



**STUDIES ON THE PRODUCTION OF  
GLUCOSE OXIDASE BY *Aspergillus terreus*  
UniMAP AA-1**

by

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## LIST OF ABBREVIATIONS

<i>A.niger</i>	<i>Aspergillus niger</i>
<i>A.terreus</i>	<i>Aspergillus terreus</i>
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
CaCO <sub>3</sub>	Calcium carbonate
CCD	Central composite design
CuSO <sub>4</sub> .5H <sub>2</sub> O	copper(II)sulfate pentahydrate
DNA	Deoxyribonucleic acid
DOE	Department of Environment
FCCCD	Face Centered Central-Composite Design
FT-IR	Fourier transform infrared spectroscopy
FeSO <sub>4</sub> .7H <sub>2</sub> O	Ferrous Sulphate Heptahydrate
GOx	Glucose oxidase
g/l	gram per volume
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
ITS	Internal transcribed spacer
KH <sub>2</sub> PO <sub>4</sub>	Potassium dihydrogen phosphate
K <sub>m</sub>	Kinetic constant
MgSO <sub>4</sub> .7H <sub>2</sub> O	Magnesium Sulphate Heptahydrate
mg	milligram
mM	millimolar
MEA	Malt extract agar
NaNO <sub>3</sub>	Sodium nitrate
NCBI	National Center for Biotechnology Information
OFAT	one-factor-at-a-time

RSM	Response surface methodology
SD	Standard deviation
SEM	Scanning electron microscopy
SSF	Solid state fermentation
U/ml	One unit is the amount of enzyme activity which will catalyse 1 micromole of the substrate per minute under standard conditions
UniMAP	Universiti Malaysia Perlis
UV/VIS	Ultra violet/visible
w/v	weight per volume

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# KAJIAN MENGENAI PENGHASILAN ENZIM GLUKOSA OKSIDASE DARIPADA *Aspergillus terreus* UniMAP AA-1

## ABSTRAK

Enzim glukosa oksidase telah diaplikasi dengan meluas dalam industri kimia, makanan, minuman, bioteknologi dan lain-lain. Umumnya enzim ini terhasil dari *Aspergillus niger* dan *Penicillium* sp. Kini wujud keperluan untuk mencari sumber-sumber alternatif enzim ini kerana beberapa kelemahan yang berkaitan dengan sumber yang sedia ada. Dalam kajian ini, pencilan mikroorganisma yang menghasilkan glukosa oksidase yang dicamkan sebagai *Aspergillus* UniMAP AA-1 telah dipencilkan dari sample tanah di Unit Penyelidikan Agrotek, Sg. Chuchuh, Perlis. Ujian penyaringan mikroorganisma yang menghasilkan glukosa oksidase telah dilakukan berdasarkan perubahan warna pada plat agar yang mengandungi *o*-anisidin dan lobak peroksidase. Mikroorganisma yang telah disaring telah dikenalpasti morfologinya dengan menggunakan mikroskop cahaya dan mikroskop pengimejan elektron (SEM) dan selanjutnya disahkan oleh pengecaman ke tahap molekul. Mikroorganisma ini telah dikenalpasti sebagai pengeluar utama glukosa oksidase yang bersifat ekstraselular dan morfologi yang bersifat pelet dalam kultur fermentasi. Penemuan ini menawarkan alternatif yang baru bagi masalah dan kelemahan yang sedia ada pada sumber-sumber glukosa oksidase terkini. Selanjutnya, pengoptimuman yang berturutan berdasarkan pendekatan statistik dan satu-faktor-pada-satu-masa (OFAT) telah dijalankan bagi mengoptimumkan penghasilan glukosa oksidase ekstraselular dari mikroorganisma yang telah dikenalpasti. Kaedah reka bentuk Plackett-Burman menunjukkan glukosa adalah pembolehubah yang paling berpengaruh diikuti oleh NaNO<sub>3</sub>, CaCO<sub>3</sub>, dan pepton kepada penghasilan enzim tersebut, sedangkan KH<sub>2</sub>PO<sub>4</sub>, MgSO<sub>4</sub>.7H<sub>2</sub>O, FeSO<sub>4</sub>.7H<sub>2</sub>O, menunjukkan kesan negatif terhadap penghasilan enzim tersebut. Berdasarkan hasil dari reka bentuk tersebut, glukosa, NaNO<sub>3</sub> dan CaCO<sub>3</sub> dipilih untuk kajian pengoptimuman dan seterusnya pengaruh dari tiga komponen medium ini diselidiki dengan OFAT dan pembolehubah ini selanjutnya dioptimasi menggunakan pendekatan reka bentuk komposit berpusat (FCCCD). Penghasilan medium optimum ditunjukkan pada glukosa 10.64% (w/v), NaNO<sub>3</sub> 1.21% (w/v) dan CaCO<sub>3</sub> 5.22% (w/v) dan enzim yang terhasil adalah sebanyak 6.72 U/ ml, iaitu sekitar tujuh kali ganda lebih tinggi daripada yang diperolehi daripada media sebelum pengoptimuman. Ciri-ciri seperti penggunaan oksigen dan glukosa serta penghasilan hidrogen peroksida dan asid glukonat daripada enzim kasar ini adalah selari dengan ciri-ciri khusus enzim glukosa oksidase. Nilai kinetik malar,  $K_m$ , enzim kasar ini, ditentukan oleh persepadanan langsung persamaan Michaelis-Menten melalui regresi bukan linear (dengan nilai korelasi atau  $R^2 = 0.98$ ) menggunakan fungsi solver dalam perisian Microsoft Excel, memberikan nilai dalam julat 7.5-15 mM. Keputusan kajian menunjukkan spesifikasi substrat dari enzim kasar ini terhadap glukosa  $\beta$ -D (substrat) dan menunjukkan kekuatan pengikatan enzim kasar ini dengan substratnya.

