EXPRESSION OF THERMOSTABLE α-AMYLASE USING FORMALDEHYDE DEHYDROGENASE PROMOTER IN Meyerozyma guilliermondii STRAIN SO

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FBSB 2018 54

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By

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Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

December 2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

EXPRESSION OF THERMOSTABLE *α*-AMYLASE USING FORMALDEHYDE DEHYDROGENASE PROMOTER IN *Meyerozyma guilliermondii* STRAIN SO

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December 2018

Chairman : Siti Nurbaya Oslan, PhD
Faculty : Biotechnology and Biomolecular Sciences

α-amylase is an enzyme that breakdown starch or glycogen to release simple molecules such as maltose and glucose. SR74 α-amylase produced from the *Geobacillus stearothermophilus* SR74 has huge potential for commercialization for industrial applications due to its thermostable and thermoactive properties. However, its expression in wild type host is too low. Thus, it was cloned and expressed in two expression systems namely, bacteria and yeast. Its expression in *Escherichia coli* was accompanied with the formation of inclusion bodies. The drawbacks of *E. coli* expression system were overcome by the *Pichia pastoris* expression system. However, SR74 α-amylase expression in *P. pastoris* under alcohol oxidase promoter (P_{AOX}) required longer fermentation time and high methanol consumption. Therefore, there is a need for a new expression system that can overproduce α-amylase extracellularly without methanol induction with shorter fermentation time. This study aims to express SR74 α-amylase in a newly developed yeast expression system, *Meyerozyma guilliermondii* strain SO using formaldehyde dehydrogenase promoter (P_{FLD}). Initially, an integration site of pFLDα in *M. guilliermondii* strain SO genome was predicted, followed by cloning and expression of α-amylase using P_{FLD}. Then, the α-amylase production in *M. guilliermondii* strain SO was optimized. *FLD* gene was determined and isolated with the help of hmmer3.1b-2 (HMM) software for prediction of integration site. Based on nucleic acid sequence analysis, it was confirmed that pFLDα was able to integrate at P_{FLD} loci in *M. guilliermondii* strain SO genome. Then, SR74 α-amylase gene was cloned into pFLDα followed by transformation into *M. guilliermondii* strain SO. Colony PCR, α-amylase screening plate and western blot analysis proved that SR74 α-amylase was successfully cloned and expressed in *M. guilliermondii* strain SO. Qualitative (screening plate) and quantitative screenings (Dinitrosalicylic acid assay) for recombinant α-amylase production were performed to assess the activity of recombinant α-amylase under P_{FLD} regulation. Optimization of extracellular SR74 α-amylase production (assayed at 65 °C) in *M. guilliermondii* strain

SO found that the highest expression was 26 U/mL at 24 h, without methanol induction. The yield was 16-fold higher than the wild-type. *G. stearothermophilus* SR74 and was comparable to the *P. pastoris* system at 5.17 times faster without methanol induction. In conclusion, the SR74 α-amylase has successfully been expressed in this newly expression with shorter cultivation time and without inducer requirements at comparable yield than established *P. pastoris* expression system. Shorter time and no inducer requirement were expected to significantly reduce the cost of production. The optimization procedure by Response Surface Methodology (RSM) software, mutational study and in-depth study on P_{FLD} regulation were suggested to increase the production and to make the enzyme more favorable to industrial application.

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

EKSPRESI α-AMILASE TERMOSTABIL MENGGUNAKAN PENGALAK FORMALDEHID DIHIDROGENASE DALAM *Meyerozyma guilliermondii* STRAIN SO

