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Kinetics and Thermodynamic Properties of Glucose Oxidase Obtained from *Aspergillus fumigatus ASF*4

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ABSTRACT

Heat instability is a major setback that prevents the broader use of glucose oxidase (GOx) in industries. This research explored the kinetic and thermodynamic parameters of *Aspergillus fumigatus ASF*4 GOx to determine its potential for biotechnological applications. *Aspergillus fumigatus ASF*4 GOx was purified 2.18-fold with a 6.25% yield after ammonium sulfate precipitation (60%), dialysis, ion-exchange chromatography, and gel filtration. The pH and temperature optima for GOx activity were 5.5 and 40°C, respectively. Metal ions, Ag^{2+} and Hg^{2+} had a remarkable inhibitory effect on GOx activity whereas Ca^{2+}, Mg^{2+}, and Mn^{2+} enhanced GOx activity. The maximum velocity (V_{max}) and Michaelis constant (K_M) were 2000 µmol/min, and 24 mM, respectively. The enzyme retained 85% and 90% of its initial activity at 40°C and 30°C, respectively after 120 min of incubation. At 50°C and 45°C, the enzyme retained more than 50% of its initial activity after 120 min of incubation. The k values at 37°C were the lowest (0.002) whereas that at 70°C was the highest (0.011). The Z-value was 0.3 and the activation energy (Ea) was 70.64 KJ/mol/K suggesting great sensitivity of GOx to temperature change. The D-value of *Aspergillus fumigatus ASF*4 GOx ranged between 115.5 to 208.4 min. The thermodynamic studies showed that glucose oxidation by *Aspergillus fumigatus ASF*4 GOx was reversible ($\Delta S<0$), endothermic ($\Delta H>0$), and non-spontaneous ($\Delta G>0$) at all temperatures tested. The results on the optimum conditions for GOx activity and stability have shown that *Aspergillus fumigatus ASF*4 GOx can find application in the industrial production of gluconic acid.

Keywords: *Aspergillus fumigatus ASF*4, characterization, glucose oxidase, thermos-stability, 18S-rDNA sequencing.

Introduction

Gluconic acid (GA) is a polyhydroxy carboxylic acid possessing both reactive hydroxyl and carboxyl groups. Gluconic acid is a non-volatile, easily biodegradable, non-toxic, and non-corrosive chemical produced from biomass using different approaches including microbial fermentation, enzymatic oxidation of glucose, and oxidation by molecular oxygen using heterogeneous catalysis.[1] GA is usually produced by glucose oxidation catalyzed by Glucose oxidase (GOx) using molecular oxygen as the electron acceptor.[2]

GOx catalyzes glucose oxidation, by a ping-pong mechanism,[2] involving glucose and molecular oxygen as the electron acceptor and yielding hydrogen peroxide and gluconic acid.[3-9] This enzyme is a member of a vast family of oxidoreductases acting on glucose, methanol and choline. GOx is a dimeric glycoprotein with two identical subunits (80kDa), linked together by disulfide bonds.[10,11] Structurally, each of the subunits contains a tightly but not covalently bound FAD moiety as a co-factor.[12] This cofactor acts as a redox carrier during catalysis.[10-14.] The ability of GOx to oxidize glucose with liberation of hydrogen peroxide underlines its use in glucose biosensors for evaluation of antidiabetic properties of some medicinal plants.

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Glucose oxidase is known to possess antimicrobial properties arising from the production of hydrogen peroxide and can be used to supplement diets and enhance pathogen defense response.[15,16] Also, the accumulation of gluconic acid due to degradation of gluconolactone lowers the pH of a solution thereby contributing to the antimicrobial activity of GOx.[6]

GOx is obtained from different sources, including citrus fruits, red algae, bacteria, plants, animals, insects, and fungi. Among these sources, fungal sources are preferred and are extensively employed in various sectors of food industries as in the production of dry egg powder, beverages, gluconic acid, and baking products.[14]

