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# *Aspergillus terreus* Unimap AA-1: A Newly Isolated Extracellular Glucose Oxidase-Producing Strain

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**Abstract:** Glucose oxidase (GOx) has found wide range of industrial applications. Commercial GOx are produced from *Aspergillus niger* and selected strains of *Penicllium* species, however, these strains are associated with some drawbacks. *Aspergillus niger* produces predominantly intracellular GOx, which requires extra recovery processes thus incurs more processing cost while *Penicillium* sp. exhibits filamentous morphology in submerged cultivation which results in poor oxygen mass transfer and in turn affects the GOx production. Therefore, it is necessary to find an alternative strain which produces predominantly extracellular GOx and exhibits pelleted morphology in submerged cultivation. The present work deals with the isolation and identification of a new isolated GOx-producing strain, *Aspergillus terreus* UniMAP AA-1, which produced both the desired traits. It was found that enzymatic properties of the crude enzyme are all in-line with the typical commercial GOx properties.

**Key words:** Enzyme · Glucose oxidase · Fermentation · *Aspergillus terreus* · Fungus

## INTRODUCTION

Glucose oxidase (GOx) has been the subject of many research studies due to its numerous applications. Glucose oxidase (Gox) catalyzes the oxidation of β-D-glucose to D-glucono-1,5-lactone, utilizing oxygen as an electron acceptor and simultaneously producing hydrogen peroxide. This enzyme has found several commercial applications in the food industry as an agent of colour, flavour, texture and shelf life improvement of food materials [1]. In addition, the enzyme is also widely utilized in the development of biofuels [2] and biosensors [3].

Despite its numerous applications, the microbial sources for commercial preparation of GOx is rather limited, predominated by *Aspergillus niger* and selected strains of *Penicillium* species. However, both species are associated with some drawbacks. *Aspergillus niger* produces an intracellular enzyme which requires opening of the cell wall to release the enzyme, thus, incurs comparatively a higher cost in the recovery steps as compared to a extracellular enzyme. On the other hand, *Penicllium* sp. produces an extracellular GOx. However, during fermentation, it produces a non-Newtonian fluid behaviour, which results in poor mass transfer at high mycelia concentrations [4]. In view of the above problems, it is necessary to find an alternative strain which produces a predominantly extracellular GOx and also capable of maintaining good mass transfer at high mycelia concentration. This condition can be achieved if the strain exhibits pelleted morphology in submerged cultivation.

In this study a newly isolated strain which predominantly produces extracellular GOx and exhibits pelleted morphology is reported. The isolated strain was identified as *Aspergillus terreus* based on the morphological characterization as well as molecular identification.

The enzymatic reactions of Gox were assayed using two methods; formation of hydrogen peroxide by *o*-anisidine reaction using uv-spectroscopy and D-glucono-1,5-lactone production using FT-IR analysis.

## MATERIALS AND METHODS

**Screening, Isolation and Identification of Glucose Oxidase-Producing Strain:** Screening of GOx-producing strains was carried out using serial dilution and the differential media method [5]. The isolates were

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subsequently tested for their ability in exhibiting pelleted morphology and producing extracellular GOx. The isolated strains were identified based on its morphological characteristic and further verified at the molecular level.

**Production of Crude Glucose Oxidase:** The culture medium employed was similar to that suggested by Nakamatsu *et al*. [6] which comprised of (g/l): $NaNO_3$, 5.0; $KH_2PO_4$, 1.0; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; peptone, 3.0; $CaCO_3$ (sterilized separately), 35; glucose (sterilized separately), 80. All growth experiments were carried out in 250 Erlenmeyer flasks with 100 ml working volume. The flasks were inoculated with 5 ml ($5.17 \times 10^7$ spores/ml) of inoculums and incubated in a rotary shaker operating at 200 rpm and 30°C for 110 hours.

**Assay of GOx Activity:** GOx activity in the supernatant was measured spectrophotometrically using the coupled *o*-anisidine-peroxidase reaction method as explained by Bankar *et al*. [7]. Briefly, this assay based on the reaction where β-D-glucose was oxidized to D-glucono-1,5-lactone and $H_2O_2$ by GOx in the presence of $O_2$. Then, the produced $H_2O_2$ was used to oxidize *o*-anisidine in the presence of peroxidase and resulted in color change, which was detected spectrophotometrically at 500 nm. Crude extracellular enzyme was prepared by removing the cell via centrifugation at 6,000 rpm for 15 min. The harvested supernatant was assayed for extracellular GOx activity. Intracellular enzyme was determined by suspending the residue in 0.1 M phosphate buffer, pH 7.0. The resulting suspension was subjected to sonication at 5°C for 15 min with sonicator (Elmasonic X-tra). Finally, the sample was centrifuged at 6,000 rpm for 15 min. The supernatant was assayed for intracellular GOx activity.

**Properties of Crude GOx from *Aspergillus terreus* UniMAP AA-1:** The properties of crude GOx were studied based on the changes of components involved in the enzymatic reaction namely D-glucono-1,5-lactone production and hydrogen peroxide formation at different concentrations of glucose.

