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DEVELOPMENT OF PROTEASE DEFICIENT Meyerozyma guilliermondii STRAIN SO FOR OVEREXPRESSION OF THERMOSTABLE T1 LIPASE

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STRAIN SO FOR OVEREXPRESSION OF THERMOSTABLE T1 LIPASE**

By

OKOJIE ESEOGHENE LORRINE

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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A special dedication to:

To my beloved husband, Mr. Williams Ehigbokan Okojie and my sons, Master Alvin James Okojie and Master Jayden Oseyimeje Okojie for their support, love, and care throughout this project, without which I would never have triumphed in my academic endeavours. Thank you all for been my source of strength and inspiration.

DEVELOPMENT OF PROTEASE DEFICIENT *Meyerozyma guilliermondii* STRAIN SO FOR OVEREXPRESSION OF THERMOSTABLE T1 LIPASE

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August 2022

Chair : **Assoc. Prof. Siti Nurbaya Oslan, PhD**
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Meyerozyma guilliermondii strain SO, a novel expression host was found to belong to the *Meyerozyma* species complex called the CTG clade yeasts that exhibited a particular genetic code where the universal leucine CUG codon was predominantly translated as serine and rarely as leucine. In previous study, *M. guilliermondii* strain SO, was used as an expression host for thermostable T1 lipase from *Geobacillus zalihae* under the regulation of an alcohol oxidase promoter (pAOX1) with a low yield recorded. This could be due to the CUG ambiguity as well as the vacuolar protease(s) of strain SO secretory system which were potential bottleneck during the production of recombinant secretory proteins in yeast systems. In this study, two strategies had been implemented to maximize the secretory potential of strain SO by firstly carrying out a codon optimization of the recombinant protein using the *M. guilliermondii* codon usage and secondly, by identification and disruption of the critical putative vacuolar proteases of strain SO using Cre-Lox recombination technique.

Transformation and expression of a codon optimized T1 lipase gene cloned onto pPICZαB vector (pPICZαB/T1SLip) was performed in strain SO, where previous recombinant plasmid pPICZαB/T1 was used as positive control. However, there was no significant difference in the expression levels between the wild type and the codon optimized T1 lipase gene. Then, hidden Markov model (HMM) software was used to search for the possible vacuolar proteases (hits) in strain SO proteome. From the results, a vacuolar aspartic protease (PEP4) with 97.55% identity to *Meyerozyma* sp.JA9 and a serine protease (PRB1) with 70.91% identity to *Candida albicans,* were found in strain SO proteome. Evolutionary analysis, further confirmed homology with other yeast vacuolar proteases. In addition, the structures of strain SO PEP4 and PRB1 were predicted using Phyre2 and validated by PROCHECK, ERRAT and Verify3D, with a comparability of 91.1% and 85.8% with their respective templates from Ramachandran plots prediction. Further structural analysis revealed their

essential catalytic residues and a protein-ligand interaction, depicted their catalytic mechanisms. Next, Cre-Lox recombination technique was initiated to delete the identified PEP4 and PRB1 genes of strain SO. The upstream and downstream of the target genes were cloned to the promoter and terminator regions of the *SAT*1 flipper cassette respectively. Next, positive transformants were obtained after 24 h of growth incubation time on a selective medium containing 200 µg/mL of nourseothrincin (NAT).

Finally, optimization of recombinant proteins (T1 and T1SLip lipase) expression with the developed mutants in shake flask was carried out using recombinant pPICZαB/T1/APM-(APMSO2), pPICZαB/T1/SPM-(SPMSO2), pPICZαB/T1/DPM-(DPMSO2), pPICZαB/T1SLip/APM-(APM_807), pPICZαB/T1SLip/SPM-(SPM_089) and pPICZαB/T1SLip/DPM-(DPM_0789). Media YPTG (Yeast extract-Peptone-Tryptic soy broth and glycerol) and YPTM (Yeast extract-Peptone-Tryptic soy broth and methanol) were used to grow and induce the recombinant strains for the expression of T1 lipase with 0.5% (v/v) methanol induction shown to be the optimum concentration with an optimum induction time of 12 h interval for 3-5 days. The highest expression yield was recorded with the APMSO2 (1.12 U/mL at 72 h). It is interesting to note that, the optimum T1 lipase expression in APMSO2 was a 1000% increase compared to the wild type SO2.

