UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION OF MEDIA FOR IMPROVED PRODUCTION OF RECOMBINANT T1 LIPASE USING LOCAL SUBSTRATES

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IB 2017 36

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By

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

December 2017

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctorate of Philosophy

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December 2017

Chairman: Raja Noor Zaliha binti Raja Abd. Rahman, D. Eng
Institute: Bioscience

Thermostable T1 lipase carries a lot of potential in industrial applications such as in diesel production and detergent formulation. However, the usage of laboratory media can cost a fortune when used at commercial scale (adding up to the final cost value of the enzyme). In order to create a cheaper enzyme product a new medium formulation from cheaper sources and readily available is crucial. This study was designed to formulate new medium and to develop an efficient large scale bioprocess strategie for thermostable T1 lipase from recombinant *E. coli* BL21. Different carbon and nitrogen sources from agro and industrial waste were screened. The compositions of the medium were optimized using response surface methodology (RSM). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and lactose capability as inducer were also studied. The kinetics of T1 lipase production by recombinant *E. coli* were evaluated using Monod and Luedeking-Piret equations. The effects of dissolved oxygen tension (DOT) level on growth of recombinant *E. coli* and T1 lipase production were investigated in batch fermentation using 7.5 L stirred tank bioreactor. Fed-batch fermentation for T1 lipase production was initially developed in 7.5 L stirred tank bioreactor and then scaled up to 30 L. A newly formulated medium for production of T1 lipase was formulated using 5th grade molasses and fish processing waste as carbon and nitrogen sources. The medium consisted of molasses (2 g/L), fish waste (12%), NaCl (5 g/L), $MgSO_4$ (0.5 g/L) and KH_2PO_4 (1 g/L). Through centre composite design (CCD), medium compositions using IPTG as an inducer showed higher T1 lipase production in predicted (172.89 U/mL) and actual run (164.37 U/mL) compared to lactose as an inducer in predicted (123.47 U/mL) and actual run (120.34 U/mL). Both R^2 values calculated using RSM showed a good fit and the proposed models for T1 lipase production by recombinant *E. coli* were sufficient to describe the processes. T1 lipase production was found to be a growth associated process and 30% showed the optimal level of DOT for production of T1 lipase. The constant feed rate for fed-batch fermentation at 160 mL/h using 50% lactose as feeding medium was found to be optimal for production of T1 lipase (260.10 U/mL) and recombinant *E. coli* growth (51.30 g/L). The fermentation employing recombinant *E. coli* for T1 lipase production was successfully scaled-up to 30 L stirred tank bioreactor using a constant DOT

approach, with DOT level controlled at 30% saturation. 50% of cost reduction was successfully achieved in production of T1 lipase when using new formulated medium and so far, this is the first report of using molasses and fish waste in the medium formulation. The information and findings obtained from this study are very useful in designing and in the preparation of standard operating procedure (SOP) for production of T1 lipase by recombinant *E. coli* at pilot plant and at industrial scale.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGOPTIMUMAN MEDIA UNTUK PENINGKATAN PENGELUARAN RECOMBINANT T1 LIPASE MENGGUNAKAN SUBSTRAT-SUBSTRAT TEMPATAN

