbalik, AMS8 lipase telah disimulasi pada kondisi optimum dengan manipulasi pada jumlah Triton X-100 molekul di dalamnya. AMS8 lipase yang disimulasi dengan 100 molekul Triton X-100 pada kondidi misel terbalik telah menunjukkan struktur AMS8 lipase yang paling stabil. Hasil kajian, membuktikan bahawa AMS8 lipase boleh diekstrak menggunakan kaedah misel terbalik pada suhu yang rendah. Justeru itu, RME diramalkan akan

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menjadi salah satu dari teknik yang penting untuk pengekstrakan lipase rekombinan tahan sejuk.

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ACKNOWLEDGEMENTS

In the Name of God, the Most Merciful and Most Beneficent

First and foremost I would like to thank Allah Subahanahu Wa Ta'ala (S.W.T), for giving me the best for all of my life, showering me with kindness and excellent care. Supporting me through the many lows during this work and without Allah S.W.T. I would not be in the position I am today.

Alhamdulillah.I wish to express my most profound sense of insightful regards from the core of my heart to my most honourable supervisor Assoc. Prof. Dr. Mohd Shukuri Mohamad Ali, for continuous guidance, valuable suggestions, and thoughtful advice and never give up on me. It is my great privilege to place my deep sense of gratitude to my co-supervisor Prof. Dato' Dr. Abu Bakar Salleh and Prof. Dr. Raja Noor Zaliha Raja Abdul Rahman.

I would like to thank from the bottom of my heart to EMTech labmates especially Aishah, Hanie, Kak su, Nadzmi, Ash, Kila, Fad, Pireya, Abg zarir, Kak dura, Kak Arilla, Kak yati, Kak Hasmah, Dr. Malihe and friends for their sincerity and helps during my time in UPM.

Finally, a most profound appreciation to my Abah, Ibu, Angah, Ude, and Adik for their love, care, endless moral support and prayers. Also, for those who involved directly or indirectly in this project, I sincerely thank you.

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I certify that a Thesis Examination Committee has met on 30 April 2019 to conduct the final examination of Fatin Nur Fauzi Ana binti Abd. Jalil on her thesis entitled "Reverse Micellar Extraction of a Recombinant Cold-Adapted AMS8 Lipase from the Antarctic *Pseudomonas fluorescens*" 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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LIST OF ABBREVIATIONS

α	Alpha
Å	Angstrom
Β	Beta
°C	Degree celcius
%	Percentage
A_{600nm}	Optical density at wavelength 600 nanometer
A_{410nm}	Optical density at wavelength 410 nanometer
APS	Ammonium persulphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATPE	Aqueous two phase system
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EC	Enzyme commission
g	Gram
h	hour
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
L	Liter
LB	Luria-Bertani
LLE	Liquid-liquid extraction
mA	milliamps
µL	microliter
µg	Microgram
µm	Micrometer
ml	milliliter
min	Minute
M	Molar
MD	Molecular dynamics
MW	Molecular weight
nmol	Nanomol
PEG	Polyethylene glycol
pH	Negative logarithm of hydrogen ion concentration
*p*NP	*p*-nitrophenol
*p*NPP	*p*-nitrophenol palmitate
RMSD	Root mean square deviation
rpm	Rotation per minute
s	Second
sp	Species
TCA	Trichloroacetic acid
U	Unit of enzyme activity
U/mg	Unit per milligram
v/v	Volume per volume

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LIST OF SYMBOLS FOR AMINO ACIDS

A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartate
C	Cys	Cysteine
E	Glu	Glutamate
Q	Gln	Glutamine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine

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CHAPTER 1

INTRODUCTION

1.1 Background

Downstream processing can be defined as a series of separation and purification activities in which together can produce a protein product fit for its intended use (Milne, 2010). An efficient series of purification and separation steps to obtain certain degree of purification of the products is needed. The biomolecules separation is usually performed by batch mode in small scale such as column chromatography, electrophoresis, and solvent precipitation. However, there are considerable scale-up problems with these methods and making them uneconomical unless the products are of high value.

There is a clear essential for scalable bioseparation process that can be operated on a continuous basis. Liquid-liquid extraction (LLE) has been acknowledged for this purpose based on ease of operation and high flexibility in its mode of operation (Hemavathi et al., 2011). LLE consists of two classes which are aqueous two-phase extraction (ATPE) and reverse micellar extraction (RME) to extract bioactive components.

RME is a promising LLE method to solubilize biological products such as amino acids, proteins, enzymes and nucleic acids (Tonova et al., 2008). The separation and/or purification of proteins/enzymes are rather achieved in two rapid and simple steps with high degree of activity. It can recover easily without loss of native function/activity. DNA (Goto et al., 2000), organelles and even entire cells have also been solubilized into the RMs (Cinelli et al., 2006). To extract biomolecules from mixture/crude extract into reverse micelles (RMs) selectively, varying the extraction parameters need to be executed (Harikrishna et al., 2002).

Some trends have been developed in carrying out enzymatic reactions by implementing the semipermeable membranes/membrane reactors, temperature phase behaviour of the microemulsions or microemulsion-based organogels (Nagayama et al., 2008). There have been relatively few studies on the application of RME for separation and purification of biomolecules are available for model systems (commercial samples of proteins and enzymes) (Zhang et al., 2002; Shin et al., 2004;Dovyap et al., 2006; Mutalik and Gaikar 2006; Raikar et al., 2007; Hebbar and Raghavarao, 2007). When compared with

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model systems, only few studies on RME of biomolecules from natural systems were reported (Gaikar and Kulkarni, 2001; Mutalik and Gaikar, 2003; Liu et al., 2004; Noh et al., 2005;Chen et al., 2006; Hasmann et al., 2007; Hebbar et al., 2007; Nandini et al., 2009; Hemavathi et al., 2007, 2008, 2010).

Psychrophilic lipase has low optimum temperature for catalysis and high activity at low temperatures, which are good for frail compounds production. It has great flexibility under low water conditions over mesophilic and thermophilic enzymes (Joseph et al., 2008). Cold-adapted lipase is increasingly studied and widely used in organic synthesis of chiral intermediates, however the details information of the adaptation of this enzyme in the RME is scarce. Since cold-adapted lipase from Pseudomonas sp. strain AMS8 has a good interaction with nonpolar solvent especially toluene (Yaacob et al., 2016), so AMS8 lipase has been chosen for this study to provide insight into their adaptation during RME. The accession number of AMS8 lipase gene deposited in NCBI is ADM87309. It has 476 amino acid residues and is rich in unique nonpeptide sequences (repeat in toxin). This gene has been recombinantly expressed in E. coli strain BL21 (DE3) (pET32b/AMS8) (Ali et al., 2013).

The objectives of the study were:

1. To extract AMS8 lipase using reverse micellar extraction approach
2. To conduct and analyse molecular dynamics simulation of AMS8 lipase in Triton X-100/toluene reverse micelles

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PUBLICATION

Abd. Jalil, F.N.F.A., Abd. Rahman, R.N.Z.R., Salleh, A.B., Ali, M.S.M. (2018). Optimization and in silico analysis of a cold-adapted lipase from an Antarctic Pseudomonas sp. strain AMS8 reaction in Triton X-100 reverse micelles. *Catalysts, 8,* 289.

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