In conclusion, the codon optimized T1 lipase (T1SLip) was sucessfully cloned into the vector backbone of pPICZαB and expressed in strain SO. The two critical putative vacuolar proteases (PEP4 and PRB1) were successfully identified in strain SO. The structures of strain SO PEP4 and PRB1 from strain SO were successfully predicted and analyzed for their catalytic functions. The deletion of the vacuolar protease genes were also successful and the developed mutants could express the codon optimized T1 lipase (T1SLip) and T1 (wild type) lipase genes with a 1000% increase recorded from APMSO2 compared to the wild type SO2.

PEMBANGUNAN STRAIN KEKURANGAN PROTEASE *Meyerozyma guilliermondii* SO UNTUK UNGKAPAN BERLEBIHAN LIPASE T1 TERMOSTABIL

Oleh

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Pengerusi : Prof. Madya Siti Nurbaya Oslan, PhD
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Meyerozyma guilliermondii strain SO, hos ekspresi novel didapati tergolong dalam kompleks spesies *Meyerozyma* yang dipanggil yis klad CTG yang mempamerkan kod genetik tertentu di mana kodon CUG leusina universal kebanyakannya diterjemahkan sebagai serina dan jarang sekali sebagai leusina. Dalam kajian terdahulu, strain *M. guilliermondii* SO, digunakan sebagai hos ekspresi untuk lipase T1 termostable daripada *Geobacillus zalihae* di bawah pengawalaturan pengaruh alkohol oksidase (pAOX1) dengan hasil yang rendah direkodkan. Ini mungkin disebabkan oleh kesamaran CUG serta protease vakuolar sistem rembesan SO yang berpotensi menjadi halangan semasa penghasilan protein rembesan rekombinan dalam sistem yis. Dalam kajian ini, dua strategi sedang dilaksanakan untuk memaksimumkan potensi rembesan strain SO dengan menjalankan pengoptimuman kodon protein rekombinan menggunakan kodon *M. guilliermondii* dan kedua, dengan mengenal pasti dan gangguan protease vakuolar kritikal SO menggunakan teknik rekombinasi Cre-Lox.

Transformasi dan ekspresi gen lipase T1 yang dioptimumkan kodonnya diklonkan pada vektor pPICZαB (pPICZαB/T1SLip) dalam strain SO, di mana plasmid rekombinan sebelumnya pPICZαB/T1 digunakan sebagai kawalan positif. Walau bagaimanapun, tidak terdapat banyak perbezaan yang ketara dalam tahap ekspresi antara T1 jenis liar dan gen lipase T1 yang dioptimumkan kodon. Kemudian, perisian model Markov tersembunyi (HMM) digunakan untuk mencari kemungkinan protease vakuolar (hits) dalam proteom SO. Keputusan, protease aspartik vakuolar (PEP4) dengan identiti 97.55% kepada *Meyerozyma* sp.JA9 dan protease serin (PRB1) dengan identiti 70.91% kepada *Candida albicans*, ditemui dalam proteom SO. Analisis evolusi, seterusnya mengesahkan homologi dengan protease vakuolar yis lain. Di samping itu, struktur strain SO PEP4 dan PRB1 telah diramalkan menggunakan Phyre2 dan disahkan oleh

PROCHECK, ERRAT dan Verify3D, dengan perbandingan 91.1% dan 85.8% dengan templat daripada ramalan plot Ramachandran. Analisis struktur selanjutnya mendedahkan asid-asid amino penting dan interaksi protein-ligan, di mana menggambarkan mekanisme pemangkin mereka. Seterusnya, teknik penggabungan semula Cre-Lox telah dimulakan untuk menghapuskan gen PEP4 dan PRB1 yang dikenal pasti dalam strain SO. Hulu dan hilir gen sasaran telah diklonkan ke kawasan pengaruh dan penamat bagi kaset flipper SAT1 masing-masing. Seterusnya, transforman positif diperoleh selepas 24 jam inkubasi pertumbuhan dalam medium terpilih yang mengandungi 200 µg/mL nourseothrincin (NAT).

Akhirnya, pengoptimuman ekspresi protein rekombinan (T1 dan T1SLip) dengan mutan yang dibangunkan dalam kelalang goncang telah dijalankan menggunakan rekombinan pPICZαB/T1/APM-(APMSO2), pPICZαB/T1/SPM-(SPMSO2), pPICZαB/T1/DPM- (DPMSO2), pPICZαB/T1SLip/APM-(APM_807), pPICZαB/T1SLip/SPM-(SPM_089) dan pPICZαB/T1SLip/DPM-(DPM_0789). Media YPTG (Ekstrak yis-Peptone-Tryptic soya sup dan gliserol) dan YPTM (Yis extract-Peptone-Tryptic soya broth and metanol) telah digunakan untuk dan mendorong strain rekombinan untuk ekspresi T1 lipase dengan 0.5% (v/v) aruhan metanol dan masa aruhan optimum selang 12 jam selama 3-5 hari. Hasil ungkapan tertinggi direkodkan adalah dengan APMSO2 (1.12 U/mL pada 72 jam). Adalah menarik untuk diperhatikan bahawa, ekspresi lipase T1 optimum dalam APMSO2 telah meningkat sebanyak 1000% berbanding strain SO jenis liar.

