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Properties of Glucose Oxidase produced from a newly isolated strain of *Aspergillus niger*

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Abstract

Glucose oxidase (GOx) is a key enzyme used in many industries worldwide. To identify the GO x producer strain, *Aspergillus niger* was isolated from the Beehive and screened for its glucose oxidase-producing capability. Glucose oxidase was mass-produced by a submerged fermentation system. The activity of glucose oxidase was ascertained by the continuous spectrophotometric rate determination method. GOx was purified by (70%) ammonium sulphate precipitation, dialyzed, and gel filtration with 2.82, 3.54, and 3.69U/mg specific activity respectively. The enzyme exhibited an optimum pH range of 5.5 and a temperature optimum of 40°C. Ag^{2+} and Hg^{2+} had a remarkably inhibitory effect on the partially purified GOx, whereas Ca^{2+}, Zn^{2+}, and Mg^{2+} do not affect the enzyme activity. Cu^{2+} and Co^{2+} have a slight inhibitory effect on GOx. Kinetic characteristics of GOx from *Aspergillus niger* display *Vmax as* 40 U/ml, and *K*m as 0.12 mM. The findings on the properties of GOx exhibited optimum conditions for industrial applications

Keywords: *Aspergillus niger*, Glucose oxidase, Characterization, Purification, Bee Hive

Introduction

Glucose oxidase (EC 1.1.3.4) is a principal commercial enzyme due to its numerous applications in diverse industries. As an oxidant, GOx is used for bread production with improved quality and loaf volume in the baking industry [1, 2]. GOx extends the shelf-life by removing oxygen and glucose from foods [3]. GOx is effective in the production of H_2O_2 for bleaching in the textile industry [4-6]. GOx is produced by some insects and fungi [7]. Glucose oxidase (EC 1.1.3.4) catalyzes the oxidation of β-D-glucose using oxygen as an electron acceptor, to yield D-gluconolactone and hydrogen peroxide [7]. The cofactor of GOx, flavin-adenine dinucleotide (FAD) is reduced along the reaction mechanism. Muller [8] was the first to isolate GOx from the mycelium of *Aspergillus niger.* A single polypeptide chain of one subunit of GOx contains 583 amino acid residues [9]. The ability of GOx to remove oxygen and generates hydrogen peroxide underline the trait utilized in food preservation. Other applications of GOx are in the food, pharmaceutical and medical industries [10]. Notable in these applications, is the fabrication of glucose biosensors for the measurement of glucose levels in serum. There are few

reports on high catalytic properties of GOx from various fungi, thus this research was designed to produce and characterize GOx from *Aspergillus species* isolated from Beehive to meet the demand for enhanced properties such as elevated catalytic activity for effective production of GOx for industrial purposes. This study aimed to produce and characterize glucose oxidase produced from *Aspergillus niger* under a submerged fermentation system for special application in biotechnology and industries.

Materials and Methods

Materials

Horseradish peroxidase, *O*-dianisidine dihydrochloride, and Folin-Ciocalteau phenol were purchased from Sigma, USA. All other analytical chemicals used in this study such as D (+)-glucose, glycerol, peptone, and ethanol were purchased from Merck (Germany), May and Baker Limited (England) and Sigma, USA.

Isolation and maintenance of the Organism

In this study, the fungi were isolated from a beehive bought from Opi market, Nsukka, Enugu State, Nigeria. Using a light microscope, the fungi were identified as *Aspergillus sp*. according to Martin *et al.* [11]. The screening of the fungi was carried out using the method of Eun-Ha *et al.* [12]. The isolated fungi were then grown on the medium of sodium acetate buffer pH 5.5 consisting of 80g Glucose, 3.0g peptone, 0.388g $(NH_4)_2$ HPO_4, 0.188g KH_2PO_4, 0.156g $MgSO_4$ $7H_2O$, 20.0g Agar. From the peripheral zone of the pure culture colony, a disc of fungal culture was taken and transferred to the middle of the Petri plate containing the above medium, one pure culture for each plate after which the plates were incubated at 35°C. After 3 days, the fungal culture was treated with a mixture (prepared in sodium acetate buffer (pH 5.5) containing (5%) glucose, (2%) glycerol, (0.1%) *O*-dianisidine, (60IU/ml) Horse radish peroxidase, (1%) agar after which the culture was incubated for one hour. The fungus that gave the highest GOx production was sustaind on Potato dextrose agar (PDA) and stored at 4°C.