Gluconic acid and its derivatives (such as sodium gluconate or calcium gluconate) as well as hydrogen peroxide are of great importance as essential intermediates in the food, pharmaceutical, building, and textile industries.[4,17] Also, derivatives of GA are added to soft drinks and dairy products to enhance and preserve their sensory properties. Sodium gluconate can remove bitterness from food and chelate metal.[4] They are attractive feedstock for green chemistry applications. The need for green and sustainable development has prompted the search for eco-friendly and cost-effective means of production of chemical feedstock such as gluconic acid. However, the eco-friendly and cost-effective bioconversion of glucose to gluconic acid and hydrogen peroxide by glucose oxidase remains a serious challenge. This is due to the dependence of the catalytic efficiency of GOx on its stability. Sufficient heat stability of enzymes is vital for enhancing product efficiency.[18] Thermal instability and little knowledge on the optimal conditions for GA production are the major setbacks preventing the use of glucose oxidase for the eco-friendly production of GA. This can be achieved by exploring locally sourced microbial heat-stable glucose oxidase and determine suitable conditions for its catalysis. In this study, we isolated and molecularly characterized GOx producing fungi, determined the effect of different

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metal ion concentrations, pH, glucose concentration, and temperature on the purified GOx activity as well as the effect of heat treatment on the stability of *Aspergillus fumigatus ASF*4 glucose oxidase.

Materials and Methods

Chemicals
All chemicals used in this study were of analytical grade and were products of BDH chemical limited (England), USA, Merck **(**Germany), May, and Baker limited (England), Sigma Aldrich.

Isolation of glucose oxidase producing fungi
Pure fungal strains were isolated from soil collected from a fruit garden in the University of Nigeria, Nsukka, Enugu State. The microorganism was identified as described by Martin *et al*.[19] The fungal strains were screened for glucose oxidase production capability according to the method explained by Park *et al*.[20] The fermentation medium containing glucose (80g), peptone (3.0g), $(NH_4)_2$ HPO_4 (0.388g), KH_2PO_4 (0.188g), $MgSO_4$ $7H_2O$ (0.156g), agar (20g), in one liter of sodium acetate buffer (0.05M, pH 5.5) was sterilized at 121^{o}C for 15min, dispensed into Petri dishes to gel. The Petri dishes were inoculated with the fungal strain using a cork borer and incubated at 35°C for 3 days. The plates were then spread with a solution containing agar 1% (w/v) in sodium acetate buffer (0.05M, pH 5.5), glucose 5% (w/v), *O*-dianisidine 0.1% (w/v), glycerol 2% (v/v), Horseradish peroxidase 60IU/ml, and incubated at 35°C for 1hr. A reddish-brown coloration indicates the presence of GOx. The strain with the highest GOx production suggested by the intensity of reddish-brown coloration was identified, selected, and maintained on the PDA slant at 4°C for further studies.

Molecular identification and characterization of the fungal strain
The genomic DNA (gDNA) of the isolated fungal strain was extracted using AccuPrep® DNA extraction kits following the manufacturer's instructions. The primer pairs ITS 1(5'-TCCGTAGGTGAACCT GCGG-3') and ITS 4 (5'-GCTGCGTTCTTCTTCATGATGC-3') were used to amplify the fungal internal transcribed spacer (ITS) regions. The conditions for PCR were: initial denaturation at 94^{o}C for 3min, denaturation at 94^{o}C for 40 sec, annealing at 54^{o}C for 40 sec, extension at 72^{o}C for 40 sec, and final extension at 72^{o}C for 10 sec. Agarose gel electrophoresis was carried out using 1.5% agarose gel (1.5g of agarose in 100ml of Tris-acetate-EDTA (TAE) buffer). Agarose gel powder was dissolved by microwaving in 1 × TAE buffer. The mixture was cooled to 55^{o}C and 12µl of ethidium bromide was added and allowed to cool for 30min at 37^{o}C. DNA ladder (6µl) and the amplicon (10µl) were loaded into the wells of agarose gel followed by electrophoresis at 100V for 1hr. The DNA bands on the gel were visualized using a UV lightbox/gel imaging system. The DNA sequences obtained were subjected to BLAST (Basic Local Alignment Search Tools for Nucleotides) search algorithm and aligned using Multiple Sequence Alignment based on Fast Fourier Transform (MAFFT) version 5. The phylogenetic analysis of the ITS sequence data was conducted using the molecular evolutionary genetic analysis (MEGA) version 7.