Kesimpulannya, kodon lipase T1 yang dioptimumkan (T1SLip) telah berjaya diklon ke dalam tulang belakang vektor pPICZαB dan dinyatakan dalam SO. Dua protease vakuolar putatif kritikal (PEP4 dan PRB1) berjaya dikenal pasti dalam strain SO. Struktur PEP4 dan PRB1 daripada SO telah berjaya diramalkan dan untuk fungsi pemangkinnya. Penghapusan gen protease vakuolar juga berjaya dan mutan yang dibangunkan boleh mengekspresikan gen lipase T1 (T1SLip) dan T1 (jenis liar) yang dioptimumkan kodon dengan peningkatan 1000% direkodkan daripada APMSO2 berbanding SO2 jenis liar.

iv

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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vii

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

cm	centimetre
$^{\circ}C$	degree celsius
µg	microgram
µL	microlitre
g	gram
g	gravity
GRAS	Generally Recognised as Safe
h	hour
i.d.	internal diameter
kPa	kilopascal
L	litre
M	Molar
m	meter
mg/mL	milligram/millilitre
mg	milligram
min	minute
mL	milliliter
mM	millimolar
mm	millimetre
OD	optical density
OD_{600nm}	optical density at 600 nm
OD_{715}	optical density at 715 nm
OD_{595}	optical density at 595 nm

rpm	rotation per minute
sec	second
U/mL	unit per milliliter
v/v	volume per volume
w/v	weight per volume
x g	relative centrifugal force
∞	infinity
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   General

Genome editing technologies have emerged rapidly and have been extraordinary implemented in various fields, ranging from basic research to applied biotechnology and biomedical research (Khalil, 2020; Li *et al.,* 2020). These genome editing tools include the transcription activator–like effector nucleases (TALENs) (Bhardwaj and Nain, 2021; Sebastian and Boch, 2021), zinc-finger nucleases (ZFNs) (Ran *et al.,* 2018; Paschon *et al.,* 2019), and the RNA-guided CRISPR-Cas nuclease system (Adli, 2018). The first two technologies employ a strategy of tethering endonuclease catalytic domains to modular DNA-binding proteins to induce targeted DNA double-stranded breaks (DSBs) at specific genomic loci. Alternately, CRISPR-Cas9 is a nuclease guided by small RNAs through Watson-Crick base pairing with target DNA (Ashwini et al., 2022). Cre/ lox is a proven and widely used site-specific recombination system that has been adapted from P1 bacteriophage for use in genetic engineering (Yarmolinsky *et al.,* 2015). It is an efficient genetic tool developed for targeted, repeated, and markerless gene integration (Wu *et al.,* 2018; Zhou *et al.,* 2021), that requires only a single selection marker and can completely excise all of the unwanted sequences.

Recombinant DNA technology allows the combination of a foreign DNA to a carrier DNA called a vector that enables the ease of transportation into a specific host. This technology has provided many benefits which includes larger production of genetically engineered proteins that are safe, easy to manipulate as compared to its natural host and conversely ease manufacturing processes. Recently, *Pichia pastoris* a yeast expression system was reported to express cloned gene from *Geobacillus zalihae* (Oslan *et al.,* 2015). More so, engineering biological systems and organisms, hold enormous potential for applications across basic, medicine, science, medicine and biotechnology. Some examples of engineered hosts that have been used to optimize recombinant protein production include *Escherichia coli* (Pramata *et al.,* 2021) and *P.pastoris* (Ergun *et al.,* 2021).

In recent times, different industrial sectors such as food and feed processing, paper and pulp production, detergent and textile amongst many others, rely heavily on the use of various recombinant proteins for diverse applications. Of the different available platforms for recombinant protein production (McKenzie and Abbott, 2018; Owczarek *et al.,* 2019; Puetz and Wurm, 2019), the yeasts system is one of the efficient protein production platforms due to its ability to produce functional recombinant proteins (Baghban *et al.,* 2019; Lestari and Novientri 2021). Hydrolytic enzymes such as protease, amylase cutinase and

1

lipase are classes of enzymes which are greatly utilized in many important industrial applications and depending on their substrates and conditions, they may be used to catalyse several reactions (Arnold, 2018; Trudeau and Tawfik 2019). Furthermore, enzymes with unique characteristics for optimum reactions such as thermoliability, cold activity, solvent tolerant and thermstability are also impacting in industries.