Production of Glucose oxidase

A submerged fermentation system was used for the production of GOx. The medium for the production consists of (80%) glucose, (0.3%) peptone, (0.04%) $(NH_4)_2HPO_4$, (0.0188%) KH_2PO_4, (0.0156%) $MgSO_4.7H_2O$, (3.5%) $CaCO_3$ in 50mM sodium acetate buffer pH 6.0 [9]. Four discs of the pure culture were inoculated into 100ml of a sterilized cultivation medium broth contained in 250ml Erlenmeyer flasks after which the flasks were incubated at 30°C. The fermented biomass in each flask was harvested, filtered and centrifuged at 15000 rpm for 15 min [13]. The supernatant constituted the crude enzyme.

Glucose oxidase assay

GOx activity was measured according to the method by Bergmeyer *et al*. [14] using continuous spectrophotometric rate determination at 500nm wavelength. Solution A (0.05M Sodium acetate buffer of pH 5.5), solution B (0.21mM *O*-dianisidine solution dissolved in 100ml with reagent A), solution C (10% β-D-glucose of Millipore water), and solution D (a mixture of reagent B [24ml] and reagent C [5ml]), solution E (a freshly prepared solution of 60 purpurogallin units/ml of horseradish peroxidase type II) were all prepared. The enzyme reaction contained 2.9ml of solution D and 5ml of solution E. Reagent E. The reaction was measured using a UV-Visible spectrophotometer at 500nm.

Purification of GOx

Ammonium sulphate precipitation and dialysis

Ammonium sulphate precipitation of GOx and dialysis was done as described by Chilaka *et al.* [15]. The suitable percentage of ammonium sulphate to precipitate GOx in this study was Seventy (70%). Dialysis was carried out on the precipitated enzyme against 0.01M sodium phosphate buffer pH 7.0 for 12 hours

with a change of buffer every 6 hours. The dialysate was assayed for GOx activity and protein concentration as earlier described.

Gel Filtration

The dialyzed enzyme (10ml) was introduced into a (2.0 × 80 cm) gel chromatographic column, pre-equilibrated with sodium phosphate buffer (50mM, pH 7.0). At room temperature, fractions were collected at a flow rate of 5ml/15min. The protein concentration and enzyme activity of each fraction were monitored as earlier described. Peaks with the highest GOx activity were pooled and stored at -10^{o}C for further studies.

Characterization of the partially purified *Aspergillus niger* GOx

Effect of pH on Aspergillus niger GOx activity

The optimal pH for GOx activity was monitored using 50mM sodium acetate (pH 3.5 – 5.5), 50mM Sodium phosphate (pH 6.0 – 7.5)) and 50mM Tris-HCL (8.0 – 10.0) as described by Simpson *et al.* [16]. The GOx activity was assayed as earlier described.

Effect of temperature on Aspergillus niger GOx activity

The optimum temperature for GOx activity was monitored at temperatures ranging from 30 – 75^{o}C at the interval of 5^{o}C in a water bath using 50mM sodium acetate pH 5.5. The reaction was initiated with the addition of GOx (0.15Uml^{-1}) as described by Singh and Verma [17]. The GOx activity was assayed as earlier described.

Effect of glucose concentration on Aspergillus niger GOx activity

The effect of glucose concentrations (4.0 – 25%, m/v) on GOx activity was monitored as described by Sandalli *et al.* [18] at pH 5.5 and 40^{o}C. The maximum velocity (Vmax) and Michaelis constant (Km) were gotten from Lineweaver–Burk plot of initial velocity data of different glucose concentrations.

Effect of divalent metal ions on GOx activity

The effect of metal ions on GOx activity was monitored as described by Yanmis *et al.* [19]. The partially purified enzyme (10ml) was incubated for 20mins in different concentrations of metal ions (10 to 30mM). The enzyme activity was assayed at the established optimum temperature and pH in this study.

Results and Discussion

Using a light microscope, the GOx-producing fungi isolated were identified as *Aspergillus* species. The chromosomal DNA of the *Aspergillus* strain was extracted after which the 18S rDNA was amplified with PCR. The product of the PCR was then subjected to agarose gel electrophoresis. A clear band was found at approximately 500bp when compared to the DNA ladder (Figure 1). The multiple alignments of the sequence were compared with 21 other known sequences of *Aspergillus* species gotten from the NCBI data bank. The phylogenetic tree confirmed the strain H1 as *Aspergillus niger* as it showed 99% similarity with *Aspergillus niger* strains *AN4, A sp-7136* and *IHBF 2920* (Figure 2).

