UNIVERSITI PUTRA MALAYSIA

STRUCTURAL INVESTIGATION OF ALCOHOL OXIDASE FROM *Meyerozyma guilliermondii* AND THE USE OF ITS PROMOTER FOR RECOMBINANT PROTEIN EXPRESSION

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By

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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Alcohol oxidase promoter (pAOX1) is a tightly regulated methanol inducible promoter in methylotrophic yeasts. The use of methanol is a vital factor in pAOX1 regulation. Locally isolated yeast strain SO (with partial characterisation) has been developed to be an expression host using pAOX1 (origin: *Pichia pastoris*). It demonstrated the capability to express recombinant bacterial lipase faster than *P. pastoris* expression system with minimal methanol induction from previous study. The pAOX1 of strain SO shared 100% similarity of pAOX1 in *P. pastoris*, but its *AOX* gene has not been identified yet. Therefore, the two main objectives of this project are to investigate the type of *AOX* gene in strain SO with its function and to use its pAOX1 in the expression of recombinant proteins (other than lipase). In addition, further characterisation (morphological and biochemical) were done to characterise this strain. The microscopy analysis (using scanning and transmission electron microscopy) and carbon assimilation results confirmed that strain SO was a *Meyerozyma guilliermondii*. Hidden Markov Model (HMM) analysis was performed to identify AOX protein from reference proteome (*M. guilliermondii* ATCC6260). The protein was identified as a long chain alcohol oxidase (LCAO). Then, the full of *LCAO* gene was amplified from *M. guilliermondii* strain SO genome *via* PCR using primer walking technique. Initial bioinformatics (structural) analysis indicated that the open reading frame (ORF) of the LCAO in this strain (referred as MgFAO1) composed of FAD-binding domain (the most conserved domain in the AOX protein). Based on the phylogenetics analysis, the MgFAO1 was clustered with other LCAOs. The function of MgFAO1 was identified by predicting the three-dimensional (3D) structure using YASARA and validated by PROCHECK, ERRAT and Verify3D. Docking analysis showed that MgFAO1 has corresponded to the long chain alcohol substrate (1-dodecanol). To achieve the second objective, two enzymes (diamine oxidase and W200R protease) were cloned into pPICZαB (driven by pAOX1) and transformed into *M. guilliermondii* strain SO *via* electroporation method. The results showed that, all the recombinant proteins were successfully expressed in both conditions (with and without methanol). This result suggested that the pAOX1 might use the MgFAO1 to facilitate the recombinant proteins expression in this strain without methanol

induction as it favoured 1-dodecanol than methanol. As a conclusion, the structure of AOX in *M. guilliermondii* strain SO was investigated for its function and different recombinant proteins were successfully expressed using its pAOX1 in the absence of methanol. This newly developed yeast system can be used for recombinant protein production particularly for food product since expression can be done without methanol induction.

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

PENYIASATAN STRUKTUR ALKOHOL OKSIDASE DARIPADA *MEYEROZYMA GUILLIERMONDII* DAN PENGGUNAAN PROMOTER UNTUK LUAHAN PROTIN REKOMBINAN

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Promoter alkohol oksidase (pAOX1) merupakan promoter yang dikawal ketat dan dicetus oleh metanol dalam yis metilotropik. Penggunaan metanol adalah faktor penting dalam pengawalan pAOX1. Yis pencilan tempatan strain SO (dengan pencirian separa) telah dibangunkan untuk menjadi hos kepada ekpresi protein menggunakan pAOX1 (asal: *Pichia pastoris*). Ia mempunyai keupayaan untuk mengekspresi lipase rekombinan dari bakteria lebih cepat daripada sistem ekpresi *P. pastoris* dengan induksi metanol yang kurang daripada kajian terdahulu. pAOX1 strain SO berkongsi 100% persamaan dengan *P. pastoris*, tetapi gen *AOX*nya masih belum dikenal pasti. Oleh itu, dua objektif utama projek ini adalah untuk menyiasat jenis gen *AOX* yang terdapat dalam strain SO berserta fungsinya dan menggunakan pAOX1 dalam ekpresi protein rekombinan (selain daripada lipase). Di samping itu, pencirian lanjut (morfologi dan biokimia) telah dilakukan untuk mencirikan strain ini. Analisis mikroskopi (menggunakan mikroskop elektron pengimbasan dan penghantaran) dan keputusan asimilasi karbon mengesahkan bahawa strain SO adalah *Meyerozyma guilliermondii*. Analisis Model Markov Tersembunyi (HMM) dilakukan untuk mengenal pasti protein AOX yang terdapat dalam proteom rujukan (*M. guilliermondii* ATCC6260). Protein ini dikenal pasti sebagai alkohol oksidase rantaian panjang (LCAO). Kemudian, gen *LCAO* penuh diamplikasi daripada genom *M. guilliermondii* strain SO melalui PCR menggunakan teknik primer berjalan. Analisa awal bioinformatik (struktur) menunjukkan bahawa kerangka bacaan terbuka (ORF) kepada LCAO dalam strain ini (dirujuk sebagai MgFAO1) yang terdiri daripada domain FAD-terikat (domain paling konservatif dalam protein AOX). Berdasarkan analisis filogenetik pula, MgFAO1 dikelompokkan bersama LCAO lain. Fungsi MgFAO1 dikenalpasti dengan meramalkan struktur tiga dimensi (3D) menggunakan YASARA dan disahkan oleh PROCHECK, ERRAT dan Verify3D. Analisis pendokkan menunjukkan bahawa MgFAO1 berpadanan dengan substrat alkohol rantaian panjang (1-dodekanol). Untuk mencapai objektif kedua, dua enzim (diamina oksidase dan protease W200R\) telah diklonkan ke dalam pPICZαB (didorong oleh pAOX1) dan ditransformasikan ke dalam *M. guilliermondii* strain SO melalui kaedah elektroporasi. Keputusan menunjukkan bahawa, semua protein rekombinan berjaya diekpresikan