Oleh

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T1 lipase tahan haba mempunyai banyak potensi dalam industri seperti dalam penghasilan diesel dan formulasi detergen. Walau bagaimanapun, penggunaan media makmal akan menambah kepada nilai kos akhir enzim tersebut apabila digunakan pada skala komersial. Oleh itu, untuk menghasilkan produk enzim yang lebih murah, formulasi baru daripada sumber-sumber yang lebih murah dan mudah didapati sangat diperlukan. Kajian ini telah dihasilkan untuk mencipta media yang baru dan membangunkan sebuah strategi bioproses yang cekap secara besar-besaran bagi menghasilkan lipase T1 tahan haba dari recombinan *E. coli* BL21. Sumber karbon dan nitrogen yang berbeza daripada sisa pertanian dan industri telah disaring. Komposisi media telah dioptimumkan menggunakan kaedah gerak balas permukaan (RSM). Isopropyl β-D-1-thiogalactopyranoside (IPTG) dan laktosa sebagai penggalak juga telah diuji. Kinetik bagi penghasilan T1 lipase oleh recombinan *E. coli* dinilai menggunakan persamaan Monod dan Luedeking-Piret. Kesan ketegangan oksigen terlarut (DOT) dalam pertumbuhan dan pengeluaran recombinan T1 lipase *E. coli* disiasat dalam fermentasi sekelompok menggunakan tangki bioreaktor berpengaduk 7.5 L. Penghasilan T1 lipase menggunakan fermentasi sekelompok suapan dilakukan di dalam 7.5 L tangki bioreaktor dan kemudian pada skala besar 30 L. Satu formulasi baru untuk penghasilan T1 lipase adalah dirumus menggunakan molas gred kelima dan sisa pemprosesan ikan sebagai sumber karbon dan nitrogen. Medium terdiri daripada molas (2 g/L), sisa ikan (12%), NaCl (5 g/L), $MgSO_4$ (0.5 g/L) dan KH_2PO_4 (1 g/L). Melalui reka bentuk kopositpusat (CCD), komposisi media yang menggunakan IPTG sebagai penggalak menunjukkan penghasilan T1 lipase yang tinggi (172.89 U/mL) dan penghasilan yang sebenar (164.37 U/mL) berbanding laktosa sebagai penggalak yang diramalkan (123.47 U/mL) dan penghasilan yang sebenar dijalankan (120.34 U/mL). Kedua-dua nilai R^2 yang dikira menggunakan RSM menunjukkan ianya sesuai dan model untuk dicadangkan bagi pengeluaran recombinan T1 lipase *E. coli*. Penghasilan T1 lipase merupakan proses yang berkaitan dengan pertumbuhan dan 30% yang menunjukkan tahap optimum DOT bagi pengeluaran T1 lipase. Melalui fermentasi sekelompok suapan sekata, di mana 160 mL/h menggunakan laktosa 50% sebagai

medium suapan, didapati optimum bagi pengeluaran T1 lipase (260.10 U/mL) dan pertumbuhan recombinan *E. coli* (51.30 g/L). Fermentasi menggunakan recombinan *E. coli* untuk penghasilan T1 lipase telah berjaya dihasilkan pada skala besar sehingga 30 L menggunakan tangki bioreaktor menggunakan pendekatan DOT berterusan, di mana tahap DOT dikawal pada 30% ketepuan. 50% dari pengurangan kos telah berjaya dikurangkan di dalam pengeluaran T1 lipase apabila menggunakan media baru digubal dan setakat ini, ini adalah laporan pertama menggunakan molas dan sisa ikan dalam formulasi media. Maklumat dan penemuan yang diperolehi daripada kajian ini adalah amat berguna dalam merekabentuk dan penyediaan prosedur kendalian standard (SOP) untuk penghasilan T1 lipase daripada recombinan *E. coli* di loji rintis dan pada skala perindustrian.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Merciful and the Most Grateful and thanks to Allah for blessing me and giving me strength to complete my study.

First and foremost, I would like to express my heartiest gratefulness and appreciation to the chairperson, Professor Dr. Raja Noor Zaliha Raja Abd. Rahman for her consistent supervision and valuable advices throughout my study. My thanks also goes to all the supervisory committee members, Professor Dato' Dr. Abu Bakar Salleh, Assoc. Prof. Dr. Rosfarizan Mohamad and Assoc. Prof. Dr. Mohd Shukuri Mohamad Ali, for their endless supports and guidance. My thanks also forwarded to all my lab-mates for their friendship and help.

Last but not least, I am grateful and indebted to my parents, and my most lovely and patient wife, Norashikin Abdul Razak, for their endless support and thoughtfulness and indulgence; and for being tolerant and uncomplaining. And to my children, Harith Nasryn, Nurqaireen Hanisah and Muhammad Hasnain for cheering and heartening my life.