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α-amilase adalah enzim yang boleh meguraikan kanji dan glikogen untuk menghasilkan molekul ringkas seperti maltosa dan glukosa. SR74 α-amilase yang dihasilkan oleh *Geobacillus stearothermophilus* SR74 mempunyai potensi yang besar untuk dikormesilkan bagi memenuhi keperluan industri disebabkan ciri-ciri termostabil dan termoaktifnya. Namun begitu, penghasilan α-amilase di dalam hos jenis liar adalah terlalu rendah. Oleh itu, ia telah diklon dan diekspres ke dalam dua jenis sistem pengekspresan protein iaitu bakteria dan yis. Namun yang demikian, penghasilannya dalam *Escherichia coli* disertai dengan jasad inklusi. Kelemahan sistem ekspresi *E. coli* telah diatasi oleh sistem pengekspresan protein iaitu *Pichia pastoris.* Walau bagaimanapun, penghasilanya dalam *P. pastoris* di bawah aruhan penggalak alkohol oksidase (P_{AOX}) memerlukan masa fermentasi yang lama dan penggunaan metanol yang banyak. Oleh itu, sistem ekspresi baru yang dapat menghasilkan ekstraselular SR74 α- amilase tanpa aruhan metanol dengan masa ferementasi yang pendek amatlah diperlukan. Kajian ini bertujuan untuk menghasilkan SR74 α-amilase dalam sistem eksperesi yis yang baru iaitu *Meyerozyma guilliermondii* strain SO di bawah aruhan penggalak formaldehida dehidrogenase (P_{FLD}). Sebagai permulaan, tapak integrasi pFLDα ke dalam genome *M. guilliermondii* strain SO diramal dan diikuti dengan pengklonan dan penghasilan α-amilase menggunakan P_{FLD}. Kemudian, penghasilan α-amilase dalam *M. guilliermondii* strain SO telah dioptimumkan. Gen *FLD* ditemukan dan dipencilkan dengan bantuan perisian hmmer3.1b-2 (HMM) untuk ramalan tapak integrasi. Analisis jujukan asid nukleik telah mengesahkan bahawa pFLDα berupaya berintegrasi di tapak lokus P_{FLD} di dalam genom strain SO. Kemudian, gen SR74 α-amilase telah diklon ke dalam pFLDα diikuti dengan transformasi ke dalam *M. guilliermondii* strain SO. PCR koloni, agar saringan dan analisis "Western blot" telah membuktikan bahawa SR74 α-amylase telah berjaya diklon dan diekspres di dalam *M. guilliermondii* strain SO. Saringan secara kualitatif (agar saringan) dan kuantitatif (esei "Dinitrosalicylic acid") telah dijalankan untuk penilaian aktiviti SR74 α-amilase di

bawah pengawalaturan $P_{FLD.}$ Analisis optimasi SR74 α-amilase (esei pada suhu 65 °C) dalam *M. guilliermondii* strain SO menunjukkan bahawa penghasilan tertingginya adalah 26 U/mL pada jam ke-24 tanpa aruhan metanol. Hasil tersebut adalah 16 kali ganda lebih banyak daripada jenis liar *G. stearothermophilus* SR74 dan lebih kurang sama dengan sistem *P. pastoris.* Namun, ia adalah 5.17 kali ganda lebih cepat tanpa aruhan metanol. Kesimpulanya, SR74 α-amylase telah berjaya diekspres dalam sistem ekspresi yis terbaru dengan masa pengkulturan yang lebih pendek tanpa keperluan aruhan dapat menghasilkan hasilan yang lebih kurang sama dengan sistem *P. pastoris*. Masa yang pendek tanpa keperluan aruhan diramal dapat mengurangkan kos penghasilan enzim dengan ketara. Analisis optimasi menggunakan perisian "Response Surface Methodology" (RSM), kajian mutasi ke atas SR74 α-amilase dan kajian terhadap aruhan pernggalak P_{FLD} dicadangkan agar dapat meningkatkan penghasilan enzim dan menjadikan ia lebih sesuai untuk aplikasi industri.

ACKNOWLEDGEMENTS

I am very grateful to Allah for giving me constant health and sustenance from the start till the end of my study. I would also like to express my gratitudes towards my mentor and supervisor Dr. Siti Nurbaya Oslan for guidance and advice throughout this study. The gratitude was extended to Prof, Dato Dr. Abu Bakar Salleh and Assoc. Prof. Dr. Adam Leow Thean Chor for their contribution in completion of this study.

A million thanks to my own self for not giving up and always be positive. Besides, I also would like to extend my thankfulness towards my beloved parents, sisters and brother for being supportive to me throughout my life and being the motivation for me in completing this study. Special thanks to 'Abang ngah' and family for always entertaining me and giving moral support during my study.

I would also like to thank IBS members and Biotech 3 labaratory mates especially Apple, Yana and Fatin for their willingness in sharing ideas, giving cooperation and being helpful during the study. Lastly, the thanks were extended to my dear bestfriends, Izzah, Sal and Hanim. Thank you for always lending your ears and giving emotional support.

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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LIST OF ABBREVIATIONS

AOX	Alcohol oxidase
Bla	beta-lactamase
bp	Base pair
BLAST	Basic local alignment search tool
DAS	Dihydroxyacetone synthase
Da	Daltons
DHA	Dihydroxyacetone
DNA	Deoxyribonucleic acid
DNS	3,5-Dinitrosalicylic acid
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
FDA	Food and Drug Administration
FDH	Formate dehydrogenase
FLD	Formaldehyde dehydrogenase
g	Gram
GAP	Glyceraldehyde-3-phophate
GRAS	Generally recognized as safe
GSNOR	S-nitrosoglutathione reductase
GSNO	S-nitrosoglutathione
h	Hour
His-tag	Histidine tag
HMM	Hidden Markov Model
HMMER	Software for Hidden Markov Model
kb	Kilo basepair
kDa	Kilo Dalton
L	Litre
LPS	Lipopolysachharides
M	Molar
mA	Miliampere
MEGA	Molecular Evolutionary Genetics Analysis
MGAs	Minimal Glycerol with Ammonium sulfate