Molecular Identification of Aspergillus nigerH1

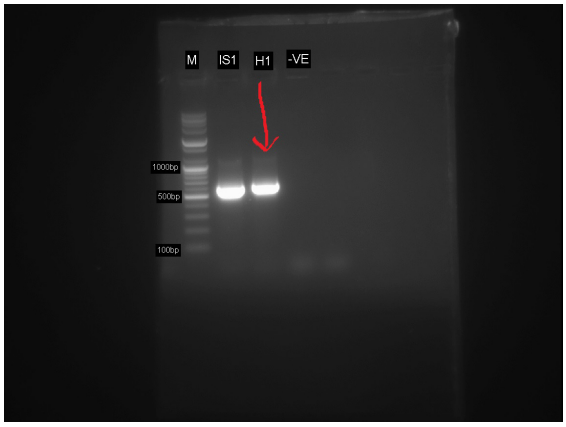

Figure 1: Agarose gel electrophoresis showing PCR amplification of *Aspergillus niger*

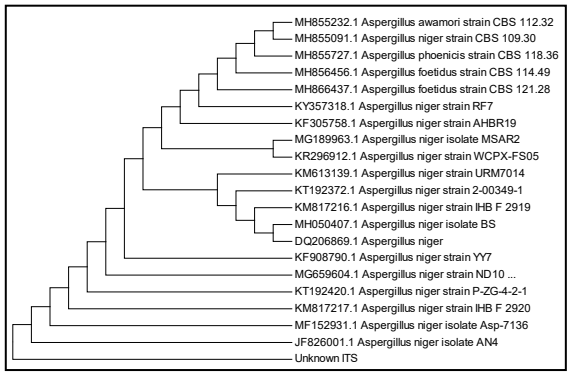

Figure 2: Phylogenetic Tree of *Aspergillus niger H1* with other *Aspergillus* species

Enzyme production

The crude enzyme has a specific activity of 3.03U/mg.

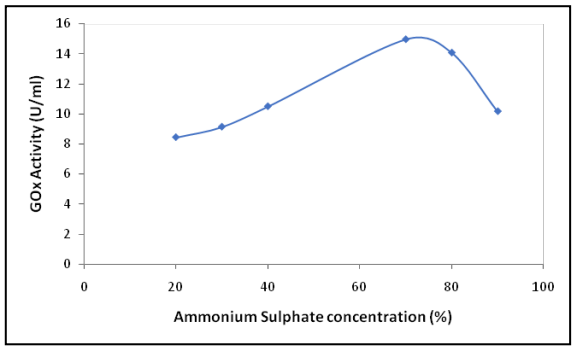

Figure 3: Ammonium Sulphate profile

Purification of Aspergillus niger GOx

Seventy-70 % (w/v) of ammonium sulphate precipitation was observed to be suitable for the first purification step with a specific activity of 2.82U/mg. The precipitated GOx was subjected to dialysis. The specific activity of 3.52 U/mg was obtained after dialysis. Dialysis of protein is encouraged after ammonium sulphate because it stimulates ionic scrambling leading to an aggregate formation with incorrect ionic bond pairing [20]. For further purification, the dialyzed enzyme underwent Gel-filtration equilibrated with 50mM sodium acetate pH 5.0. In the final purification step of Gel-filtration, specific activity increased from 3.54 to 3.69U/mg protein indicating the removal of impurities.

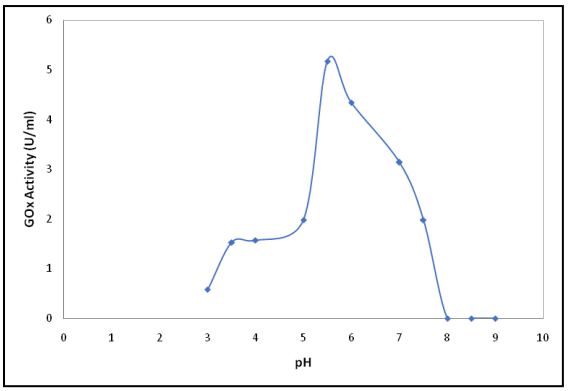

Figure 4: Effect of pH on GOx activity

Optimum pH and temperature determination

In this study, optimum pH of 5.5 was obtained. An increase in pH from 3.0 to 5.5 accompanied by an increase in GOx activity was observed after which the GOx activity started declining. High GOx activity was maintained from 5.0 - 7.0 beyond which, it decreases rapidly as shown in figure 4. GOx has many amino and carboxyl groups which are sensitive to different

