UNIVERSITI PUTRA MALAYSIA

ENHANCING RECOMBINANT T1 Lipase PRODUCTION IN Pichia guilliermondii

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FBSB 2017 19

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By

ABU MARY LADIDI

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

July 2017

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DEDICATION

I dedicate this research work to God Almighty the giver and sustainer of life and to my family who have being by my side.

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

ENHANCING RECOMBINANT T1 *Lipase* PRODUCTION IN *Pichia guilliermondii*

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ABU MARY LADIDI

July 2017

Chairman : Professor Abu Bakar Salleh, PhD
Faculty : Biotechnology and Biomolecular Sciences

Lipases are hydrolytic enzymes, ranked as third most relevant industrial enzymes with 5 % share in global enzyme market. Thermostable T1 lipase from *Geobacillus zalihae* was previously expressed under the regulatory control of alcohol oxidase promoter 1 (AOXp 1) in the methylotrophic yeast *Pichia guilliermondii*, isolated from spoiled orange. Methanol was found to be a vital compound to induce the promoter activity in *Pichia pastoris*. In this study *P. guilliermondii* has shown the potential to express the recombinant lipase without methanol under the regulation of AOXp 1. This study sought to optimise medium conditions of thermostable lipase with and without methanol as inducer. The expression of T1 lipase without methanol was expected to reduce cost and toxicity effect of methanol.

Buffered and non-buffered media compositions were studied for T1 lipase production, the media were first, supplemented with methanol then without methanol. Buffer complex methanol medium was observed to be optimum for T1 lipase production with a 3-fold increase over non-buffered methanol medium. *One-factor-at-a-time*, conventional method of optimisation was used to identify significant data range for medium parameters. Using the observed data range, eight parameters which includes temperature, pH, inoculum size, biomass concentration, incubation time, shaking speed, culture volume and methanol concentration, were screened for lipase production in methanol medium using Plackett-Burman Design. Temperature, inoculum size, culture volume and incubation time, were observed to exert significant effect on lipase production. These parameters were optimised using Box-Behnken Design of Response Surface Methodology. Optimum levels of these parameters were predicted at temperature 34 ^{o}C, culture volume 190 mL, inoculum size 4 v/v and incubation time 24 h with an experimented lipase activity of 9.26 U/mL. Over 2-fold increase before optimization in methanol medium was observed and 6-fold increase over previous research work.

On the other hand, six parameters which include temperature, pH, inoculum size, incubation time, shaking speed and culture volume were screened for T1 lipase production in medium without methanol. Three parameters were observed to have significant effect on lipase production, then, these parameters were further optimised using Box-Behnken Design and their optimum levels were achieved at pH of 6, inoculum size 2 v/v and incubation time of 24 h as was predicted and the experimented lipase activity of 2.012 U/mL was observed. This result gave 4-fold increase over lipase production before optimisation in medium without methanol. Recombinant T1 lipase production was further scaled up to 3 L in the bioreactor for 128 h in methanol medium and lipase activity observed was 12 U/mL at 120 h incubation time. Meanwhile, scale up in medium without methanol yielded a lipase activity of 3.5 U/mL at 118 h incubation time. The result has proven that, the time taken for optimum lipase production without methanol was faster from the production with methanol in the bioreactor.

In conclusion, temperature, pH, inoculum size, incubation time and culture volume for recombinant T1 lipase production were optimised in shake-flask and applied to the bioreactor level. Lipase was also produced in medium without methanol both at the shake flask level and the bioreactor. Thus, medium parameters optimisation has proven to be very useful in enhancing thermostable lipase production in recombinant *P. guilliermondii*. Although T1 lipase has been expressed in medium without methanol but it is lower compared to medium with methanol.

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

PENINGKATAN PENGELUARAN *Lipase* RENTAN HABA REKOMBINAN DALAM *Pichia guilliermondii* STRAIN SO

Oleh

ABU MARY LADIDI

Julai 2017

Pengerusi : Profesor Abu Bakar Salleh, PhD
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Lipase adalah enzim hidrolitik, tersenarai sebagai enzim ketiga paling relevan dalam industri dengan berkongsi sebanyak 5% dalam pasaran enzim global. Lipase T1 rentan panas dari *Geobacillus zalihae* sebelum ini telah dihasilkan di bawah kawalan penganjur alkohol oksidase 1 (AOXp 1) di dalam *Pichia guilliermondii* yang merupakan yis metanoltropik yang mana telah dipencilkan dari buah limau busuk. Metanol merupakan komponen penting untuk mengaruh aktiviti penganjur AOXp 1 dalam *Pichia pastoris*. Dalam kajian ini *P. guilliermondii* telah menunjukkan potensi untuk menghasilkan lipase rekombinan tanpa metanol di bawah aruhan AOXp 1. Kajian ini bertujuan untuk mengoptimumkan penghasilan lipase T1 rentan haba dengan kehadiran metanol dan tanpa methanol sebagai pengaruh. Penghasilan T1 lipase rekombinan tanpa aruhan metanol diharapkan akan mengurang kos dan kesan tosik daripada metanol.

