

OPTIMIZATION OF RECOMBINANT AMYLASE EXPRESSION USING  
RESPONSE SURFACE METHODOLOGY (RSM)

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*Specially dedicated to my beloved parents and brothers*

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## ABSTRACT

The *Anoxybaccilus* DT3-1 is a newly found bacterium that is able to express amylase. The gene that encodes the amylase was recently cloned and expressed in *E. coli* system. However, the expression level was far too low to be used. The main objective of this study is to enhance the recombinant amylase expression level using pET-22b vector. Another objective of this study is to determine the end product release by the reaction of this amylase. The media optimization was carried out with five different media i.e. LB, TB, SB, CDM 1 and CDM 2. Medium LB was found to be the best medium to support the cell growth and amylase production (72 U/ml). Relevant factors such as the inducer (IPTG) concentration, yeast extract concentration and induction time ( $OD_{600nm}$ ) were optimized through two Response Surface Methodology (RSM) methods, which were the Two-level factorial and Central Composite Design (CCD). After the final optimization using CCD, 83 U/ml of amylase activity was obtained with the optimal condition of 0.007 mM IPTG, 0.3% of yeast extract and induction should be done when the cells optical density was at 1.52. Upon achieving the optimal conditions, the end products were determined using High Performance Liquid Chromatography (HPLC). The amylase was able to degrade various starches like rice, corn, wheat and soluble starch and produced a wide variety of oligosaccharides such as the glucose, maltose and isomers of maltose.

## ABSTRAK

*Anoxybacillus* DT3-1 adalah bakteria yang baru ditemui yang mampu mengekspres amilase. Gen yang mengkod amilase ini telah diklonkan dan diekspres dalam sistem *E. coli*. Namun, tahap ekspresi terlalu rendah untuk digunakan. Tujuan utama projek ini adalah untuk meningkatkan tahap ekspresi amilase rekombinan menggunakan vektor PET-22b. Selain itu, tujuan lain dalam projek ini adalah untuk menentukan penghasilan produk akhir dari reaksi amilase ini. Optimasi media dilakukan dengan menggunakan lima media yang berbeza iaitu LB, TB, SB, CDM 1 dan CDM 2. Didapati, media LB merupakan media yang terbaik menengah untuk pertumbuhan sel dan pengeluaran amilase (72 U / ml). Faktor lain yang relevan seperti kepekatan induser (IPTG), kepekatan ekstrak ragi dan masa induksi (OD600nm) telah dioptimumkan melalui dua kaedah 'Response Surface Methodology (RSM)' iaitu 'Two-level Factorial' dan 'Central Composite Design (CCD)'. Setelah pengoptimuman terakhir menggunakan CCD, 83 U/ml aktiviti amylase dapat diperoleh dengan keadaan optimum IPTG 0.007 mM, 0.3% ekstrak ragi dan induksi harus dilakukan ketika sel ketumpatan optik berada pada 1.52. Setelah mencapai keadaan yang optimum, penghasilan produk akhir ditentukan dengan menggunakan Kromatografi Cair Kinerja Tinggi (HPLC). Amilase tersebut mampu mendegradasi pelbagai jenis kanji dari beras, jagung, keladi dan gandum di mana ia dapat menghasilkan pelbagai oligosakarida seperti maltose, glukosa dan isomer maltose yang lain.

## TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	TITLE	i
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGEMENTS	iv
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	x
	LIST OF FIGURES	xi
	LIST OF ABBREVIATIONS	xiii
	LIST OF APPENDICES	xiv
<b>1</b>	<b>INTRODUCTION</b>	<b>1</b>
	1.1 Introduction	1
	1.2 Problem Statement	3
	1.3 Objectives	3

1.4	Scopes of the research	4
2	<b>LITERATURE REVIEW</b>	5
2.1	Starch	5
2.2	Thermostable Bacteria	7
2.2.1	Thermostable enzyme	8
2.2.2	Applications of Thermophilic Enzymes	9
2.3	$\alpha$ -amylase ( amylase family and characteristic)	11
2.3.1	Reaction Mechanisms of Amylase	12
2.4	Design of Experiment	15
2.4.1	Response Surface Methodology (RSM)	16
2.4.2	Central Composite Design (CCD)	17
3	<b>MATERIALS AND METHODS</b>	19
3.1	Preparation of Bacterial Stock	19
3.2	Bacteria Revival and Culture	21
3.3	General Media Optimization	21
3.3.1	Composition of Each Medium	22
3.3.2	Overnight culture preparation	23
3.3.3	Optimization and Expression of E.coli BL21 Carrying Amylase in pET 22-b Vector	24
3.4	Further Optimization of Media	25
3.4.1	Intracellular Enzyme Extraction	25
3.4.2	Enzyme Assay	26
3.5	Optimization of Other Factors Using DoE	27
3.5.1	Analysis	30
3.6	End Product Analysis	31
3.6.1	High Performance Liquid Chromatography (HPLC)	31