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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LIST OF ABBREVIATIONS

A	Acetate concentration
A_m	Maximum acetate accumulation
BSA	Bovine serum albumin
C	Oxygen concentration
C*	Oxygen concentration at 100% DOT
CCD	Central composite design
CL	Oxygen concentration at DOT<100%
dCO_2	Dissolved CO_2
DCW	Dry cell weight
DO	Dissolved oxygen
DOT	Dissolved oxygen tension
E/x	Lipase activity/cell concentration
F	Feed flow rate
HCDC	High cell density culture
HSD	Honestly Significant Difference
IPTG	Isopropyl β-D-thiogalactoside
IU	International units
kLa	Mass transfer coefficient
KS	Saturation constant
LB	Luria-Bertani
LPM	Litre per minutes
m	Growth associated sugar consumption constant
n	Non-growth associated sugar consumption constant
OD	Optical density

OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
P	Product
P/V	Constant power per unit volume
PB	Plackett-Burman
PBS	Phosphate Buffered Saline
Pg/VL	Power input/volume
Pm	Maximum product formed
Q/V	Volumetric airflow rate per unit volume
qO	Specific oxygen consumption rate
qP	Specific product formation rate
qS	Specific substrate consumption rate
RMSE	Root mean square error
RSM	Response surface methodology
S	Substrate
Sm	Maximum substrate consumed
So	Initial substrate concentration
SOP	Standard operating procedure
SSE	Sum of squares error
STR	Stirrer tank reactor
STDEV	Standard Deviation
SUDS	Scale-up-deteriorated production syndrome
t	Time
V	Volume
Vo	Initial volume

X	Cell concentration
Xm	Maximum cell concentration
Xo	Initial cell concentration
$Y_{P/S}$	Lipase yield
$Y_{P/X}$	Cell productivity
$Y_{X/S}$	Cell yield
α	Growth associated lipase production constant
β	Non-growth associated lipase production constant
π	Lipase production rate in fed-batch mode
σ	Substrate consumption rate in fed-batch mode
μ	Specific growth rate
μm	Maximum specific growth rate

CHAPTER 1

INTRODUCTION

Enzymes played a significant part in mankind's history to employ biological components aimed at a range of productions. Developments such brewing and wine production, which the roots could be traced to the beginning of history, it is ultimately dependent by the enzymes to complement of the fermenting yeast cells that mediating the transformation of substrates into desired products (Shuler & Kargi, 2002). Traditionally, most enzymes are obtained by ways of fermentation using GRAS-listed microorganisms and some enzymes are obtained from plant and animal sources (Casteleijn et al., 2013).

Lipases or acylglycerol hydrolases (E.C. 3.1.1.3) are enzymes that catalyse the hydrolysis of long chain triglyceride into the formation of diacylglyceride, monoglyceride, glycerol and free fatty acids at the interface between the insoluble substrate and water. Aside from their natural substrates, lipases catalyze the enantio- and regioselective hydrolysis and synthesis of a broad range of natural and non-natural esters. Lipases are prevalent throughout the earth's flora and fauna, most abundant found in bacteria, fungi and yeasts (Borrelli & Trono, 2015; Andualema & Gessesse, 2012; Treichel et al., 2010).

In industry, lipases are produced through microorganism cultivation. Lipases are often produced by bacteria, yeast and fungi such as several species of *Bacillus* sp. (Renge et al., 2012), *Pseudomonas* sp. (Haddar et al., 2010), *Staphlococcus* sp. (Khoramnia et al., 2010), *Aspergillus* sp. (Jia et al., 2015; Salihu et al., 2016), *Candida* sp. (Emond et al., 2010; Liu et al., 2012) and *Rhizopus* sp. (Iftikhar et al., 2010). Microbial lipases are capable of catalysing series of reactions for various industrial applications (Treichel et al., 2010; Andualema & Gessesse, 2012; Zhao et al., 2015). Lipases work in mild reaction conditions over a range of temperatures and pressures that minimize the formation of unwanted products. Microbial lipases are usually stable and have unique characteristics as compared to plant and animal lipases (Singh & Mukhopadhyay, 2012).