*GOx production from Aspergillus fumigatus ASF*4
The fermentation broth was charged for GOx production with glucose 80% (w/v), peptone (0.3%), $MgSO_4.7H_2O$ (0.0156%), KH_2PO_4 (0.0188%), $(NH_4)_2HPO_4$ (0.04%), $CaCO_3$ (3.5%) in 100ml of 50mM sodium acetate buffer pH 6.0.[21] The flasks were sterilized and inoculated with four discs (10 mm) of pure strains of *Aspergillus fumigatus ASF4*. The flasks were incubated at 30°C on an orbital shaker at 150rpm for seven days, filtered, and centrifuged at 15000 rpm for 15 min. The supernatant was assayed for GOx activity.

Glucose oxidase assay and protein determination
GOx activity was assayed using glucose as a substrate, and *O*-dianisidine as a coupling reagent.[22] Reagents A, B, C, D, and E were prepared for the assay. Reagent A was 0.05M sodium acetate buffer of pH 5.5; reagent B was an *o*-dianisidine (0.21mM) solution dissolved in 100ml of reagent A; reagent C was β- D-glucose solution (10%); solution D was a mixture of reagent B (24ml) and 5ml of reagent C; reagent E was a freshly prepared solution of horseradish peroxidase type II. The reaction mixture contained 2.9ml of solution D, 0.1ml of reagent E, and 0.1ml of enzyme solution. The absorbance was measured every 15 sec for 5 min using a UV-Visible spectrophotometer at 500nm. One unit of glucose oxidase activity is defined as the amount of enzyme that catalyzes the conversion of 1µmole of β-D-glucose to D-gluconolactone and H_2O_2 per minute at 35°C and pH 5.5. The concentration of protein was determined using the Lowry method and Bovine serum albumin (BSA) as standard.[23]

Purification of Aspergillus fumigatus ASF4 GOx
The crude GOx was brought to 60% saturation using $(NH_4)_2SO_4$ salt as described by Chilaka *et al*.[24] The precipitated enzyme (20ml) was dialyzed for 12 hr against sodium phosphate buffer (0.05 M, pH 7.0). The dialysate was loaded into a (2.0 x 14 cm) DEAE chromatographic column equilibrated with 50 mM sodium acetate buffer (pH 5.5). The unbound proteins were washed using the same buffer. A stepwise elution with 50 mM sodium acetate buffer containing 0.05 to 1M NaCl was carried out at a flow rate of 2.5 ml/min. The active fractions were pooled together and introduced into a (2.0 x 80 cm) Sephadex- G-200 gel chromatographic column, pre-equilibrated with 0.05 M sodium acetate buffer (pH 5.5). enzyme fractions were collected at a flow rate of 5ml/15min and assayed for GOx activity. The active fractions were combined and stored at -10°C for further studies.

Effect of pH and temperature on Aspergillus fumigatus ASF4 GOx activity
The pH optimum for GOx activity was monitored using 0.05M sodium acetate (pH 3.5-5.5), 0.05M sodium phosphate (pH 6.0-7.5) and 0.05M Tris-HCl (8.0-10.0). The GOx activity was assayed using each buffer as described above. The temperature optimum for GOx activity was evaluated by incubating the reaction mixture excluding the enzyme at a temperature range of 30-75^{o}C (5^{o}C interval) before initiating the reaction. The reaction was initiated by adding GOx ($0.15Uml^{-1}$) as described by Singh and Verma.[25] The GOx activity was assayed and plotted against temperature.

Effect of glucose concentration and metal ions on GOx activity
The effect of different glucose concentrations (4.0-25%, w/v) on GOx activity was determined at pH 5.5 and 40^{o}C as described by Sandalli *et al*.[26] The maximum velocity (V_{max}) and Michaelis constant (K_M) were obtained from the Lineweaver-Burk plot of initial velocity values at varying glucose concentrations. The effect of different metal ions (Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ag^{2+}, and Hg^{2+}) on GOx activity was determined as described by Yanmis *et al*.[27] The purified enzyme (100µl) was incubated with 0.9ml of different concentrations (10, 20, and 50 mM) of metal ions for 20 min. After incubation, GOx activity was assayed. The GOx activity in each case was compared with the activity obtained without metal ions.

