



UNIVERSITI PUTRA MALAYSIA

***EXPRESSION AND CHARACTERIZATION OF RECOMBINANT
THERMOSTABLE L2 LIPASE IN PICHIA PASTORIS***

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**EXPRESSION AND CHARACTERIZATION OF
RECOMBINANT THERMOSTABLE L2 LIPASE IN
*PICHA PASTORIS***

SURIANA SABRI

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**EXPRESSION AND CHARACTERIZATION OF RECOMBINANT
THERMOSTABLE L2 LIPASE IN *PICCHIA PASTORIS***

By

SURIANA SABRI

**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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A special dedication to

Mak, Abah, K. Long, K. Ngah, Abg. Hosni, Abg. Halim, K. Ani, Abg. Jamal, K. Uji, Abg. Yunus and Sarah, who believe in me,

To my nephews and nieces; Syafiqah, Hafiz, Zafirah, Syahirah, Hazirah, Samirah, Suraya, Safia, Nabil and Idham, for their presence, that light up my life,

To Leow; for his unfaltering support and always being there for me...



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**EXPRESSION AND CHARACTERIZATION OF RECOMBINANT
THERMOSTABLE L2 LIPASE IN *PICHIA PASTORIS***

By

SURIANA SABRI

April 2007

Chairman: Professor Raja Noor Zaliha Raja Abd Rahman, PhD

Faculty: Biotechnology and Biomolecular Sciences

The gene encoding mature thermostable L2 lipase from *Bacillus* sp. L2 was cloned into *Pichia pastoris* expression vectors and placed under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter and methanol inducible alcohol oxidase (*AOX*) promoter. In inducible system, recombinant L2 lipase was efficiently secreted into the culture medium driven by the *Saccharomyces cerevisiae* α -factor signal sequence, compared to the constitutive system. The optimization of the recombinant L2 lipase production (from inducible system) in 100 mL culture was done for the best clones pP α S3 and pP α G2 from *Pichia* strains SMD1168H and GS115, respectively. The effect of media formulation, methanol concentration and induction time on L2 lipase production from inducible system was evaluated. A time course profile of recombinant lipase production in 500-mL flasks with the optimized conditions



was performed and 15.3 mg/mL and 14.25 mg/mL of dry cell weight were produced after 144 h of induction time from recombinant pP α S3 and pP α G2, respectively. The lipase activities detected from both clones were 91 U/mL and 125 U/mL for pP α S3 and pP α G2, respectively.

The recombinant L2 lipase was purified to 1.8-fold with 63.2% yield and with specific activity of 458.1 U/mg using affinity chromatography. The enzyme was in a monomeric form, non-glycosylated with a molecular weight of 44.5 kDa. The optimum pH and temperature were 8.0 and 70°C, respectively. The enzyme was stable in the pH range of 8.0-9.0 and at 65°C for 60 min where it retained more than 70% of its residual activity. The metal ions Ca²⁺, Na⁺, Cu²⁺ and Mn²⁺ activated the lipase at 1 mM, whereas Mg²⁺ and Zn²⁺ inhibited it. Lipase showed a notable preference for medium to long chain triacylglycerols (C10-C16), with the highest activity toward tripalmitin (C16). It hydrolyzed all the natural oil tested, with the highest hydrolysis rate on corn oil and the least was on sunflower oil. L2 lipase was inhibited by EDTA, PMSF, pepstatin A and all the surfactants tested. It showed random positional specificity towards triolein. CD spectral analysis of L2 lipase revealed a T_m of around 67.2°C.



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

**PENGEKSPRESAN DAN PENCIRIAN LIPASE L2 TERMOSTABIL
REKOMBINAN DALAM *PICHIA PASTORIS***

Oleh

SURIANA SABRI

April 2007

Pengerusi: Profesor Raja Noor Zaliha Raja Abd Rahman, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Gen yang mengekod lipase L2 termostabil dari *Bacillus* sp. L2 telah diklonkan di dalam vektor pengepresan *Pichia pastoris* dan diletakkan di bawah kawalan promoter-promoter gliseraldehida-3-fosfat dehydrogenase (GAP) konstitutif dan alkohol oksidase (AOX) teraruh metanol. Di dalam sistem teraruh, lipase L2 rekombinan telah dirembeskan ke dalam media kultur oleh jujukan isyarat α -faktor *Saccharomyces cerevisiae* dengan lebih berkesan berbanding sistem konstitutif. Pengoptimuman penghasilan lipase L2 (dari sistem teraruh) di dalam kultur 100 mL telah dilakukan untuk klon-klon terbaik iaitu pP α S3 dan pP α G2 daripada strain *Pichia* SMD1168H dan GS115, masing-masing. Kesan formulasi media, kepekatan metanol, dan masa aruhan ke atas penghasilan rekombinan lipase L2 dari sistem teraruh telah dinilai. Kajian profil masa terhadap penghasilan lipase L2 dengan keadaan optimum telah dijalankan dengan menggunakan kelalang 500-mL dan sebanyak 15.3 mg/mL dan 14.25



mg/mL berat sel kering telah dihasilkan selepas 144 j masa aruhan dari klon-klon pP α S3 dan pP α G2 masing-masing. Aktiviti lipase untuk kedua-dua klon adalah 91 U/mL dan 125 U/mL untuk pP α S3 dan pP α G2, masing-masing.