dalam kedua-dua keadaan (dengan dan tanpa metanol). Hasil ini mencadangkan bahawa pAOX1 mungkin menggunakan MgFAO1 untuk membantu ekpresi protein rekombinan tanpa induksi metanol kerana ia menyukai 1-dodekanol daripada metanol. Sebagai kesimpulan, struktur AOX dalam *M. guilliermondii* strain SO disiasat untuk fungsinya dan protein rekombinan yang berlainan telah berjaya diekpresikan dengan menggunakan pAOX1 tanpa kehadiran metanol. Sistem ragi yang baru dibangunkan ini boleh digunakan untuk pengeluaran protein rekombinan terutamanya untuk produk makanan kerana ekpresi boleh dijalankan tanpa induksi metanol.

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

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Å	Armstrong
AAO	Aromatic alcohol oxidase
AAO1	AAO from *Pleurotus eryngii*
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)
ADP	Adenosine diphosphate
AOD1	AOX from *Candida boidinii*
AOX	Alcohol oxidase
AOX1	Alcohol oxidase 1 from *Pichia pastoris*
AOX2	Alcohol oxidase 2 from *P. pastoris*
BLAST	Basic local alignment search tool
bp	Base pair
CAT	Catalase
CDH	Cellobiose dehydrogenase
CHD	Choline dehydrogenase
cm	Centimeter
CM	Cell membrane
CHOx	Cholesterol oxidase
CO	Choline oxidase
CW	Cell wall
DAO	Diamine oxidase
DAO_{BL21}	DAO/pET102/D-TOPO in *Escherichia coli* BL21
DAO_{KM71H}	Diamine oxidase/pPICZαB in *P. pastoris* KM71H
DAO_{SO}	Diamine oxidase/pPICZαB in strain SO
DAO_{TOP10}	DAO/pPICZαB in *E. coli* TOP10
DAS	Dihydroxyacetone synthase
DHA	Dihydroxyacetone
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
FAD	Flavin adenine dinucleotide
FAO	Fatty alcohol oxidase
FDH	Formate dehydrogenase
FLD/ FLD1	Formaldehyde dehydrogenase
g	Gram
g	Centrifugal force
GAP	Glyceraldehydes-3-phosphate
GMC	Glucose methanol choline
GOX	Glucose oxidase
GSH	Gluthathione
g/L	Gram per litre
h	Hour/s
HBsAg	Hepatitis B virus surface antigen

HMM	Hidden Markov Model
HNL	Hydroxynitrile lyase
HRP	Horseradish peroxidase
HSA	Human serum albumin
H_2O_2	Hydrogen peroxide
kcal/mol	Kilocalorie per mol
kDa	Kilo Dalton
LB	Luria bertani
LCAO	Long chain alcohol oxidase
M	Molar
MEGA7	Molecular evolutionary genetics analysis
mFAD	Modified Flavin adenine dinucleotide
MgFAO1	Long chain alcohol oxidase from *Meyerozyma guilliermondii* strain SO
mL	Millilitre
Mut^+	Methanol utilisation plus
Mut^s	Methanol utilisation slow
Mut^-	Methanol utilisation minus
mg	Milligram
NAD	Nicotinamide adenine dinucleotide
ng	Nanogram
µg	Microgram
µl	Microlitre
MCS	Multiple cloning site
min	Minute/s
mM	Millimolar
MOX	Methanol oxidase
MSA	Multiple sequence alignment
MUSTANG	Multiple structural alignment algorithm
nm	Nanometer
ω	Omega
OD	Optical density
ORF	Open reading frame
PAO	Polyvinyl alcohol oxidase
pAOX	Alcohol oxidase promoter
PCR	Polymerase chain reaction
PDB	Protein data bank
pGAP	Glyceraldehydes-3-phosphate promoter
pMOD1	Promoter of methanol oxidase 1 from *Pichia methanolica*
pMOD2	Promoter of methanol oxidase 2 from *P. methanolica*
pMOX	Promoter of methanol oxidase from *Hansenula polymorpha*
psi-blast	Position-specific iterated-basic local alignment search tool
RE	Restriction enzyme
RNA	Ribonucleic acid