Komposisi media yang berpenimbal dan tanpa penimbal dikaji untuk penghjasilan T1 lipase. Pada permulaannya, media tersebut disertakan dengan metanol, kemudian tanpa metanol. Media berpenimbal kompleks dengan metanol didapati telah menghasilkan T1 lipase yang optimum dengan peningkatan sebanyak 3 kali ganda berbanding media yang tidak berpenimbal dan bermetanol. Satu-faktor-pada-satu-masa merupakan kaedah perngoptimuman konvensional telah digunakan untuk mengenalpasti julat data penting untuk parameter dalam media. Berdasarkan julat data yang diperolehi, lapan parameter seperti suhu, pH, saiz inokulasi, kepekatan biomas, masa pengeraman, kelajuan penggolak, isipadu kultur dan kepekatan metanol telah dicerap untuk pengeluaran lipase dalam medium metanol menggunakan Reka Bentuk Plackett-Burman. Suhu, saiz inokulasi, isispadu kultur dan masa pengeraman didapati memberikan kesan yang penting kepada penghasilan lipase. Parameter-parameter ini dioptimumkan menggunakan yang reka bentuk Box–Behnken daripada kaedah respon permukaan. Tahap optimum parameter ini telah diramalkan pada suhu 34 ^{o}C,

kandungan cecair 190 mL, saiz inokulum 4 v/v dan masa inkubasi 24 jam dengan hasil lipase 9.26 U/mL dengan eksperimen. Lebih 2 kali ganda peningkatan diperhatikan sebelum pengoptimuman dalam medium metanol dan 6 kali ganda peningkatan ke atas kerja penyelidikan yang sebelumnya.

Sebaliknya, enam parameter termasuk suhu, pH, saiz inokulasi, masa pengeraman, kelajuan penggolak dan isipadu kultur telah disaring untuk penghasilan lipase dalam media tanpa metanol. Tiga parameter telah diperhatikan mempunyai kesan ketara dalam pengeluaran lipase maka, parameter ini seterusnya telah dioptimumkan menggunakan Reka bentuk Box–Behnken dan tahap optimum mereka dicapai pada pH 6 saiz inokulum 2 v/v dan masa inkubasi 24 jam seperti yang dijangkakan dan hasil lipase eksperimen 2.01 U/mL direkodhan. Keputusan ini memberikan kenaikan 4 kali ganda ke atas pengeluaran lipase sebelum pengoptimuman dalam media tanpa metanol. Pengeluaran T1 lipase rekcmbinan bertambah pada skala besar sehingga 3 L bioreaktor pada 128 jam di dalam media metanol dan penghasilan lipase yang diperolehi adalah 12 U/mL pada 120 h masa inkubasi. Sementara itu, media tanpa methanol pada skala besar menghasilkan aktiviti lipase sebanyak 3.5 U/mL ketika masa inkubasi 118 jam. Hasilnya telah terbukti bahawa, masa yang diambil untuk pengeluaran optimum lipase tanpa methanol adalah lebih cepat daripada pengeluaran dengan methanol di dalam bioreaktor.

Kesimpulannya, parameter media untuk pengeluaran lipase T1 rekombinan dioptimumkan dalam kelalang pengoncang dan digunakan ke peringkat bioreaktor. Lipase juga dihasilkan dalam media tanpa metanol di kedua–dua peringkat kelalang pengoncang dan di dalam bioreaktor. Oleh itu, parameter media yang optimum telah terbukti menjadi sangat berguna dalam meningkatkan pengeluaran lipase rekombinan rentan haba *P. guilliermondii*. Walaupun enzim lipase T1 telah berjaya dihasilkan dalam media tanpa metanol namun pengasilannya adalah ia lebih rendah berbanding media dengan metanol.

ACKNOWLEDGEMENTS

To God Almighty who is able to do exceedingly, abundantly, above all I ever ask, think or imagine, to him be glory and honor for the successful completion of this research work forever (Amen). I thank my supervisor Prof. Dato' Dr. Abu Bakar Salleh, for his counsel and expository during my research work, for I have gained knowledge. With a grateful heart I say thank you to my co-supervisor Dr. Siti Nurbaya Oslan, for her tireless efforts and guidance to the success of this work. Thank you Assoc. Prof. Dr. Rosfarizan Mohammad (HOD Bioprocess) for your contribution.

I appreciate members of the department of Biochemistry, Faculty of applied and natural sciences, Ibrahim Badamasi Babangida university Lapai, Niger state, Nigeria for the opportunity to study in Malaysia. Thanks to Enzyme and Microbial Research group for contributing to this research during the weekly group meetings. To members of Enzyme technology laboratory, Institut of Biosains, thanks for providing an enabling working environment for me to carry out my research work. Contributions made by Hisham and Atieah cannot be forgotten. To my friends, God bless you all. Words fail me to express my profound gratitude to my family Mrs Salamatu Comfort Abubakar, Mr and Mrs Iliya Joseph, Mr and Mrs Shillishi, Lydia Abu, Mr and Mrs Maikarfi, Mercy Abu, and Comfort Abu.