Most recent technology of recombinant DNA has eased the production of enzymes and other different proteins from foreign species. As the low levels of naturally produced precluded their widespread industrial use, this technology is probably going to have the greatest impact on enzymes production that have important therapeutic usage. Since enzymes are environmentally friendly, therefore, over 75% of the hydrolysis processes were conducted by using enzymes rather than by acids (Singh et al., 2016).

Considering most biotechnological products processes need a lot of capital, obtaining an optimum yield of product at the lowest expenses through usage of low cost material is vital (Agarwal et al., 2006). Yet, very small quantities of enzymes are usually produced by microorganisms natively and expensive raw materials which near to 30% of the total production expenditure. This explains the reason most of the marketable products based on enzymes are costly. Hence, it is crucial to decrease the production costs of enzyme production through the use of economical and renewable component (Haddar et al., 2010; Luo & Mu, 2014; Souza et al., 2015; Losordo et al., 2016). Multiple carbon and nitrogen sources have been tested for enzyme production, however depending on the substrate composition particularly the carbon source which may results varies on the level of enzyme production (Andersson et al., 2007; Sabri et al., 2013). In addition to usual water soluble carbon sources, a variety of unusual carbon sources such as blended gasoline, ethanol, hydrocarbons like hexadecane and heptadecane have also been tested (Deutscher, 2008).

The increase in public awareness on environmental related issues, intensely influence the development of technologies that assists in cleaning the contaminants. This has given rise to the opportunities for finding relevant yet cheap sources that can be used for enzyme production. Novelli et al., (2016) proposed a substitute approach using solid state fermentation to gain more cost-effective and viable production process worth employing on a commercial scale. Some of the recommended strategies included the use of more inexpensive materials, optimization of environmental conditions and screening for over producing strain to achieve the maximum productivity (Renge et al., 2012; Weuster-Botz et al., 2007).

Farming and food processing are the two massive industries that most of its wastes are environmentally plight. These industrial activities have continuously created a lot of pollution, such as wastewater, gaseous and solid waste pollution. Even though several agricultural residues are often disposed of within the environment (due to biodegradable nature), the large quantities of residues generated as a result of various agricultural and industrial practices, it is crucial to find alternative wherever these residue might be utilized for other useful application. Since these are rich in organic nutrient, they represent one of the most energy-rich resources on the planet. The negligence of the potential of these biomass may result in loss of prised material that may yield valuable added. By using waste as medium can minimize the cost of waste management and subsequently can reduce the overall manufacturing capitals (Jegannathan & Nielsen, 2013).

One of novel lipase enzyme that has high potential to be marketed is T1 lipase, proved to be thermostable alkaliphilic enzyme that is secreted by *Geobacillus zalihae* strain T1 (Leow et al., 2007). During this study, T1 lipase that is expressed by *Escherichia coli* BL21 are grown in a custom-made medium optimized using Response Surface Methodology (RSM) software that consists of local industries waste to lower the cost of production. Meanwhile, the fermentation process will also be optimized. The performance of the fermentation was evaluated using Monod and Luedeking-Piret equations. Shake flask, batch and fed-batch fermentation evironment was applied to collect the data to assess the most optimum conditions for used in the scale-up of 30 L fermentation.

This study aims to improve the yields of T1 lipase and to formulate low cost medium composition for productions of T1 lipase at a lower price. Hence, it will help to boost local biotechnology industry in Malaysia. The specific objectives of this research are as follows:

1- To investigate the influence of different substrates on the expression of T1 enzyme that will lower the cost production for industrial application.
2- To optimize the production of T1 lipase using RSM and compare the effect of IPTG and lactose on the process.
3- To study the kinetic and modelling of T1 lipase production in shake flask and batch bioreactor.
4- To develop T1 lipase production in 7.5 L bioreactor using batch and fed-batch mode.
5- To scale-up the T1 lipase production in fed-batch fermentation based on constant DOT in 30 L bioreactor.

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