Effect of temperature on Aspergillus fumigatus ASF4 GOx stability
The effect of temperature on *Aspergillus fumigatus ASF*4 GOx stability was determined by incubating *Aspergillus fumigatus ASF*4 GOx at different temperatures (37 to 60 ^{o}C) without substrate for 2 h.[28] Aliquots (0.1ml) of the enzyme were collected at different intervals, quickly cooled in ice for 20 min, and assayed for GOx activity. The initial activity was assumed 100 % and was used to calculate the percentage residual activity after every incubation period. The first order inactivation constant, *k* values were obtained from the gradient of the first-order enzyme inactivation equation as follows:

$$Log(\%\ residual\ activity) = -(k/2.303)t$$

Where t is the time of enzyme inactivation.
The activation energy of the purified GOx was obtained from the Arrhenius plot of lnk against 1/T. Arrhenius law is usually used to explain the temperature dependence of the rate of inactivation constants (*k*) and is given by

$$lnk = Ae^{-Ea/RT}$$

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Where:
Ea = activation energy of inactivation,
A=Arrhenius constant,
R= Universal gas constant (8.314 J/mol.K) and
T= Absolute temperature.
The activation energy (Ea) values and the Arrhenius rate constant (k) were used to obtain the Gibbs free energy change (ΔG), the entropy change (ΔS), and the enthalpy change (ΔH) using equations below:

$$\Delta G_{inact} = -RTln(\frac{kh}{K_B T})$$
$$\Delta S_{inact} = (\Delta H_{inact} - \Delta G_{inact})/T$$
$$\Delta H_{inact} = Ea_{inact} - RT$$

Where t, E_a, R, T, K_B, and h are the time of inactivation, activation energy, universal gas constant (8.314J/mol.K), absolute temperature, Boltzmann's constant (1.3806 x 10^{-23} J/K), and Plank's constant (6.6260 x 10^{-34} J.s).
The half-life of inactivation ($t_{1/2}$) was calculated from:

$$t_{1/2} = ln(2)/k$$

The D-value (the time required for 90% reduction of the initial activity) was obtained using the equation:

$$D - value = ln10/k$$

The Z-value was obtained by plotting log D values at different treatment times against the respective temperature. The slope of the line is equal to the negative reciprocal of the Z-value.

Results and Discussion

The glucose oxidase-producing fungi were isolated and identified as *Aspergillus* species based on morphological and microscopic characteristics. The fungal strain was further confirmed as *Aspergillus fumigatus ASF*4 using 18S rDNA sequencing technique. The result of the agarose gel electrophoresis showed a single band at approximately 500bp when compared to the DNA ladder which indicates that the isolate mostly likely belongs to *Aspergillus* sp (Figure 1). The phylogenetic analysis revealed that the *Aspergillus* sp is very closely related to *Aspergillus fumigatus ASF*4 (Figure 2). Also, from the NCBI blast result of the query sequence and as shown on the phylogenetic tree of evolutionary history, the organism had a bootstrap score of 99 % with *Aspergillus fumigatus* strains *MG991595.1*, *MK719925.1*, and *MH378448.1*.

Bootstrap score often shows the level of relatedness between the query sequence and other homologous sequences from the NCBI Genbank. A bootstrap score of 99 % indicates a strong relatedness of the query sequence and *Aspergillus fumigatus*. Identification of fungi based on morphology and microscopic characteristics is useful but quite ambiguous, open-ended, and does not give accurate information about the genus or species of the organism. The nuclear rDNA internal transcribed spacer (ITS) region is the crucial fungal barcode marker used to identify distinct strains and analyze fungal diversity in a sample because the sequence (ITS) can be easily amplified from most DNA samples using universal primers.[29] This method is a precise technique for fungal identification and has the highest probability of identifying the widest range of fungi. GOx was produced from *Aspergillus fumigatus* AFS4 under a submerged fermentation system with a specific activity of 409 U/mg protein (Table 1). Sixty percent (60%) ammonium sulfate saturation was suitable to precipitate GOx with a specific activity of 602U/mg. Zia *et al.*[30] reported 60 to 85% ammonium sulfate saturation as suitable for commercial GOx preparation. After dialysis, the specific activity was 647 U/mg. Two prominent peaks indicating two different isoforms of GOx were observed after ion-exchange chromatography (Figure 3). Simpson *et al.*[28] reported that the intra- and extracellular fractions of GOx contained isoenzymes. Dialysis of enzyme solutions after ammonium sulfate precipitation can encourage ionic scrambling leading to the formation of aggregates with incorrect ionic bond pairing.[24] *Aspergillus fumigatus* AFS4 GOx was purified 2.6 fold with a yield and specific activity of 8.75% and 1062 U/mg after ion-exchange chromatography, respectively. Gel filtration purified the enzyme 2.85 fold with a percentage yield of 6.25 and a specific activity of 1167 U/mg (Table 1). The gel filtration elution profile had a single peak of GOx activity (Figure 4).