I certify that a Thesis Examination Committee has met on 28 July 2017 to conduct the final examination of Abu Mary Ladidi on his thesis entitled "Enhancing Recombinant T1 *Lipase* Production in *Pichia guilliermondii*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

%	Percentage
YPD	Yeast extract Peptone Dextrose
OD_{600}	Optical Density at 600 nanometer
BMMY	Buffered complex - methanol medium yeast extract
BMY	Buffered complex - l medium yeast extract
BMGY	Buffered complex – glycerol medium yeast extract
YPT	Yeast extract Peptone Tryptic soy
YPTG	Yeast extract Peptone Tryptic soy Glycerol
YPTM	Yeast extract Peptone Tryptic soy Methanol
mL	Milliliter
Rpm	Rotation per minute
^oC	Degree Celsius
h	hour
Df	Degree of freedom
CV	Coefficient of variation
R^2	Regression coefficient
Std Dev	Standard deviation
SS	Sum of Square
w/v	Weight per volume
v/v	Volume per volume

mg	Milligram
PO_2	partial pressure of oxygen
mM	Millimole
sec	Second
IT	Incubation time
AG	Agitation speed
CV	Culture volume
IS	Inoculum size
M	Molar
L	Liter
µg	Microgram
PBD	Plackett- Burman Design
BBD	Box-Behnken Design
$CaCl_2$	Calcium Chloride

CHAPTER 1

INTRODUCTION

Yeasts provide an alternative platform for recombinant protein production (Gellissen *et al.,* 2005). Yeasts like the prokaryotic system are easy to modify genetically, with a simple fermentation profile. Furthermore, they secrete large amount of glycosylated proteins, a feature peculiar to the eukaryotic system (Gellissen *et al.,* 2005; Böer *et al*., 2007).

Recently, a limited number of yeasts species able to metabolise methanol as their energy and carbon source were identified. They are being applied in the field of biotechnology for improved yield and cost-effective production of glycosylated recombinant proteins and secretion (Gellissen, 2000; Maleki *et al.,* 2010). These methanol metabolising yeasts are called the methylotrophic yeasts belonging to four genera. These are: *Pichia pastoris* (*Pichia*), *Hansenula polymorpha* (*Hansenula*), *Candida boidinii* (*Candida*) and *Pichia methanolica* (*Torulopsis*) (Hartner & Glieder, 2006).

Pichia pastoris is the model organism for the study of methanol utilization in the methylotrophic yeasts and is the most frequently used methylotrophic yeast for recombinant protein production (De Schutter *et al.,* 2009). Its ability to successfully express recombinant proteins is linked to the presence of a tightly regulated promoter system (alcohol oxidase 1) derivable from its methanol utilization pathway genes (Gellissen *et al.,* 2005; Hartner & Glieder, 2006; Böer, *et al*., 2007; Ahmad *et al*., 2014). The activation of AOX 1 promoter is fully dependent on methanol and its derivatives but is repressed by other carbon sources in *P. pastoris* (Inan & Meagher, 2001; Ahmad *et al*., 2014). Methanol is hazardous and flammable in nature making it undesirable for the production of certain foods, pharmaceuticals and industrial products (Ahmad *et al*., 2014).

A new yeast belonging to the genera *Pichia* of the methylotrophic yeast - *Pichia guilliermondii* (*Meyerozyma guilliermondii*), was isolated from spoiled orange recently in Malaysia (Oslan *et al.,* 2012). Thermostable lipase gene, strain T1, isolated from *Geobacillus zalihae* (Leow *et al.,* 2004), was cloned and expressed in *P. guilliermondii* using *P. pastoris* expression vector (pPICZαB-Invitrogen) (Appendix A). This led to the development of a recombinant host system in *P. guilliermondii* (Oslan *et al.,* 2015). *Pichia guilliermondii* is being developed as an alternative recombinant host to *P. pastoris*.

This study aim at enhancing thermostable T1 lipase production, in recombinant *P. guilliermondii*. The study also, seeks to address the cost of using methanol for gene expression and to optimise fermentation processes involve in protein production in *P. guilliermondii.*

1.1 Specific objectives

1. To study and evaluate buffered and non-buffered media compositions suitable for optimum growth of recombinant *P. guilliermondii* and T1 lipase expression. Also, to carryout univariate analysis of *one-factor-at-a-time* for generation of data units, for protein production in recombinant *P. guilliermondii* using the observed optimum medium.
2. To screen and optimise medium parameters in methanol supplemented medium and non-methanol supplemented medium using, Plackett-Burman Design (PBD) and Box-Behnken Design (BBD) of Response Surface Methodology (RSM).
3. To scale up thermostable lipase production at the bioreactor.

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