UNIVERSITI PUTRA MALAYSIA

METABOLIC PROFILING OF Meyerozyma guilliermondii STRAIN SO AND A RECOMBINANT STRAIN SO2 EXPRESSING LIPASE

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METABOLIC PROFILING OF *Meyerozyma guilliermondii* STRAIN SO AND A RECOMBINANT STRAIN SO2 EXPRESSING LIPASE

By

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METABOLIC PROFILING OF *Meyerozyma guilliermondii* STRAIN SO AND A RECOMBINANT STRAIN SO2 EXPRESSING LIPASE

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Faculty: Biotechnology and Biomolecular Sciences

A locally isolated yeast, *Meyerozyma guilliermondii* strain SO is capable of acting as a host to express heterologous protein under the regulation of methanol-dependence alcohol oxidase promoter (P_{AOX}). Methanol is a potent compound to induce the P_{AOX}. However, *M. guilliermondii* strain SO has shown its ability to express the bacterial recombinant thermostable lipase from *Geobacillus zalihae* strain T1 without methanol induction. Metabolite profiling could facilitate in understanding the distinctive compounds of the metabolic pathway in this system after the lipase was expressed. This study aims to investigate and identify the metabolites responsible for P_{AOX} auto-induction in this newly developed expression system. Initially, the time point where the lipase was expressed optimally without methanol in Yeast extract-Peptone-Tryptic soy broth (YPT) medium was determined, followed by metabolites extraction. Then, the metabolites were detected using gas chromatography-mass spectrometry (GC-MS). A multivariate statistical analysis (MVA) was performed and biosynthetic pathways for the respective metabolites were determined from the KEGG database. The results showed that the optimum time for lipase expression without methanol was detected after 60 h cultivation with 3.34 U/mL activity. In contrast, no lipase activity was detected in the commercial system, *Komagataella pastoris* without methanol as the inducer. In this study, MVA namely principle component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were used to determine the relationship between metabolites present in wild-type SO and recombinant strain SO2 carrying T1 lipase. Upon evaluation of four different samples at 0 and 60 h, numbers of primary metabolites such as fatty acids, amino acids and organic acids were significantly present based on the separation trend and the contribution of metabolites in PCA and PLS-DA. Further interpretation using variable importance in projection (VIP) scores of PLS-DA showed that eicosanebioic acid and benzeneacetic acid were the most significant compounds present in four different sets of intracellular and extracellular samples, respectively. In addition, the heatmap analysis and showed a slightly abundance of fatty acids (eicosanoic acid, eicosanebionic acid, octadecenoic acid and hexadecenoic acid) produced throughout the cultivation period. The pathway analysis showed the significant number of hits for fatty acid and unsaturated fatty acid

biosynthesis from the compounds detected. Finally, using the available data, a biosynthetic pathway was reconstructed and the metabolites responsible for auto-induction of P_{AOX} were found to be unsaturated fatty acids. In conclusion, metabolites of strain SO and its recombinant SO2 were successfully profiled and identified. This finding was significant where these unsaturated fatty acids could be used as the alternative inducers for P_{AOX} in *M. guilliermondii* strain SO expression system.

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

PEMPROFILAN METABOLIK *Meyerozyma guilliermondii* STRAIN SO DAN REKOMBINAN SO2 DALAM PENGHASILAN LIPASE