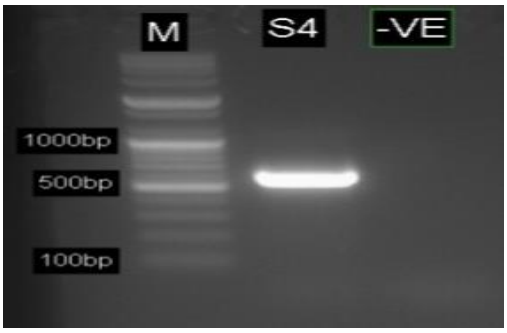

Figure 1: Agarose gel electrophoresis of the (M) DNA marker and (S4) amplicons.

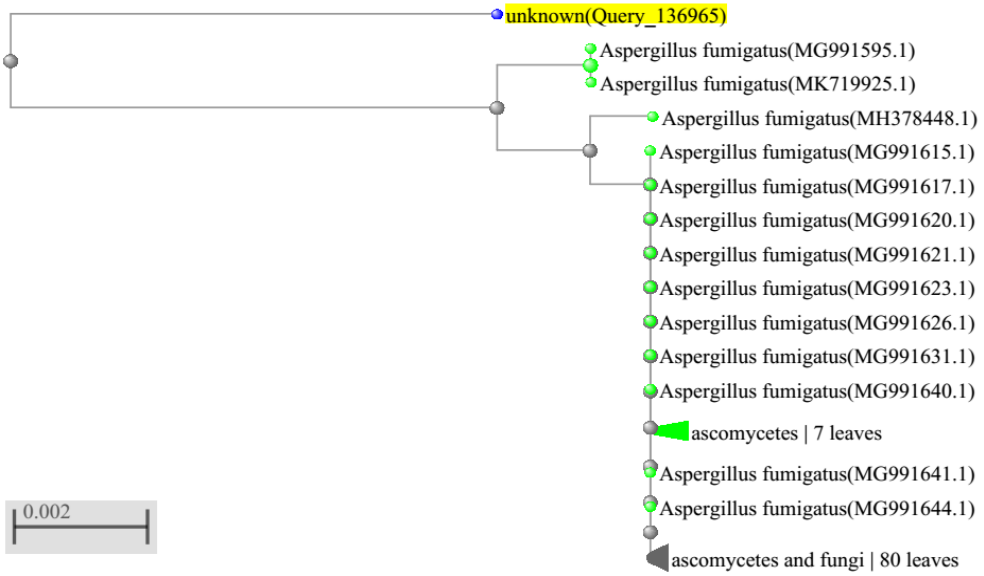

Figure 2: Phylogenetic tree of *Aspergillus fumigatus* AFS4 with other species of *Aspergillus*

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Table 1: Purification table of GOx from *Aspergillus fumigatus AFS4*

Purification Step	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Activity %	Yield
Crude Enzyme	400	1556	636400	409	1.00	100	
$(NH_4)_2SO_4$ Saturation	55	91.9	55330	602	1.47	13.75	
Dialysis	35	74.6	48270	647	1.58	8.75	
DEAE-Cellulose column	35	30.1	31990	1062	2.61	8.75	
Gel column chromatography	25	17.0	19850	1167	2.85	6.25	