Lipase L2 rekombinan telah ditulenkan kepada 1.8 kali ganda, dengan penghasilan sebanyak 63.2% dan aktiviti spesifik sebanyak 458.1 U/mg dengan menggunakan kromatografi afiniti. Enzim tersebut berada dalam bentuk monomer, tidak diglikosilasikan dan mempunyai berat molekul sebanyak 44.5 kDa. pH dan suhu optimum enzim ini adalah 8.0 dan 70°C, masing-masing. Enzim ini stabil pada pH 8.0-9.0 dan pada 65°C selama 60 min di mana ia mengekalkan lebih daripada 70% aktivitinya. Ion-ion logam seperti Ca²⁺, Na⁺, Cu²⁺ dan Mn²⁺ pada kepekatan 1 mM boleh mengaktifkan lipase L2, manakala Mg²⁺ dan Zn²⁺ menyahaktifkannya. Lipase L2 lebih memilih untuk menghidrolisiskan triasilgliserol berantai sederhana ke panjang (C10–C16), dengan aktiviti yang paling tinggi ke atas tripalmitin (C16). Ia juga menghidrolisiskan kesemua minyak semulajadi yang diuji dengan kadar hidrolisis yang tertinggi pada minyak jagung, dan yang terendah pada minyak bunga matahari. Lipase L2 dinyahaktifkan oleh EDTA, PMSF, pepstatin A dan kesemua surfaktan yang telah diuji. Ia menunjukkan kespesifikan posisi rawak terhadap triolein. Analisis spektra CD terhadap lipase L2 menunjukkan nilai T_m sebanyak 67.2°C.

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I certify that an Examination Committee met on 20th April 2007 to conduct the final examination of Suriana Sabri on her Master of Science thesis entitle “Expression and Characterization of Recombinant Thermostable L2 Lipase in *Pichia pastoris*” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the Master of Science.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at other institution.

SURIANA SABRI

Date: 22 MAY 2007



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LIST OF ABBREVIATIONS

APS	ammonium persulphate
bp	base pair
BSA	bovine serum albumin
CTAB	cetyltrimethylammonium bromide
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Da	dalton
kDa	kilo dalton
g/L	gram per liter
pmol	picomole
N	normal
rpm	rotation per minute
xg	gravity
UV	ultraviolet
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis



LB	Luria Bertani
TCA	trichloroacetic acid
OD	optical density
OD ₆₀₀	optical density at 600 nm
A ₂₆₀	absorbance at 260 nm
A ₂₈₀	absorbance at 280 nm
ms	milisecond
SLS	sodium lauryl sulphonate
TEMED	N,N,N,N-Tetramethylenediamide
TSB	tripticase soy broth
YNB	yeast nitrogen base
U/mL	unit per milliliter
U/mg	unit per milligram
v/v	volume per volume
w/v	weight per volume
V	Volt
V/cm	volt per centimeter
μF	Microfarad
MD	minimal dextrose
MM	minimal methanol
kb	kilo base
Mut ⁺	methanol utilization phenotype plus



Mut ^S	methanol utilization phenotype slow
BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
RT	room temperature
sp	species
U	unit
YPD	yeast extract, peptone and dextrose media
YPDS	yeast extract, peptone, dextrose and sorbitol media
CD	circular dichroism
MW	molecular weight



CHAPTER 1

INTRODUCTION

The global market for industrial enzymes has already achieved the USD 2 billion mark, and it is sure to grow. A report from McKinsey & Co., recently indicated that the future for sustainable development is clearly a bright one, and enzyme technology will play a major role, along with the use of microorganisms, both natural and engineered (Wood and Scott, 2004). To date, approximately 80% of all industrial enzymes are hydrolytic in nature and used for depolymerization of natural substances. Of these enzymes, 60% are proteolytic enzymes used by the detergent, dairy and leather industries. Thirty percent are carbohydrases used in baking, distilling, brewing, starch, and textile industries. This leaves lipases and highly specialized enzymes for use in pharmaceutical, oleochemical, and analytical industries (Kirk *et al.*, 2002). However, this share has the potential to increase dramatically via a wide range of lipases' new applications (Jaeger and Eggert, 2002; Pandey *et al.*, 1999).

Lipases are efficient catalysts for lipolytic reactions initiating the catabolism of fats and oils by hydrolyzing the fatty acyl ester bonds of acylglycerols (Vulfson, 1994). Lipases have tremendous potential for further exploitation in biotechnology. Their ability to catalyze a wide variety of reactions allow numerous applications in industry such as the removal of oils and fats from



fabrics, machinery and waste water, the production of mono- and diglycerides for food emulsifiers and stereospecific synthesis of compounds including precursors for biologically active therapeutics, herbicides or pesticides (van Kuiken and Behnke, 1994; Haas *et al.*, 1992).

Enzymes from thermophiles have been found to be the most practical commercial used biocatalysts to date because of their overall inherent stability which are better suited to the harsh conditions of industrial processes (Kirk *et al.*, 2002). There are many efforts directed at improving enzymes involved in industrial processes in order to decrease cost and increase energy efficiency. One of the most promising methods to obtain better enzymes is via recombination DNA technology to produce the enzymes in large quantities with desired properties which will make them economically viable.

Cloning and characterization of lipases from thermophilic bacteria and the expression of the biologically active proteins in *Escherichia coli* had been reported (Rahman *et al.*, 2005, Sinchaikul *et al.*, 2001). This protein shows high activity at high temperature and this feature offers several interesting advantages in term of biotechnological applications. Although the protein obtained from recombinant *E. coli* was sufficient to perform a variety of experiments, the low production together with the complex purification procedures were proven unsuitable for industrial production of the enzyme.