rpm	Rotation per minute
SAO	Secondary alcohol oxidase
SCAO	Short chain alcohol oxidase
SEM	Scanning electron microscopy
TBM	Template-based modelling
TEM	Transmission electron microscopy
T1lipase$_{SO}$	T1lipase/pPICZαB in strain SO
U	Unit
U/mg	Unit per milligram
U/ml	Unit per ml
W200R$_{KM71H}$	W200R/pPICZαB in *P. pastoris* KM71H
W200R$_{SO}$	W200R/pPICZαB in strain SO
W200R$_{TOP10}$	W200R/pPICZαB in *E. coli* TOP10
Xu_5P	Xylulose-5-phosphate
2D	Two-dimensional
3D	Three-dimensional

CHAPTER 1

INTRODUCTION

Recombinant protein production has been studied over a decade ago because of the multibillion-dollar market value as an efficient strategy for more applications to obtain the high amounts of high-quality proteins (Mattanovich *et al*., 2012). The choice of a production host is a first step in developing a new product. Several expression hosts ranging from bacteria to higher eukaryotic, including the mammalian cell are commercially available nowadays. Expression of proteins in yeast is a mutual alternative to prokaryotic and higher eukaryotic. The commonly available of yeast protein expression systems are from genera *Pichia, Saccharomyces, Hansenula, Yarrowia* and *Kluyveromyces* (Madzak *et al*., 2004; van Ooyen *et al.,* 2006; Mattanovich *et al*., 2012). Yeast cells offer several advantages over the others for recombinant protein expression. Yeast is a single cell, with ease of genetic modification. It can grow faster than other eukaryotic cells. The most crucial feature in yeast expression system is the ability to perform post-translational modification which does not exist in the prokaryotic system. This modification improves the protein structure and diversifies its functions, and dynamically coordinate the signal integration and physiological states (Wang *et al*., 2014**)**. Glycosylation, proteolytic processing, disulphide bond formation and multimeric assembly are the list of available protein modifications (Chen *et al*., 2012). The improvement of protein structure is important since it greatly influence almost all aspects of pathogenesis and many cellular processes such as protein degradation, cellular differentiation signaling, regulatory processes, regulation of gene expression, and protein-protein interactions (Geiss-Friedlander and Melchior, 2007; Grotenbreg and Ploegh, 2007; Mattanovich *et al*., 2012). Yeast systems promote high level of protein expression and have the possibility of high cell densities cultivation with simple medium formulation by the process of fermentation (Chen *et al*., 2012; Adrio and Demain, 2014). Besides, strong and tightly regulated promoters present in yeast system make it preferable as an expression host (Pornpimon *et al*., 2010). Moreover, several yeasts also meet all the safety aspects and hold the *Generally Recognized as Safe* (GRAS) status such as *Saccharomyces cerevisiae* as it is not a pathogen and does not harbor the viral inclusion (Boer *et al*., 2007).

Locally isolated yeast strain SO was previously isolated from a spoiled orange. Partial identification through DNA barcoding has identified this strain as *Pichia* sp. strain SO (GenBank accession no.: JN084128) (Oslan *et al*., 2012). Yet, the morphological and biochemical characteristics are still unknown. Previous research done by Oslan *et al*., (2015) showed that, the host can be used to express recombinant lipase. The optimum expression of recombinant T1 lipase under the regulation of alcohol oxidase promoter (pAOX1) in yeast strain SO has proven to be faster than *Pichia pastoris* system with minimal methanol induction. pAOX1 is known as an inducible promoter where the expression of recombinant protein in *P. pastoris* using pAOX1 strictly requires methanol induction (Zhang *et al*., 2010). Currently, only one protein has been expressed in this system. Therefore, in order to test the yeast system, other recombinant proteins need to be expressed in this system. In addition, previous research indicated that pAOX in yeast strain SO was 100% similarity to *P. pastoris*, but the AOX gene has not been investigated yet (Oslan *et al*., 2015). Therefore, the research questions here are: 1) what are the

morphological and biochemical characteristics of the yeast strain SO, 2) what is/ are the type(s) and characteristic(s) of *AOX* gene present in yeast strain SO, and 3) can this yeast system express other recombinant proteins using pAOX1 vector system from *P. pastoris*. Thus, this research was conducted with the following objectives:

1. To characterise the morphological and biochemical characteristics of yeast strain SO.
2. To investigate the structure and function of AOX gene present in yeast strain SO.
3. To express two recombinant proteins using AOX promoter in yeast strain SO.

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