Oleh

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Yis pencilan tempatan, *Meyerozyma guilliermondii* strain SO mampu bertindak sebagai perumah untuk mengungkap protein heterologus di bawah kawalan penggalak (P_{AOX}). Methanol adalah sebatian penting untuk mendorong penggalak dalam sistem yis. Manakala, *Meyerozyma guilliermondii* strain SO telah menunjukkan keupayaan untuk mengungkap lipase rekombinan tahan haba bakteria daripada *Geobacillus zalihae* strain T1 tanpa aruhan methanol. Pemprofilan metabolit boleh memudahkan pemahaman sebatian istimewa dalam laluan metabolik di dalam sistem ini apabila lipase rekombinan telah diungkapkan. Kajian ini bertujuan untuk mengaji dan mengenalpasti metabolit yang bertanggungjawab untuk aruhan otomatik P_{AOX} dalam sistem ekspresi yang baru dibangunkan ini. Pada mulanya, titik masa di mana lipase dihasilkan secara optimum tanpa metanol dalam medium YPT ditentukan, diikuti oleh penghasilan metabolit menggunakan kaedah pengekstrakan alkohol. Kemudiannya, metabolit dikesan menggunakan kromatografi gas spektrometri jisim (GC-MS). Analisis statistik multivariat (MVA) telah dilakukan dan laluan bagi metabolit masing-masing ditentukan daripada pangkalan data KEGG. Hasil kjian menunjukkan bahawa masa yang optimum ekspresi lipase tanpa methanol dikesan selepas pengeraman 60 jam dengan 3.34 U / mL Manakala, aktiviti ini tidak dikesan dalam sistem ekspresi komersial, *Komagataella pastori*s apabila methanol sebagai induksi tidak dibekalkan. Dalam kajian ini, analisis statistik multivariat (MVA) iaitu analisis komponen utama (PCA) dan analisis diskriminan-separa kuasa dua terkecil (PLS-DA) digunakan untuk menentukan hubungan antara metabolit yang berada dalam jenis liar SO dan rekombinan SO2 yang mempunyai T1 lipase. Penilaian empat set sampel yang ditetapkan pada 0 h dan 60 h, sebilangan metabolit hadir dengan ketara seperti asid lemak, asid amino dan asid organik berdasarkan sumbangan metabolit dan corak pemisahan yang diperhatikan dalam PCA dan PLS-DA. Untuk analisis lebih lanjut, kepentingan pembolehubah dalam unjuran (VIP) PLS-DA menunjukkan bahawa asid eikosanebioik adalah sebatian paling banyak terdapat dalam empat set sampel. Di samping itu, analisis peta haba menunjukkan kehadiran asid lemak (asid eikosanebioik, asid oktadecanoik dan asid heksadekanoik) sepanjang tempoh.

Analisis jalur menunjukkan kepentingan dalam laluan yang berkaitan dengan asid lemak seperti biosintesis asid lemak dan biosintesis asid lemak tak tepu. Akhir sekali, dengan menggunakan hasil kajian yang ada, laluan biosintetik telah dibina secara manual dan metabolit yang bertanggungjawab untuk menyebabkan aruhan otomatik P_{AOX} adalah asid lemak tak tepu. Kesimpulannya, metabolit SO strain dan rekombinannya SO2 telah berjaya diprofilkan dan dikenalpasti dengan sewajarnya. Hasil kajian ini menunjukkan kepentingan bahawa asid lemak tak tepu ini boleh digunakan sebagai induksi alternatif untuk P_{AOX} dalam sistem ekspresi *M. guilliermondii* strain SO.

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LIST OF ABBREVIATIONS

%	percent
% (v/v)	percent concentration volume / volume
% (w/v)	percent concentration weight / volume
^{o}C	degree celsius
µg	microgram
µL	microliter
µm	micrometer
x *g*	relative centrifugal force (times gravity)
cm	centimetre
et al.,	and colleagues
g	gram
GRAS	Generally Recognised as Safe
h	hour
i.d.	internal diameter
kPa	kilopascal
L	litre
M	Molar
m	meter
mg	milligram
min	minute
mL	milliliter
mM	millimolar
mm	millimetre
NSB	non-salt-based
OD_{600nm}	optical density at 600 nm
rpm	rotation per minute
sp.	species (singular)
sec	second
U/mL	unit per milliliter
v/v	volume per volume
w/v	weight per volume
YPD	yeast extract, peptone, and dextrose
YPT	yeast extract, peptone, tryptic soy broth and biotin
YPTG	yeast extract, peptone, tryptic soy broth, biotin and glycerol
YPTM	yeast extract, peptone, tryptic soy broth, biotin and methanol

CHAPTER 1

INTRODUCTION

1.1 Background Study

Meyerozyma guilliermondii is a model organism for flavinogenic yeasts. It has an ability to oversynthesis riboflavin during starvation of iron (Tanner et al., 1945). Some of *M. guilliermondii* can convert xylose into xylitol, which is an anti-carries sweetener (Rosa et al., 1998). *M. guilliermondii* is classified as GRAS organism. It utilizes hydrocarbon compounds as the sole carbon source. Hence, a lot of studies were done on *M. guilliermondii* for overproduction of riboflavin, enhancing the xylitol production and bio-control of postharvest disease (Abbas and Sibirny, 2011; Zou et al., 2011; Sangwanich et al., 2013).

