UNIVERSITI PUTRA MALAYSIA

EXPRESSION OF RECOMBINANT T1 LIPASE IN E. coli STRAIN W USING FIFTH GRADE MOLASSES AS CARBON SOURCE

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EXPRESSION OF RECOMBINANT T1 LIPASE IN *E. coli* STRAIN W USING FIFTH GRADE MOLASSES AS CARBON SOURCE

By

SITI HAJAR BINTI YUSOFF

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

November 2019

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

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By

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November 2019

Chair : Suriana Sabri, PhD
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T1 lipase is suitable for various industrial applications due to its thermoalkaliphilic property. However, for industrial purposes normally large scale cultures are used and the current plasmid-based expression system raised concerns for the cost of antibiotics, dissemination of antibiotics and metabolic burden that could lead to reduction of recombinant protein productivity. Furthermore, the overall production cost depends greatly on the source of carbon. Therefore, many attempts have been made to produce recombinant proteins in *E. coli* from molasses, a cheap carbon source. However, most *E. coli* cannot utilize sucrose which is the major sugar in molasses. The aim of this study is to solve these problems by expressing the recombinant lipase using genome-based expression in *E. coli* W. *E. coli* W was chosen because it is the only safe laboratory strain that can utilize sucrose and shows fast, highly oxidative sucrose metabolism with low acetate production. The objectives of this study are to clone and integrate lipase gene into *E. coli* W genome, to express the recombinant lipase and to optimize the production of the recombinant product. The T1 lipase gene cassettes with different promoters (*tac* and *trc* promoters) were successfully cloned and integrated into *E. coli* W for plasmid based and genome based expression respectively. The expression of T1 lipase in *E. coli* W were higher in plasmid-based expression system compared to genome-based expression system but both systems have higher expression under *trc* promoter (thus, it was chosen for further study). Optimization study recorded the highest expression of T1 lipase at induction temperature of 16°C, 0.8 mM of IPTG concentration and 3% of molasses in M9 medium. A combination of the approaches described above may permit the industrial scale utilization of *E. coli* for bioconversion of low-cost starting

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materials (sucrose-molasses) into industrially important enzymes. Furthermore, production of recombinant lipase from the stable expression in the genome can be a model for production of other industrially important recombinant proteins.

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

EKSPRESI REKOMBINAN LIPASE T1 DARI *E. coli* STRAIN W MENGGUNAKAN MOLASES GRED LIMA SEBAGAI SUMBER KARBON

Oleh

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Lipase T1 sesuai digunakan untuk pelbagai kegunaan industri kerana sifat thermoalkalifiliknya. Walau bagaimanapun, untuk kegunaan industri, biasanya kultur skala besar digunakan dan sistem ekspresi berasaskan plasmid yang sedia ada menimbulkan kebimbangan untuk kos antibiotik, penyebaran antibiotik dan beban metabolik yang boleh membawa kepada pengurangan produktiviti protein rekombinan. Selain itu, kos pengeluaran secara keseluruhan sangat bergantung kepada sumber karbon. Oleh itu, banyak percubaan telah dibuat untuk menghasilkan protein rekombinan dalam *E. coli* dari molases, sumber karbon murah. Walau bagaimanapun, kebanyakan *E. coli* tidak dapat menggunakan sukrosa yang merupakan gula utama dalam molases. Tujuan kajian ini adalah untuk menyelesaikan masalah ini dengan mengeksperi lipase rekombinan menggunakan ekspresi berasaskan genom dalam *E. coli* W. *E coli* W dipilih kerana ia merupakan satu-satunya strain makmal selamat yang boleh menggunakan sukrosa dan menunjukkan metabolisme sukrosa yang sangat cepat, oksidatif dengan pengeluaran asetat yang rendah. Objektif kajian ini adalah untuk mengklon dan mengintegrasikan gen lipase ke genom *E. coli* W, untuk mengekspresikan lipase rekombinan dan mengoptimumkan penghasilan produk rekombinan. Kaset gen lipase T1 dengan promoter yang berlainan (promotor tac dan trc) telah berjaya diklon dan diintegrasikan ke *E. coli* W untuk ekspresi berasaskan plasmid dan genom. Ekspresi T1 lipase dalam *E. coli* W lebih tinggi dalam sistem ekspresi berasaskan plasmid berbanding sistem ekspresi berasaskan genom tetapi kedua-dua sistem mempunyai ekspresi yang lebih tinggi di bawah promoter *trc* (dengan itu, ia dipilih untuk kajian lanjut). Kajian pengoptimuman mencatatkan ekspresi tertinggi T1 lipase pada suhu induksi 16°C, 0.8 mM kepekatan IPTG