## STUDIES ON THE PRODUCTION OF GLUCOSE OXIDASE BY *Aspergillus terreus* UniMAP AA-1

### ABSTRACT

Glucose oxidase (GOx) has found a wide range of applications in chemical, food, beverage, biotechnology and other industries. It is commonly extracted from *Aspergillus niger* and selected strains of *Penicillium* sp. Currently there is a growing need to find alternative sources of this enzyme due to some drawbacks associated with *A.niger* and *Penicillium* sp. In this work, a novel GOx-producing strain, *Aspergillus terreus* UniMAP AA-1, was isolated from soil of Agrotech Research Centre, Sg Chuchuh, Perlis. The screening tests for the GOx-producing strain were carried out on the basis of color development test by using agar plate containing *o*-anisidine and horseradish peroxidase. The screened strain was identified morphologically using light microscope and Scanning Electron Microscope (SEM) and further verified by molecular level identification. The strain was identified as a predominant extracellular GOx producer and exhibits a pelleted morphology in fermentation culture. These findings offer a new alternative to the existing GOx-producing strains which are known to be associated with few drawbacks. Subsequently, a sequential optimization based on statistical design and one-factor-at-a-time (OFAT) method was employed to optimize the production of extracellular GOx from the potential strain. Plackett-Burman design indicated glucose as the most influential variable followed by NaNO<sub>3</sub>, CaCO<sub>3</sub>, and peptone on the GOx activity; while KH<sub>2</sub>PO<sub>4</sub>, MgSO<sub>4</sub>.7H<sub>2</sub>O and FeSO<sub>4</sub>.7H<sub>2</sub>O showed negative main effect on the enzyme activity. Based on the result, glucose, NaNO<sub>3</sub> and CaCO<sub>3</sub> were selected for further optimization studies. The influences of the three medium components were investigated with one-factor-at-a-time (OFAT) and these variables were subsequently optimized using a face centered central composite design (FCCCD). The optimum conditions were found to be 10.64% (w/v), 1.21% (w/v) and 5.22% (w/v) for glucose, NaNO<sub>3</sub> and CaCO<sub>3</sub> respectively and the enzyme activity was found to be 6.72 U/ml, which was about seven fold higher than that obtained in media before optimization. The oxygen and glucose consumption as well as hydrogen peroxide and gluconic acid production profiles of the crude enzyme are all in-line with typical GOx properties. The kinetic constant, K<sub>m</sub> of the crude enzyme for its substrate, determined by direct fits of Michaelis–Menten equation through nonlinear regression (with correlation value or R<sup>2</sup> =0.98) using solver function in Microsoft Excel software, gave the value of within the range of 7.5-15 mM. The result indicates substrate specificity of the crude enzyme towards β-D glucose (substrate) and demonstrated the tight binding of the crude enzyme with its substrate.

## CHAPTER 1

### INTRODUCTION

#### 1.1 BACKGROUND

Enzyme is a protein that catalyzes a large number of biochemical reactions. To date, enzyme is utilized for diverse applications ranging from the manufacture of various industrial products to diagnostics and therapeutic agents. Although the world demand of enzyme is increasing, production of enzyme is yet to flourish in developing countries like Malaysia due to the high production cost and high capital investment (Ibrahim, 2008). Most of the industrial enzymes used in the country are imported from Denmark, Netherlands, Belgium and other countries amounting to about USD 3.5 millions annually with the quantum of more than 1 million kg of crude enzyme preparations (Ibrahim, 2008).

Glucose oxidase (GOx) is one of the enzyme which has gained an importance and popularity in industry. GOx catalyzes the oxidation of  $\beta$ -D-glucose to gluconic acid, utilizing oxygen as an electron acceptor and simultaneously producing hydrogen peroxide. This enzyme has found several commercial applications in food and beverage industry including glucose removal from dried egg; improvement of color, flavor, texture and shelf life of food materials; oxygen removal from fruit juices, canned beverages and mayonnaise to prevent bacterial growth (Wong, Wong & Chen, 2008 and Bankar, Bule, Rekha & Ananthanarayan, 2008). Besides, it has also been used in biofuel cells (Kim, Parkey, Rhodes & Gonzalez-Martin, 2009) and widely in glucose biosensors for clinical applications (Yoo & Lee, 2010).

