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.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
$CaCl_2$	Calcium chloride
CCD	Central Composite Design
CDM 1	Chemically Defined Medium 1
CDM 2	Chemically Defined Mediun 2
DNA	Deoxyribonucleic acid
DoE	Design of Experiment
E.coli	*Escherichia coli*
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

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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-α-D-Glucan glucanohydrolase) are enzymes that hydrolyze starch molecules into smaller compounds such as oligosaccharides and dextrins. 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 find 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)