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dan 3% molases dalam medium M9. Gabungan pendekatan yang dinyatakan di atas mungkin membenarkan penggunaan skala industri *E. coli* untuk biokonversi bahan permulaan kos rendah (sukrosa-molases) ke dalam enzim penting industri. Tambahan pula, penghasilan lipase rekombinan dari ekspresi stabil dalam genom boleh menjadi model untuk menghasilkan protein rekombinan penting yang lain secara industri.

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TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iii
ACKNOWLEDGEMENTS	v
APPROVAL	vi
DECLARATION	viii
LIST OF TABLES	xiv
LIST OF FIGURES	xv
LIST OF ABBREVIATIONS	xix

CHAPTER

1	**INTRODUCTION**		
	1.1	Background of study	1
	1.2	Problem statement	2
	1.3	Objectives	2
2	**LITERATURE REVIEW**		
	2.1	*E. coli* as microbial cell factories for recombinant protein production	3
	2.2	Features of *E. coli* for production of recombinant proteins	3
		2.2.1 *E. coli* strains used in recombinant protein production	4
		2.2.2 Promoters	7
	2.3	Molasses as an alternative carbon source	7
	2.4	*E. coli* W	8
		2.4.1 Background of *E. coli* W	8
		2.4.2 *E. coli* W an efficient sucrose utilizer	9
	2.5	Stable expression of recombinant protein	11
		2.5.1 Approaches for genome based expression system	11
	2.2	Thermostable T1 lipase as reporter gene	12
3	**MATERIALS AND METHODS / METHODOLOGY**		
	3.1	Chemicals and equipment	13
	3.2	Bacterial strains and primer used	13
	3.3	Bacterial growth condition	13
	3.4	Confirmation of *E. coli* W	16
		3.4.1 Growth on selective media	16

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	3.4.2	Colony PCR for verification of *csc* genes	16
	3.4.3	Agarose gel electrophoresis	16
3.5	Confirmation of plasmid carrying T1 lipase		18
	3.5.1	Recombinant *E. coli* culture preparation	18
	3.5.2	Plasmid extraction	18
	3.5.3	Plasmid digestion using *Pvu*I	18
3.6	Cloning of T1 lipase in *E. coli* W		19
	3.6.1	Preparation of competent cells	19
	3.6.2	Transformation of *E. coli* W with pGEX/T1 and pTrcHis/T1	19
	3.6.3	Verification of positive transformants using colony PCR	19
3.7	Integration of T1 lipase gene cassette into *E. coli* W genome		20
	3.7.1	Amplification of T1 lipase gene cassette containing *tac* and *trc* promoters	22
	3.7.2	Cloning T1 lipase gene cassette in pKIKOarsBCm plasmid	22
	3.7.3	Verification of *E. coli* BW25141 carrying pKIKOarsBCm_trcT1 and pKIKOarsBCm_tacT1	22
	3.7.4	pKIKOarsBCm_trcT1 and pKIKOarsBCm_tacT1 recombinant plasmids digestion profile and sequencing	23
	3.7.5	Preparation of pKIKOarsBCm_trcT1 and pKIKOarsBCm_tacT1 for recombination	23
	3.7.6	Transformation of *E. coli* W with pKD46	24
	3.7.7	Knock in of T1 lipase into *E. coli* W	24
	3.7.8	Verification of T1 lipase integration	25
	3.7.9	Removal of antibiotic resistance gene	25
3.8	Expression of T1 lipase		26
	3.8.1	Culture preparation	26
	3.8.2	IPTG induction	26
	3.8.3	Harvesting	26