**Crude Glucose Oxidase Analysis by Fourier Transforms Infrared Spectroscopy (FT-IR):** GOx converts glucose and oxygen to D-glucono-1,5-lactone which is then hydrolyzed to gluconic acid. The formation of D-glucono-1,5-lactone as result of the enzymatic reaction was analyzed qualitatively using Spectrum 65 Perkin-Elmer FT-IR spectrophotometer. The rate of D-glucono-1,5-lactone formation was followed by monitoring the intensity.

**Initial Velocity of Crude GOx in Response to Different Concentrations of Glucose:** The hydrogen peroxide formation was tracked spectrophotometrically using the coupled *o*-anisidine-peroxidase reaction method as described in the previous section. The initial velocity of the crude GOx was plotted against different concentrations of glucose. The kinetic constant ($K_m$) for this substrate was determined by direct fits of Michaelis-Menten equation through nonlinear regression using the solver function in Microsoft Excel.

## RESULT AND DISCUSSION

**Screening, Isolation and Identification of GOx-Producing Strain:** Among 13 isolates studied, only one isolate exhibits pelleted morphology in submerged cultivation after 48 hours of incubation. Analysis of the pellet using an Olympus BX51 microscope (5x) showed a pellet diameter of approximately 200 μm. According to previous studies [8] pellets with diameter smaller than 400 um in diameter will promote the ability of cell to get sufficient oxygen. Additionally, smaller pellet size promotes a lower viscosity medium resulting in easier nutrient transport and oxygen uptake for cellular utilization [9]. This isolate was identified as *Aspergillus terreus* and specified as *Aspergillus terreus* UniMAP AA-1. Table 1 shows the comparison between extracellular and intracellular GOx produced by *Aspergillus terreus* UniMAP AA-1. It is apparent that the strain produces extracellular enzyme predominantly. This is in contrast with *A.niger* [4] which produces a predominantly intracellular GOx although they are both from the same genus. The fact that this strain produces predominatly extracellular GOx, share simillar properties with that of *Penicillium* sp. commmonly used in commmercial GOx production [10]. However as compared to *Penicillium* sp., this newly isolated strain offers some advantages related to its pelleted behaviour.

**Properties of the Crude GOx from *Aspergillus terreus* UniMAP AA-1**

**Crude GOx Analysis by Fourier Transform Infrared Spectroscopy (FT-IR):** The ability of the crude GOx to catalyze the oxidation of β-D-glucose into D-glucono-1,5-lactone was analyzed by FT-IR spectra. The spectra in Figure 1 indicate that the product formation resulted from

Table 1: Location of GOx in *Aspergillus terreus* UniMAP AA-1

| Location of enzyme | U/ml |
|---|---|
| Extracellular | 4.520* |
| Intracellular | 2.828* |

*Data are expressed as means ± SD ($n$ = 3)