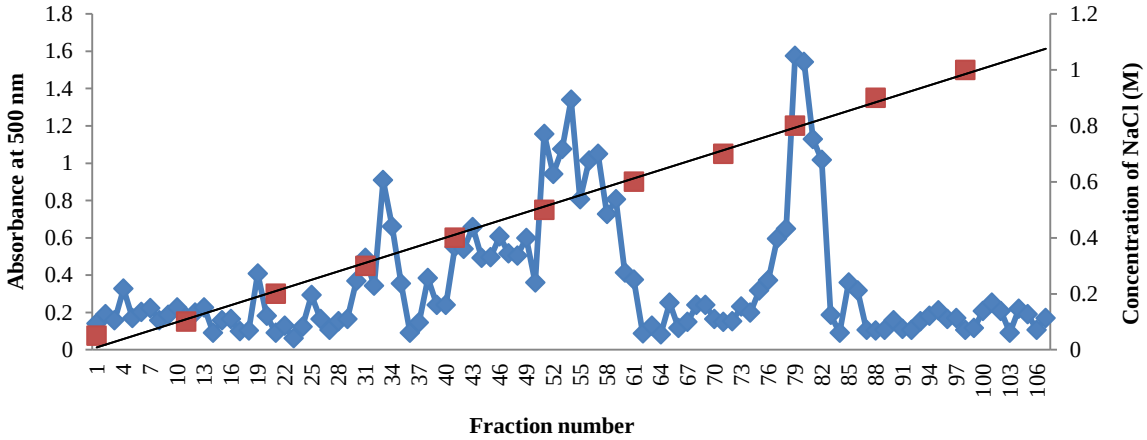

Figure 3: Ion-exchange chromatogram for *Aspergillus fumigatus* AFS4 GOx

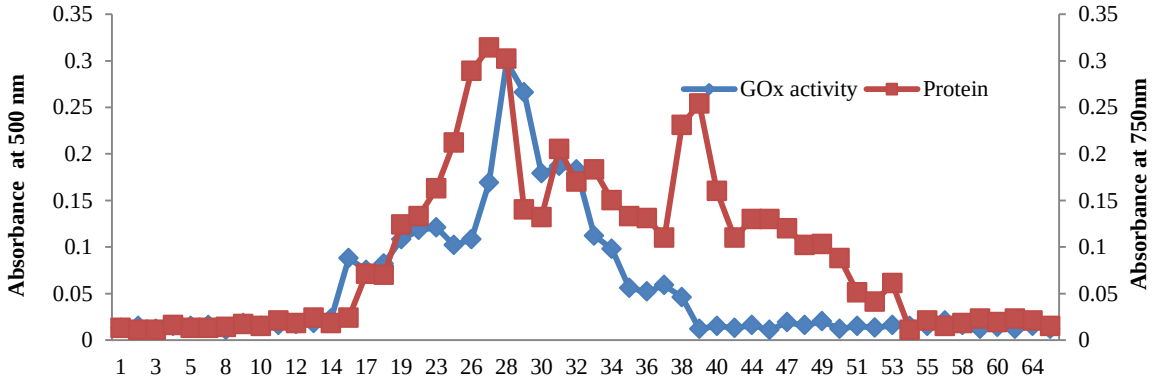

Figure 4: Gel filtration chromatogram for *Aspergillus fumigatus* AFS4 GOx

Our studies on the effect of pH on GOx activity showed that, as the pH was increased from 3 to 5.5, the GOx activity was increased beyond which the enzyme activity decreased making 5.5 the optimum pH (Figure 5). As a protein, GOx has many ionizable groups such as amino and carboxyl groups. These groups contribute to the sensitivity of the enzyme to different pH values thereby affecting the protein conformation, enzymes, and substrate ionization state. Jithendar *et al.*[31] and Belyad *et al.*[32] reported 6.0 and 7.0 as the optima pH for glucose oxidases produced from *Aspergillus niger* PIL7 and *Aspergillus niger* ATCC 9029, respectively. Also, Simpson *et al.*[28] reported that GOx activity from *Penicillium sp.* CBS 120262 was maximum at pH 7 and showed a wide pH profile with more than 70% of the highest activity between a pH range of 4.9-8.9. The result of this study is per the result of Yuan *et al.*[12] who reported pH optimum of 5.5 for recombinant glucose oxidase.