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3.8.4 Determination of protein concentration 27
3.8.5 Standard curve of free fatty acids 27
3.8.6 Determination of lipase activity 27
3.8.7 SDS-PAGE 28
3.8.8 Activity staining 30
3.9 Optimization of T1 lipase expression in LB 30
3.9.1 Temperature 30
3.9.2 IPTG concentration 30
3.10 Optimization of T1 lipase expression in M9 + 2% sucrose 30
3.10.1 Temperature 30
3.10.2 IPTG concentration 31
3.11 Optimization of T1 lipase expression in M9 + molasses 31
3.11.1 Temperature 31
3.11.2 IPTG concentration 31
3.11.3 Molasses concentration 32
3.12 Statistical analysis 32

4 RESULTS AND DISCUSSION
4.1 Confirmation of *E. coli* W 33
4.2 Successful transformation of *E. coli* W with recombinant plasmids 35
4.3 Screening of T1 lipase activity 37
4.4 Integration of T1 lipase into *E. coli* W genome 39
4.4.1 Amplification of T1 lipase gene cassette 39
4.4.2 Cloning of T1 lipase gene cassette into pKIKOarsBCm 41
4.4.3 Successful integration of T1 lipase gene cassette into *E. coli* W genome 46
4.5 Expression of T1 lipase 55
4.6 Optimization of T1 lipase in *E. coli* WΔarsB::trc_T1 58
4.6.1 Temperature 58
4.6.2 IPTG concentration 60
4.6.3 Molasses concentration 62
4.6.4 Statistical analysis 64
4.7 Comparison of T1 lipase expression between plasmid and genome based expression systems 65

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5	**SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH**	67
REFERENCES		68
APPENDICES		77
BIODATA OF STUDENT		85

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LIST OF TABLES

Table		Page
2.1	*E. coli* strains used for recombinant proteins production	5
3.1	Bacterial strains used in this study	12
3.2	3.2: Plasmids used in this study	12
3.3	Primers used in this study	14
3.4	Composition of 12 % resolving gel and 5 % stacking gel for SDS-PAGE.	22

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LIST OF FIGURES

Figure		Page
2.1	Chromosomally encoded sucrose catabolism (*csc*) genes in *E. coli* W.	8
3.1	Summary of the methodology for integration of T1 lipase gene cassettes (with *tac* and *trc* promoters) using homologous recombination with the aid of pKIKO vector.	17
4.1	Confirmation of the ability of *E. coli* W to utilize sucrose as its carbon source.	26
4.2	Confirmation of successful transformation of *E. coli* W/pGEX_T1 and E. coli W/pTrcHis_T1 using colony PCR.	28
4.3	Restriction enzyme profile for confirmation of recombinant plasmids pGEX_T1 and pTrcHis_T1 into *E. coli* W.	28
4.4	Screening of T1 lipase activity in *E. coli* W/pGEX_T1 and *E. coli* W/pTrcHis_T1.	30
4.5	Gel electrophoresis of T1 lipase gene cassettes with *trc* promoter and *tac* promoter.	32
4.6	Gel electrophoresis of digested pKIKOarsBCm before ligation.	34
4.7	Gel electrophoresis of amplified and *Eco*RI and *Spe*I-digested T1 lipase gene cassette with *tac* promoter and *trc* promoter.	34
4.8	Confirmation of cloned pKIKOarsBCm_tac_T1 in *E. coli* BW25141 using colony PCR.	35

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4.9 Confirmation of cloned pKIKOarsBCm_trc_T1 in *E. coli* BW25141 using colony PCR. 35