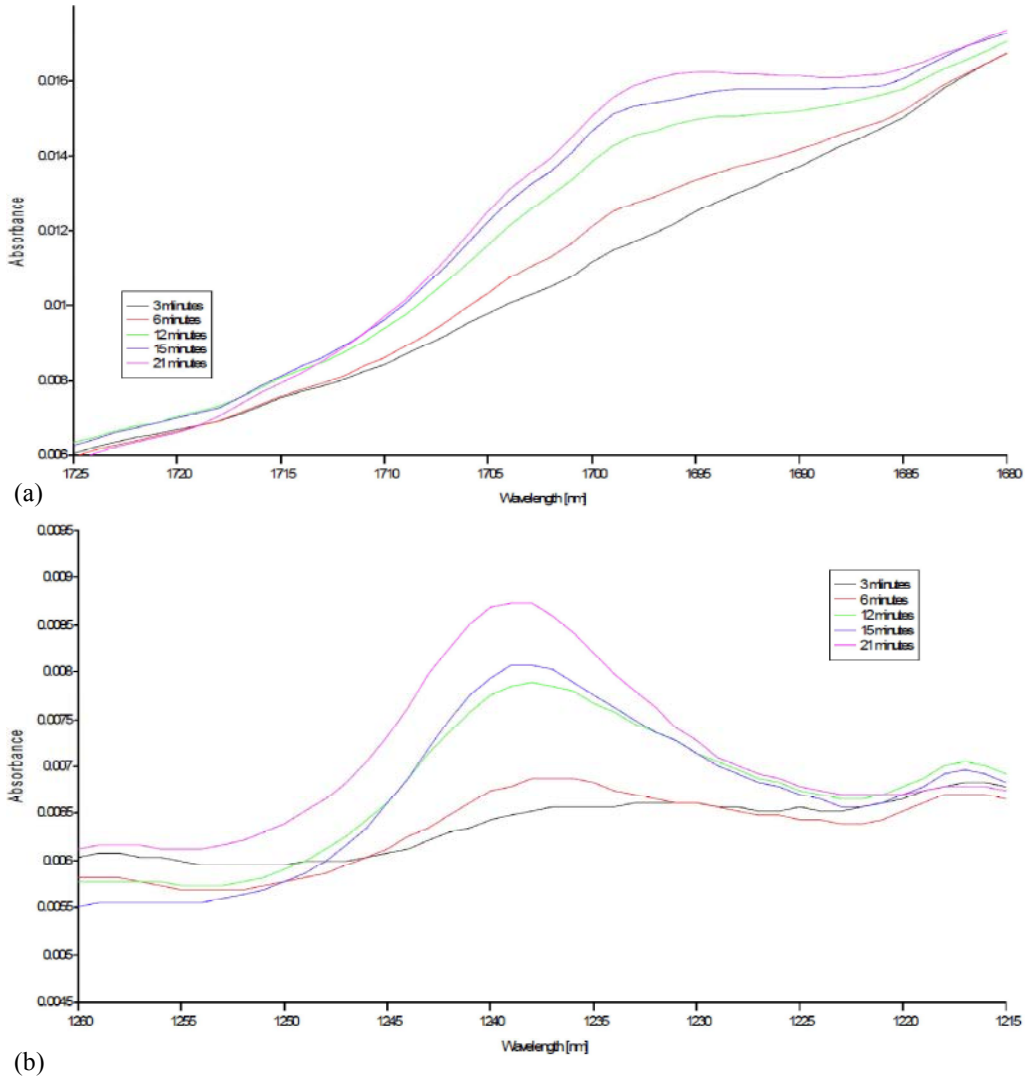


Fig. 1: FT-IR spectra of the product (D-glucono-1,5-lactone) at band 1697 $cm^{-1}$ (a) and 1239 $cm^{-1}$ (b) recorded at several time intervals

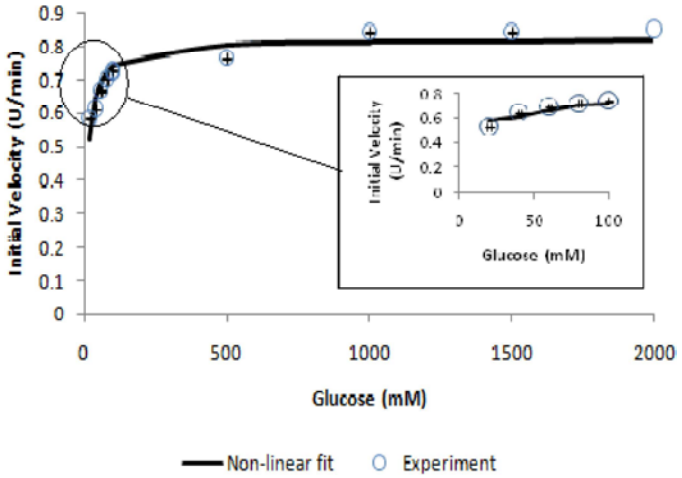


Fig. 2: Initial velocity of crude GOx in response to different glucose concentrations

the enzymatic reaction of crude glucose oxidase with β-D-glucose. The increase in the intensity of the C=O band at 1697 $cm^{-1}$ and C-O band at 1239 $cm^{-1}$ of the D-glucono-1,5-lactone are shown in Figure 1a and in Figure 1b respectively. D-glucono-1,5-lactone absorbs strongly on both bands which demonstrates the ability of the crude enzyme to perform the enzyme reaction.

**Initial Velocity of Crude GOx in Response to Different Concentrations of Glucose:** Figure 2 shows both the actual data and their non-linear fit graph of initial velocity of crude Gox against various glucose concentrations. It is apparent from insert in the Figure 2, the initial velocity of the crude glucose oxidase is increased proportionally with the increased in concentration of glucose. However, when concentration of glucose reached above 500 mM, the initial velocity of the crude GOx started to stabilize which can be attributed to limited number of enzymes. The kinetic constant, $K_m$ for this substrate is in the range of 7.5- 15 mM with a 95% confidence interval and with a correlation determination value ($R^2$) calculated to be 0.98. This indicates a good binding between substrate and enzyme. In addition to that, the $R^2$ value shows good correlation between actual and calculated data. The $K_m$ value is close to that of GOx from *Penicilum* sp. which is in the range of 9.6- 15 mM and lower compared to that of *A. niger* which is 33 mM [11].

## CONCLUSION

This study shows that the newly isolated strain *Aspergillus terreus* UniMAP AA-1 carries two interesting properties. Firstly, it produces predominantly extracellular GOx enzyme as is noticeable from the ability of the strain's cell-free extract to convert glucose into glucono-1,5 lactone and hydrogen peroxide (Figure 1 and 2). Secondly, the strain exhibits pelleted morphology in fermentation culture with the size smaller than 400 um in diameter will promote good oxygen transfer in the culture. These two properties will prove beneficial for commercial production of GOx since it reduce the recovery processing cost as well as improve the productivity. In this research an alternative approach is introduced to follow the enzymatic properties of the newly isolated strain by using two different assays. It was found that enzymatic properties of the crude enzyme are all in-line with the typical commercial GOx properties by using this approach.

## ACKNOWLEDGMENTS

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