Table 1: Purification table of *Aspergillus niger* GOx

	Volume (ml)	Activity (Units/ml)	Total Activity (Units)	Protein Conc. (mg/ml)	Total Protein (mg)	Specific Activity (U/mg)	Yield	Purification fold
Crude enzyme	1000	16.22	16220	5.36	5360	3.03	100	1
NH_4SO_4 Precipitate	120	7.70	924	2.73	327.6	2.82	12	0.931
Dialyzed	50	9.62	481	2.72	136	3.54	5	1.170
Gel Filtration	30	7.85	236	2.13	63.9	3.69	3	1.220

pH values, thus affecting its conformation. Ozyilmaz *et al*. [21] also reported the optimum pH of GOx to be 5.5. An optimum of 5.5 for GOx produced from *Aspergillus fumigatus* AFS4 was also reported by Onosakponome *et al.* [20]. Also, the increase from 20 to 40°C in temperature was accompanied by a rise in GOx activity after which there was a decrease in GOx activity making 40°C the optimum temperature. In this study, GOx maintained high activity over a temperature range of 30 - 70°C as shown in figure 5. The rapid decline in GOx activity after the optimum temperature may be due to subunits dissociation. Like other multimeric enzymes, the subunits dissociation may be involved in *Aspergillus niger* GOx inactivation. Sukhacheva *et al.* [22] reported optimum temperature range of 40–60 °C for GOx from *Aspergillus niger* and *Pencillium amagasakiense* ATCC 28686.

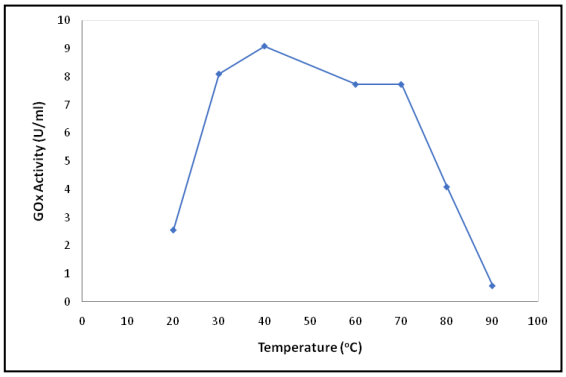

Figure 5: Effect of temperature on GOx activity

Metal ions on GOx activity

The effect of metal ions on *Aspergillus niger* GOx activity showed that Ag^{2+} and Hg^{2+} had a notably inhibitory effect on partially purified GOx, whereas Ca^{2+}, Zn^{2+} and Mg^{2+} do not affect the enzyme activity. Cu^{2+} and Co^{2+} have a modest inhibitory effect on GOx. This study is by Kusai *et al.* [23] who reported that Ag^{2+} and Hg^{2+} are inhibitors of GOx. The Inhibitory Effect of GOx by Ag^{2+} ions may be a result of the reaction of Ag^{2+} with the thiol group of GOx essential for catalysis, which is in proximity to the FAD-binding region of GOx (20). Yuan *et al.* [24] reported a similar result for recombinant GOx.

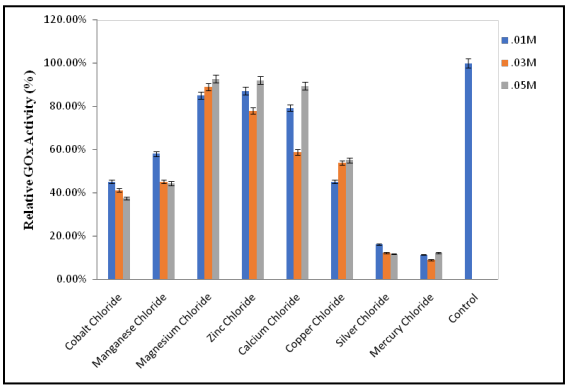

Figure 6: Effect of divalent metal ions on GOx activity

Kinetic characterization

The Michaelis mentens constant (Km) and maximum velocity (Vmax) were 0.12mM and 40U/ml respectively. This property indicated that GOx produced from *Aspergillus niger* has a high affinity for β D-glucose oxidation.

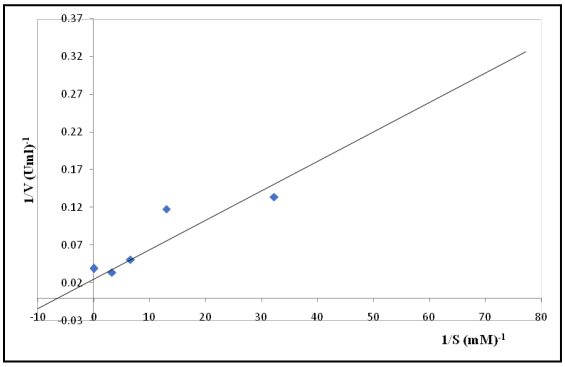

Figure 7: Lineweaver-Burk of initial velocity values at various glucose concentrations

Conclusion

The findings from this study suggest that the *Aspergillus niger* strain isolated from a local beehive was capable of producing glucose oxidase with an optimum pH and temperature of 5.5 and 40°C respectively. The wide range of optimal temperatures (30 - 70°C) and pH (5 - 7.5) give this enzyme an edge in industrial applications, especially in diagnostic applications.

Conflict of interest

No conflict of interest was declared

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