The wide application of GOx has increased the demand of GOx in the world market. According to the report by Global Industry Analysts, Inc., the global market for GOx based-biosensors and strips will reach USD 11.5 billion by 2012 (Yoo & Lee, 2010).

The most common microbial sources for GOx production are selected strains of *Aspergillus* and *Penicillium* genera. Among these sources, *Aspergillus niger* is the most commonly utilized microorganism for commercial production of GOx (Bankar, Bule, Rekha & et al., 2009).

However, these two fungal sources for producing GOx have been known to be associated with some drawbacks. *Aspergillus niger* produces intracellular GOx (Hamid, Kalil-ur-Rehman, Zia & Asgher, 2003) which incurs comparatively more cost in the recovery steps as compared to extracellular enzyme. Extracellular enzyme is preferable in industry because the downstream process is simpler and cheaper as compared to intracellular enzyme (Ibrahim, 2008). Intracellular enzyme is located in the cell, thus, the cell need to be disrupted in order to release the enzyme. This characteristic requires extra downstream processes to recover the enzyme, hence it will incur more processing cost (Headon & Walsh, 1994). Furthermore, some extracellular enzymes are more stable than their intracellular counterparts because they are glycosylated and have a broad pH range for activity. In addition, the enzymes have some resistance to degradation due to proteases activity (Burns & Wallenstein, 2010).

On the other hand, although *Penicillium* sp is known as extracellular GOx producer (Sabir, Bhatti, Zia, & Sheikh, 2007), however it produces non-Newtonian fluids behaviour during fermentation which results in high viscous cultivation broth (Clarke, Johnstone-Robertson, Price & Harrison, 2006). The high viscosity and pseudo-plasticity of the suspension caused many problems during cultivation which include

decreasing the mass transfer, heat transfer, and requiring more power input for mixing (El-Enshasy, 2007). Conversely, pelleted morphology offers an alternative growth form for the culture of fungi. It exhibits Newtonian fluids which produce less viscous culture broth and good mass and heat transfer properties which offers easier separation of the biomass from the broth (Suijdam, Kossen & Paul, 1980).

Considering the above two drawbacks, it is necessary to find alternative microbial sources for GOx production which are free from the above drawbacks. In line with that, in order to increase the production efficiency, it is necessary to optimize the production of GOx. As common to enzyme production, the most crucial factors is medium composition, since it affects the production in terms of cost and its productivity (Schmidt, 2005). Hence, it is important to consider the optimization of fermentation medium in order to maximize the production efficiency and profits eventually.

Although optimization of GOx production was reported widely, however, most of it was achieved by using conventional method like one-factor-at-one-time (OFAT) rather than statistical tools like Plackett-Burman design and Response Surface Methodology (RSM). Conventional method like OFAT modifies one factor while maintaining other factors at a specified constant level. This practice is time consuming as it requires a large number of experiments. It is also less effective since it does not consider the interaction between factors involved. By contrast, statistical experimental design offers considerable advantages as compared to OFAT for fermentation improvement. Plackett-Burman design allows a reliable short listing of medium components in fermentation prior to optimization study while Response Surface Methodology (RSM) allows studying the optimum conditions of the selected factors and studying interaction between the factors in limited number of experiment (Vaidya, Shah, Vyas & Chhatpar, 2001).

In this study, we report the isolation and identification of a novel GOx-producing strain from soil samples taken from different places of Perlis area, Malaysia. The isolated strain was identified as *Aspergillus terreus* based on the morphological characterization and molecular identification. To our knowledge, there has been no report regarding the production of GOx from *Aspergillus terreus*. Furthermore, this novel GOx-producing strain showed a predominant extracellular GOx and exhibits pelleted morphology which offers a better alternative to the existing sources of GOx which are known to be associated with some drawbacks.

Since there has been no reported work on the production of GOx from this strain, it is necessary to optimize the growth conditions of the strain for optimal production and study the properties of the crude GOx. The optimization studies on composition of media components were carried out in three stages as follows:

1. Plackett–Burman design was applied to address the most significant media components which affect GOx production.
2. The one-factor-at-a-time (OFAT) approach was used to obtain the most possible optimum level of selected factors.
3. The central composite design (CCD) was employed to determine the optimal condition and to study the interaction among the significant media components for the production of GOx.

Finally, studies on the enzymatic properties of the crude GOx produced from the optimized media conditions have been attempted. The properties of crude GOx were studied based on the change of components involved in the enzymatic reaction of the crude enzyme. These are oxygen consumption, glucose oxidation, hydrogen peroxide formation and gluconic acid production.