<b>4</b>	<b>RESULTS AND DISCUSSIONS</b>	<b>33</b>
4.1	Optimization and Expression of E.coli BL21 Carrying Amylase in pET 22-b Vector	33
4.2	Further Comparison of CDM 2 and LB media	37
4.2.1	Cell Growth Profiles	37
4.2.2	Enzyme Activity	38
4.3	Optimization of Relevant Factors Using DoE	44
4.3.1	Adequacy of The Model	49
4.3.2	Optimal Design from Two-level Factorial	52
4.4	Expression Optimization Using Central Composite Design (CCD)	54
4.4.1	Selection and Validation For Significant Effect	54
4.4.2	Analysis of Variance (ANOVA)	60
4.4.3	Model Validation	63
4.4.3.1	Normal Probability Plot	63
4.4.3.2	Residual Versus Predicted Plot	64
4.4.3.3	Outlier T Plot	65
4.4.3.4	Box-Cox Plot	66
4.4.4	Optimal Design Based on CCD	67
4.5	End Product Analysis	69
4.5.1	High Performance Liquid Chromatography	69
<b>5</b>	<b>CONCLUSION</b>	<b>72</b>
5.1	Conclusion	72
5.2	Future Work	74
	<b>REFERENCES</b>	<b>75</b>
	<b>APPENDICES</b>	<b>80</b>



## LIST OF TABLES

<b>TABLE NO.</b>	<b>TITLE</b>	<b>PAGE</b>
2.1	Thermophiles and their common habitat	8
2.2	Applications of the thermophilic enzymes	10
3.1	Experimental factors for Two-level Factorial	28
3.2	Experimental factors and levels for Two-level Factorial	29
3.3	Experiment factors for CCD	29
3.4	Experimental factors and levels for second CCD	30
4.1	Process parameters and their levels for Two-level Factorial	45
4.2	Experiment factors and responses	46
4.3	Model and coded factors	47
4.4	Comparison between actual values and predicted values	49
4.5	ANOVA analysis for extracellular activity	51
4.6	ANOVA analysis for intracellular activity	51
4.7	Process parameters and their levels for CCD	56
4.8	Experiment factors and responses for amylase activity	58
4.9	ANOVA for amylase activity	60
4.10	Model and coded factor of CCD	62
4.11	Comparison between enzyme at unoptimized condition, Two-level factorial and CCD	68

## LIST OF FIGURES

<b>FIGURE NO.</b>	<b>TITLE</b>	<b>PAGE</b>
2.1	Structures of starch	7
2.2	Different enzymes involve in starch degradation	13
2.3	The double displacement mechanisms	14
3.1	Overview of total work flow	20
4.1	Profiles of microbial growth in five media	35
4.2	Fuwa assay on three final intervals samples in all five media	35
4.3	Profiles of microbial growth in LB broth and CDM 2 for 68 hours at 25°C	37
4.4	The extracellular and intracellular amylase activity in LB medium for 68 Hours	39
4.5	The extracellular and intracellular amylase activity in CDM 2 medium for 68 hours	41
4.6	The comparison of extracellular amylase activity between LB and CDM 2 media for 68 hours	43
4.7	The comparison of intracellular amylase activity between LB and CDM 2 media for 68 hours	43
4.8	Predicted vs. actual data for extracellular activity	47

4.9	Predicted vs. actual data for intracellular activity	48
4.10	Ramp of extracellular amylase	52
4.11	Ramp of intracellular amylase	53
4.12	Half-normal plot of two-level factorial (extracellular activity)	55
4.13	Response surface of first CCD design	57
4.14	Contour plots and response surfaces for the effect of IPTG concentration	59
4.15	Predicted versus actual in CCD	62
4.16	Normal plot of residual for amylase production in second CCD	64
4.17	Residual versus predicted plot for amylase expression	65
4.18	Plot of Outlier T of amylase production	66
4.19	Box-Cox plot for generated model of amylase expression	67
4.20	Ramps of various factors in CCD	68
4.21	Chromatogram of separation of standards (oligosaccharides)	69
4.22	Chromatogram of separation of sugar components for various kind of starch after degraded with recombinant amylase	70

## LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CaCl <sub>2</sub>	Calcium chloride
CCD	Central Composite Design
CDM 1	Chemically Defined Medium 1
CDM 2	Chemically Defined Medium 2
DNA	Deoxyribonucleic acid
DoE	Design of Experiment
<i>E.coli</i>	<i>Escherichia coli</i>
HPLC	High Performance Liquid Chromatography
LB Broth	Luria-bertani broth
mL	Mililiter
NaOH	Sodium hydroxide
OFAT	One Factor At Time
PCR	Polymerase chain reaction
rpm	rotary per minute
RSM	Response Surface Methodology
TAE	Tris-acecate-EDTA
Tris	Tris (hydroxymethyl) aminomethane

**LIST OF APPENDICES**

<b>APPENDIX</b>	<b>TITLE</b>	<b>PAGE</b>
A1	Process parameters and responses (amylase activity) for first CCD	80
A2	ANOVA analysis of first CCD	81
A3	Selected Model Validation Analysis for First CCD	82
A4	Optimal Design Based on First CCD	84
B1	Sugar Separation of Various Kind of Starch After Degraded with Recombinant Amylase	85

## **CHAPTER 1**

### **INTRODUCTION**

#### **1.1 Introduction**

Enzymes are biological catalyst that reduces the activation energy of a reaction by providing an alternative pathway for the reaction to occur. These enzymes are the key component in many industries that revolve around biotechnology industries. This is mainly due to the ability of enzymes to convert the substrates to desired product with minimal conditions at relatively lesser time and money (Gupta *et al.*, 2003).

Starch degrading enzymes such as amylases have been in high amount for their industrial benefits. Amylases (1, 4- $\alpha$ -D-Glucan glucanohydrolase) are enzymes that hydrolyze starch molecules into smaller compounds such as oligosaccharides and dextrans. Amylases are also able to hydrolyse starch to the very basic sugar component which is glucose. Amylases are one of the most important enzymes in many industries

such as food, textiles and paper industries. Recent discoveries also reveal that amylases have potential useful in pharmaceutical industry as well if amylases are prepared with suitable properties (Hmidet *et al.*, 2008).

There are many sources of amylase which varied from animal to plant and can be found vastly in microorganisms. Current mode in industries requires the usage of microorganisms as biotechnological sources of industrially relevant enzymes. This is because, microbial enzymes are significantly more economical and environmental friendly compared to chemicals. The major advantages of using microorganisms for the production of amylases is the cost effective bulk production capacity, less time and space required for production and microbes are relatively easy to manipulate to obtain enzymes of desired characteristics (Pandey *et al.*, 2000, Gupta *et al.*, 2003 and Asgher *et al.*, 2007).

Due to the increasing demand for amylase enzymes in various industries, there is enormous interest in developing enzymes with novel properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques. This increases the discovery and researches on the exploration of extracellular enzymatic activity in several microorganisms (Gupta *et al.*, 2003).

The classical procedure in revelation of novel species that able to produce useful enzyme is the isolation of microbial species. By using this method, it is able to produce novel enzymes from uniquely extreme environments such as extreme temperature or extreme pH environment. This would also able to offer a competitive advantage over the existing products which are more common. Eventually, characterization of these novel extreme environmental enzymes under fermentation conditions to optimize the enzyme production properties plays crucial role in evaluation of their industrial and economic significance (Prakasham *et al.*, 2007).

One of these novel discoveries are the founding of newly emerge species of *Anoxybaccilus* from one of the hot-spring in Malaysia known as the Dusun Tua hot-spring. The *Anoxybaccilus* which are currently named as the *Anoxybaccilus* DT3-1 are found by the research team of Universiti Teknologi Malaysia (UTM), where this microorganism is able to produce thermostable amylase enzyme.

## **1.2 Problem Statement**

Optimizing the best recombinant amylase production is important as this is a novel enzyme from a newly found thermostable microorganism. Once the highest enzyme expression through optimal condition able to obtain, the industrial value for this enzyme will increased. This ultimately provides an alternative to currently available enzymes with less expenditures and high productivity.

## **1.3 Objectives**

1. To determine the best media for amylase expression.
2. To optimize relevant factors that involve in amylase expression such as absorbance value and induction time through Two-level factorial and Central Composite Design (CCD)
3. End product determination using HPLC



## **1.4 Scopes of the research**

The scopes of research are as follow:

- a) General optimization of the best media
- b) Precise optimization of the best media
- c) Enzyme assay, protein assay and localization of cell
- d) Other factors optimization using 2-level factorial
- e) Further optimization using Central Composite Design (CCD)
- f) High Performance Liquid Chromatography (HPLC)