Over time, thermostable enzymes have attracted huge attention due to their high reaction rate at higher temperatures and inherent stability (Boyce and Walsh, 2018; Fusco *et al.,* 2018; Singh N., *et al.,* 2021). They are mostly isolated from thermophilic microorganism. However, reports showed that they could be obtained from mesophiles and psychophiles (Shariff *et al.,* 2007). In 2007, Rahman *et al.,* isolated a thermostable T1 lipase from a thermophilic *G. zalihae* strain T1, a bacterium isolated from palm oil mill effluent in Malaysia with an activity of 0.15 U/mL and its properties were characterized (Leow *et al.,* 2007). With prokaryotic expression system, thermostable T1 lipase was expressed intracellularly with an activity of 42 U/mL and extracellularly with an activity of 28 U/mL facilitated by bacteriocin release protein (BRP) (Rahman *et al.,* 2005).

However, Oslan *et al.,* (2015) reported the expression of thermostable T1 lipase in a eukaryotic expression system. They reported the use of an alternative yeast expression host called *Meyerozyma guilliermondii* strain SO. Yeast expression system aids to eliminate several limitations faced in prokaryotic expression system such as toxic acetate that prevents cells to reach higher cell density, formation of inclusion bodies (mis-folded proteins) (Abdel-Fattah and Gaballa, 2008) and requires tedious downstream purification processes. Yeasts are easy to be modified genetically, with a simple fermentation profile. Nonetheless, they secrete large amount of glycosylated proteins, a typical feature to the eukaryotic system (Huerta and Michan, 2019).

Interestingly, *M. guilliermondii* belongs to the *Meyerozyma* species complex called the CTG-clade yeast and this group of yeast is reported to be ambiguous in nature (Corte *et al.,* 2015). In *M. guilliermondii,* an anamorph of *Candida guilliermondii,* its CUG codon is translated into serine residue instead of leucine (Santos e*t al.,* 2011), which occurs during the translational phase where the mRNA alters the decoding rules and thus changes the amino acids composition (Butler *et al.,* 2009). This is distinty fascinating because erroneons production of proteins is generally seen as a nuisance to biological systems (Kapur and Ackerman, 2018; Santos *et al.,* 2018). However, this phenomenon only occurs 3-5% when under normal or mild stress conditions, respectively (Gomes *et al.,* 2007, Ueda *et al.,* 1994, Massey *et al.,* 2003).

The expression of thermostable T1 lipase in *M. guilliermondii* strain SO was reported to be very low with an activity of 14 (U/ml) compared to commercial yeast expression system *P. pastoris* which gave an activity of 88 (U/ml) (Oslan

et al., 2015). This low level of expression may be due to one out of several limiting factors which is the activities of the vacuolar proteases, reported to be a bottleneck in heterologous protein expression in yeast host (Forgac *et al.,* 2000 and Li *et al.,* 2009) and also due to the CUG ambiguity presented by the CTG clade yeasts (Gomes *et al.,* 2007). It is possible to express the recombinant protein in CTG clade yeast by conducting codon optimization to reduce/eliminate the mistranslation due to CUG ambiguity.

1.2   Problem Statement

As a CTG clade yeast*,* the CUG ambiguity as well as the vacuolar protease(s) of *M. guilliermondii* strain SO secretory system, may have contributed to the low expression of thermostable T1 lipase.

1.3   Hypothesis

Synthezing of a codon optimized gene with the codon usage of *M. guilliermondii* and deletion or disruption of the vacuolar proteases of strain SO could help to improve the recombinant protein expression.

1.4   Main Objective

To develop protease deficient *M. guilliermondii* strain SO with improved expression for thermostable T1 lipase production.

1.4.1 Specific Objectives

- ➢ To clone and express the codon optimized T1 lipase gene in strain SO.
- ➢ To determine the protease activity and identify the vacuolar proteases in strain SO
- ➢ To predict and analyze the targeted vacuolar proteases structures using bioinformatic tools
- ➢ To construct the protease deficient strain(s) SO using homologous recombination technique (Cre-Lox)
- ➢ To express the codon optimized T1 lipase gene in protease deficient strains under optimized conditions.

3

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135