mL	Millilitre
MMAs	Minimal Methanol with Ammonium sulfate Medium
MUT	Methanol utilization pathway
MUT^-	Methanol utilization minus phenotype
MUT^S	Methanol utilization slow phenotype
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrate
nm	Nano metre
$OD_{600\ nm}$	Optical density at 600 nm
PCR	Polymerase chain reaction
pH	Measure of the hydorgen ion concentration of a solution
RNA	Ribonucleic acid
RSM	Response Surface Methodology
S-HMGSH	S-(hydroxymethyl)glutathione dehydrogenase
SO	Spoiled orange
TCA	Trichloroacetic acid
U	Unit
V	Voltage
YIP	Yeast integrative Plasmid
YPD	Yeast Peptone Dextrose medium
YPT	Yeast Peptone Tryptic soy broth medium
YNB	Yeast Nitrogen Base
µF	Millifarad
Ω	Ohm
x g	Gravity

CHAPTER 1

INTRODUCTION

α-amylase is an enzyme that belongs to glycosyl hydrolase family that randomnly hydrolyses polysaccharides such as starch or glycogen by cleaving internal α-1,4-glycosidic bond to release simple sugar molecule such as maltose or glucose (Metin et al., 2010). α-amylase has been widely used in textile, starch processing, detergent, textile and pharmaceutical industries (Mehta & Satyanarayana, 2016). In industrial process, enzymes are used in extreme condition in respect to temperature as well as pH (Prakash & Jaiswal, 2010). Therefore, thermostability is one of main issue for industrial amylase. Thermophilic microorganisms are favored for the production of thermostable amylases (Kavanagh, 2017). *Geobacillus stearothermophilus* SR74 is a thermophilic microorganism isolated from Slim River, Perak and it is able to produce thermoactive extracllular α-amylase (Kassaye, 2009). The α-amylase from *G. stearothermophilus* SR74 has potential to be commercialized for industrial application due to its thermoactive and thermostable properties. Therefore, it has to be produced in large amounts for its commercialization purposes. However, its production in wild-type *G. stearothermophilus* SR74 is too low (Kassaye, 2009). Therefore, experimental studies have been done to maximize its production. To date, it has been cloned and expressed into *Escherichia coli* and *Pichia pastoris* expression systems (Kassaye, 2009; Gandhi et al., 2015).

In general, recombinant protein production in *E. coli* system is high however it comes with a few bottlenecks such as proteins produced are intracellular, prone to aggregation and accompanied with formation of inclusion body (form as a result of protein aggregation) that lead to low protein recovery (Espargaró et al., 2012). Formation of inclusion bodies has been the hindarance to the large-scale production of soluble proteins in *E. coli* (Indicula-Thomas & Balaji, 2007). Hence, it was cloned again into *P. pastoris* (methylotrophic yeast) due to its lower allergenic potential than *S. cerevisiae* (Gellissen et al., 2005), the presence of strong promoters like alcohol oxidase promoter (P_{AOX}) that contributes to higher protein yield (Hartner & Glieder, 2006) and its ability to produce protein extracellularly. Nevertheless, the production of SR74 α-amylase in *P. pastoris* under P_{AOX} regulation is inconvenient for commercial application due to longer fermentation time as well as its strong dependency on methanol as inducer (Gandhi et al., 2015).

P_{AOX} is the most commonly used promoter for foreign protein expression in *P. pastoris*. Nonetheless, its dependency on methanol is challenging in certain aspects. Therefore, promoter such as formaldehyde dehydrogenase promoter (P_{FLD}) that does not rely on methanol is considered as an alternative promoter (Shen et al., 1998). P_{FLD} was reported to produce foreign protein in comparable yield as P_{AOX} in *P. pastoris* (Shen et al., 1998). It is a promising alternative to P_{AOX} (Cos et al. , 2005). Moreover, a newly developed expression host, *Meyerozyma guillermondii* strain SO has potential to produce foreign protein at faster rate than *P. pastoris* with minimal methanol induction

(Oslan et al., 2015). *M. guillermondii* strain SO requires 30 h whereas *P. pastoris* requires 60 h to produce foreign protein at optimum level (Oslan et al., 2015). P_{FLD} is found to be present in this host. Hence, P_{FLD} regulation in *M. guilliermondii* strain SO is expected to overproduce the SR74 amylase extracellularly in minimal or without methanol induction at faster rate. Since this is the first study to report on the use of P_{FLD} in strain SO, determination of integration site in the genome needs to be done. Therefore, this study comes out with the following objectives;

1) To determine the integration site of pFLDα in *M. guilliermodii* strain SO genome.
2) To clone and express the α-amylase gene into *M. guilliermodii* strain SO under the regulation of $P_{FLD.}$
3) To optimize the related parameters for α-amylase expression in *M. guilliermodii* strain SO.

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