4.10 Confirmation of cloned T1 lipase gene cassette with *tac* promoter and *trc* promoter into pKIKOarsBCm. 37

4.11 Confirmation of successful transformants of *E. coli* W/pKD46 using colony PCR. 39

4.12 Linearized and treated pKIKOarsBCm_tac_T1 and pKIKOarsBCm_trc_T1. 41

4.13 Confirmation of successful integration of T1 lipase (tac promoter) into *E. coli* W genome. 42

4.14 Confirmation of successful integration of T1 lipase (*trc* promoter) into *E. coli* W genome. 42

4.15 The transformation plate (LB+ampicillin) for *E. coli* WΔarsB::tac_T1 and *E. coli* WΔarsB::trc_T1. 44

4.16 The differential plate (LB+tributyrin) for *E. coli* WΔarsB::tac_T1 and *E. coli* WΔarsB::trc_T1. 44

4.17 Integration of T1 lipase gene cassette with *tac* promoter at *ars*B gene site in *E. coli* W genome in silico. 45

4.18 Confirmation of *E. coli* WΔarsB::tac_T1 using colony PCR. 45

4.19 Integration of T1 lipase gene cassette with *trc* promoter at *ars*B gene site in *E. coli* W genome in silico. 46

4.20 Confirmation of *E. coli* WΔarsB::trc_T1 using colony PCR. 46

4.21 Lipase activity of all recombinant strains. 48

4.22 SDS-PAGE results for T1 lipase expression in plasmid based expression. 49

4.23 Zymogram of Native-PAGE overlaid on tributyrin agar plate for qualitative analysis of T1 lipase activity. 49

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4.24 Optimisation of T1 lipase expression in *E. coli* WΔarsB::trc_T1 using three culture medium (LB media, M9 + 2% sucrose and M9 + molasses) at different temperatures. 51

4.25 Optimisation of T1 lipase expression in *E. coli* WΔarsB::trc_T1 using three culture media (LB, M9 + 2% sucrose and M9 + molasses) at different IPTG concentrations. 53

4.26 Optimization of T1 lipase expression in *E. coli* WΔarsB::trc_T1 using M9 + molasses with different molasses concentration. 55

4.27 A comparison of T1 lipase expression between plasmid and genome based expression systems under optimized conditions in molasses. 57

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LIST OF APPENDICES

Appendix		Page
A	List of chemicals used and the manufacturers.	66
B	List of equipment used and the manufacturers.	67
C	Construction of pGEX_T1 *in silico* using CloneManager Software	68
D	Construction of pTrcHis_T1 *in silico* using CloneManager Software	69
E	Summary of results for statistical analysis One-way ANOVA done for *E. coli* WΔarsB::trc_T1 in optimization using LB medium	70
F	Summary of results for statistical analysis One-way ANOVA done for *E. coli* WΔarsB::trc_T1 in optimization using M9 + molasses	71
G	Summary of results for statistical analysis One-way ANOVA done for *E. coli* WΔarsB::trc_T1 in optimization using M9 + 2% sucrose	72

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LIST OF ABREVIATIONS

UPM	Universiti Putra Malaysia
α	alpha
β	beta
°C	degree celsius
%	percentage
A_{600nm}	optical density at wavelength 600 nanometer
µL	microliter
µm	micrometer
µmoles	micromoles
APS	Ammonium persulfate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bp	base pair
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
Crp	cAMP receptor Protein
csc	chromosomally encoded sucrose catabolism genes
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
g	Gram

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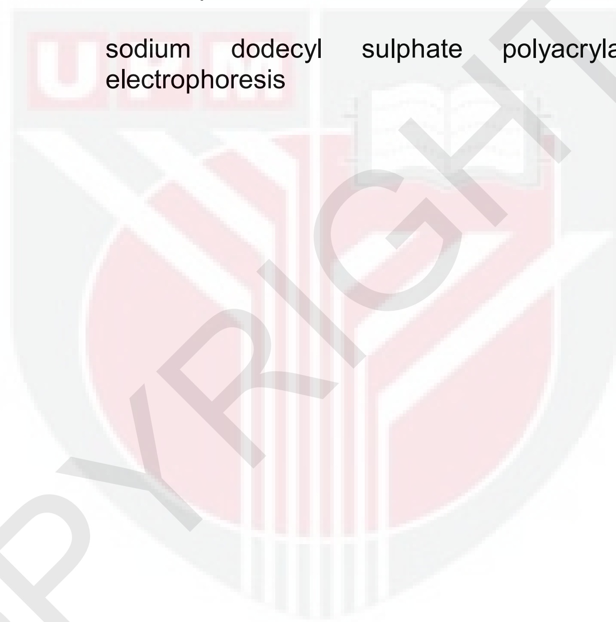
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilobase
kDA	kilodaltons
L	Litre
LB	Luria-Bertani
M	Molar
mM	milli Molar
mA	milli Amps
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis



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CHAPTER 1

INTRODUCTION

1.1 Background of study

Escherichia coli is a well-known microbial host for production of myriad products from recombinant pharmaceuticals to commercial enzymes. The central aspect of *E. coli* fermentation is the development of cost-efficient, abundant, and readily available carbon source to obtain maximal production yield of product of interests. The choice of carbon source for production of recombinant proteins in *E. coli* fermentation is a critical step as feedstock price represents 30.7% of the total cost (Alper and Stephanopoulos, 2009). Thus, feedstock price is a major contributor to the total bioprocess cost. Molasses, which is a waste product of sugar production from sugarcane and sugar beet, can be a suitable candidate for the requirement for biotechnological production through microbial fermentation. It is an inexpensive raw material and the costs is 33-50 % less than pure glucose (Zhang et al., 1994).

Many approaches were established to utilize inexpensive and easily available molasses as alternate feedstock in *Escherichia coli* with intention to bring down the production cost. Yet, the system was not productive for the reason that most of the *E. coli* strains are unable to utilize sucrose, which is the major sugar content present in molasses. In addition, majority of the sucrose utilizing *E. coli* strain are enteropathogenic (Jahreis et al., 2002). *E. coli* W is one of the five strains that is categorized as safe and it is the only strain that can utilize sucrose as its carbon source (Lee and Chang, 1993). Additionally, *E. coli* W showed fast, highly oxidative sucrose metabolism with low acetate production unlike other commercially used *E. coli* strains (Arifin *et al,* 2014). Therefore, it is an attractive host option to be used to produce recombinant protein industrially using a cheap waste product (molasses – major sugar is sucrose) as its carbon source.

For industrial purposes normally large-scale recombinant protein production is driven by *E. coli* carrying recombinant plasmids. A central issue during plasmid-based expression of recombinant proteins is plasmid loss due to plasmid instability (Friehs, 2004), high cost for antibiotics supplementation, as well as concerns on the probability of dissemination of antibiotic resistance organism to the environment (Pruden *et al.,* 2013). In addition, the presence of plasmids can impose a metabolic burden on the host cell, which can lead to decreased plasmid copy number in host cells and thus negatively affect the recombinant protein productivity (Mairhofer *et al*, 2013).

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Alternatively, recombinant protein can be expressed in plasmid free systems. Product formation rates generated in these systems are comparable to plasmid-based ones (Streidner *et al,* 2010). The gene of interest can be integrated into a defined locus of the *E. coli* genome using a few strategies. This includes the use of pKO3 vector and homologous recombination in *rec*-proficient strains (Link *et al*, 1997), λ-site specific recombination (Landy, 2015), *recET* system (Zhang *et al.* 1998), and the more recent technique, CRISPR optimized MAGE recombineering technique (Ronda *et al,* 2016). Lastly, a study has shown an efficient and easy way to insert whole gene cassettes into *E. coli* genome with the aid of pKIKO plasmids using lambda Red recombination technique (Sabri *et al*., 2013).

T1 lipase is a well characterized thermostable enzyme isolated from *Geobacillus zalihae* strain T1 (Leow *et al*, 2007). Many expression systems were developed using several promoters such as pBAD expression system (araBAD promoter), pRSET expression system (T7 promoter), pET22b (+) expression system (T7 *lac* promoter) and pGEX-4T1 expression system (*tac* promoter). However, the highest expression levels were recorded at 20.02 U/mg and 15.48 U/mg under *tac* promoter using *E. coli* BL21 (De3) and *E. coli* BL21 (De3) plysS, respectively (Leow *et al*., 2007).

1.2 Problem statement

Molasses is a preferred substrate for fermentation. However, most commonly used *E. coli* strain (BL21) cannot utilize molasses efficiently because it cannot ferment sucrose (the major sugar in molasses).
Therefore, *E. coli* W which is the only safe laboratory *E. coli* strain that can catabolize sucrose might be able to efficiently utilize molasses as carbon source for stable expression of recombinant protein product. Furthermore, *E. coli* W, an efficient molasses utilizer, is a wildtype strain where recombinant enzyme production remained less explored.

1.3 Objectives

The aim of this study is to produce a stable recombinant T1 lipase expression in *E. coli* W. The specific objectives of this study are:
1) To clone and express T1 lipase in *E. coli* strain W and
2) To optimize the production of T1 lipase in minimal medium using molasses as carbon source.

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