In 2015, Oslan et al., conducted the first study on the capability of *M. guilliermondii* SO as a host to express thermostable T1 lipase from *Geobacillus zalihae*. It has an alcohol oxidase (AOX) and a formaldehyde dehydrogenase (FLD) promoters in the yeast genome. The difference between *M. guilliermondii* strain SO and *K. pastoris* is that *M. guilliermondii* strain SO does not require methanol to induce alcohol oxidase promoter, P_{AOX} (Abu et al., 2017). Besides, Oslan et al. (2015) reported that the recombinant yeast took less time duration (30 h) to reach optimal production as compared to *K. pastoris*, which took longer time (144 h) to reach its optimal condition. In addition, the protein produced is safe for food production because methanol is not added during expression. *M. guilliermondii* strain SO may be commercialized better than *K. pastoris*, due to the advantage of food safety production.

Metabolomics is the comprehensive and quantitative assessment of endogenous metabolites and attempts to systematically identify and quantify metabolites from a biological sample (Zhang et al., 2012). The sampling and sample treatment techniques have to be reliable, reproducible due to the rapid turnover of intracellular metabolites (Tredwell et al., 2011). Biochemical pathways such as metabolic, regulatory or signal transduction pathways can be viewed as inter-connected processes. There are three major biochemical pathways; signal transduction pathways (STPs), gene regulatory networks (GRNs) and metabolic pathways (Liu, 2005).

1.2 The Current Status of the Research

A number of metabolomics studies were done on *K. pastoris*, but neither targeted nor non-targeted metabolomics studies was done on *M. guilliermondii*. The protocols of metabolomics sampling for *K. pastoris* was developed to improve the baseline metabolome data and to reduce the leakage of intracellular metabolite (Tredwell et al., 2011; Carnicer et al., 2012). Genome-scale metabolic network models (GMEs) of *K. pastoris* has been reconstructed and expanded for a better understanding of metabolic

network. Besides, a few possible approaches for strain improvement was developed through *in silico* simulations focusing on the metabolic effects of recombinant protein production (Caspeta et al., 2012). Unrean (2014) constructed and analysed metabolic pathway of *K. pastoris* to interpret methanol metabolism and its regulation for production of recombinant proteins by elementary mode analysis (EMA). Analysis of all the identified pathways led to the determination of the metabolic capacities as well as the optimum metabolic pathways for recombinant protein synthesis during methanol induction. The understanding of the metabolic behaviour in *K. pastoris* was enhanced by applying the quantitative metabolomics coupled with ^{13}C-based metabolic flux analysis (^{13}C-MFA) (Jordà et al., 2014). The combination of ^{13}C-MFA methodology and quantitative metabolomics shows how multi-level -omic studies can bring new understandings on key elements of the relationship between cell metabolism and recombinant protein production.

The ability of *M. guilliermondii* strain SO to express recombinant T1 lipase using alcohol oxidase promoter has not been fully understood. In methylotrophic yeast, the protein expression system strictly require methanol as inducer. In *M. guilliermondii* strain SO, the recombinant protein can be expressed without methanol induction. The metabolites in *M. guilliermondii* strain SO are unknown. By comparing metabolic profiles of SO and its recombinant carrying bacterial lipase (SO2) at the highest lipase expression may discover the metabolites responsible for auto-induction of the P_{AOX}. Thus, in order to find a way to understand *M. guilliermondii* strain SO, the research was conducted with the following objectives:

1. To determine the optimum time for recombinant T1 lipase expression in recombinant *M. guilliermondii* strain SO2 without methanol induction.
2. To extract and profile the intra- and extracellular metabolites within *M. guilliermondii* wild-type SO and recombinant SO2 without methanol induction.
3. To identify the metabolites responsible for auto-induction of alcohol oxidase promoter (P_{AOX}) in recombinant SO2 from the constructed metabolic pathways.

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