Also, an increase in temperature from 30 to 40°C was accompanied by a rise in GOx activity beyond which the enzyme activity decreased (Figure 6). The *Aspergillus fumigatus* AFS4 GOx was optimally active at 40°C and maintained a high activity over a wide range of temperatures (30-65°C) (Figure 4b). The rapid decrease in GOx activity beyond 40 °C could be because of subunits dissociation. The wide range of optimal temperature of activity observed with *Aspergillus fumigatus* AFS4 GOx could be attributed to the tightly bound FAD co-factor, which held the two protein subunits together. As a dimeric enzyme,[33] the concentration of GOx may affect the enzyme stability. Subunits dissociation may play a key role in *Aspergillus fumigatus* AFS4 GOx inactivation as seen in other multimeric enzymes.[34] Yuan *et al.*[12] also, reported a temperature optimum of 35ºC for recombinant glucose oxidase whereas Belyad *et al.*[32] reported 50 ºC for GOx obtained from *Aspergillus niger* ATCC 9029. The Michaelis Menten constant (K_M) and maximum velocity (V_{max}) of glucose oxidation obtained from the Lineweaver-Burk plot of initial velocity values at various glucose concentrations (Figure 7) were 24 mM and 2000 µmol/min, respectively. This indicates that *Aspergillus fumigatus* AFS4 GOx had a high affinity for β D-glucose during the oxidation process. The high affinity of *Aspergillus fumigatus* AFS4 GOx for D-glucose and its wide range of optimal temperature and pH are promising properties for its application in

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gluconic acid production, and food industry as a bio-preservative agent. Sukhacheva *et al.*[35] reported that *Pencillium. amagasakiense* ATCC 28686 and *Pencillium. funiculosum* 433 glucose oxidases showed low K_M values of 5.7 mM and 3.3 Mm, respectively.

The result on the effect of metal ions on *Aspergillus fumigatus* AFS4 GOx activity showed that Ag^{2+} and Hg^{2+} had a remarkably inhibitory effect on purified GOx, whereas Ca^{2+}, Zn^{2+}, and Mg^{2+} enhanced GOx activity. Cu^{2+} and Co^{2+} had a slight inhibitory effect on GOx (Figure 8). This is in line with the report that Ag^{2+} and Hg^{2+} are inhibitors of GOx.[10,36] These inhibitors may precipitate the enzyme making it lose its three-dimensional structure required for catalysis. Divalent metals bind more strongly to ribose and pyrophosphate than monovalent metals. Therefore, enzyme inhibition by divalent metal ions is greater than the monovalent metal ions. Inhibition of GOx by Ag^{2+} ions may be due to the reaction of Ag^{2+} with the thiol group of the enzyme essential for catalysis, which is in proximity to the FAD-binding region of the enzyme.[28,37] A similar result was obtained for recombinant GOx.[12] on the other hand, the increase in GOx activity by metal ions could be attributed to the ability of the ions to act as an electron donor or Lewis acid as they may participate directly in the catalytic mechanism of the enzyme.[37]

An enzyme's thermostability is the ability of molecule of an enzyme to withstand thermal unfolding in the absence of a substrate. Thermostability differs from thermophilicity which is the ability of enzymes to act optimally at increased temperatures in the presence of substrate. Thermostability studies on *Aspergillus fumigatus ASF*4 GOx were carried out by incubating the enzyme at a temperature range of 37 to 60 °C for 120 min. *Aspergillus fumigatus ASF*4 GOx retained 85 and 90 % of its initial activity at 37 and 40 °C, respectively after 120 min. At 45 and 50 °C, the enzyme retained more than 50% of its activity after 120min of incubation. 25% of the original activity was retained after 120min of incubation at 60°C (Figure 9). The plot of residual GOx activity against time resulted in multiphasic inactivation curves as shown in Figure 9. The enzyme appeared to be stable at 30, 40, 45, and 50°C. The multiphasic inactivation curves may be due to the formation of thermostable aggregates, recovery, and regeneration of activity, the existence of different isoforms of GOx.[38] More so, the rapid loss of activity in the first phase on inactivation curves might be due to the inactivation of heat-labile isoforms of GOx.

The plots of log (% residual activity) against inactivation time (t) was linear at all temperatures tested (Figure 10). The increase in the inactivation rate constant (k) at higher temperatures suggests less thermal stability at a higher temperature. The enzyme was stable at 40°C with a half-life of 231.03 min. However, at 60°C it was less stable and showed a half-life of 63.01 min under similar conditions (Figures 9 and 10). Half-life (which is the time required to lose 50% of enzyme original activity), is a crucial economic parameter in the industrial application of enzymes. The higher the half-life value the more stable the enzyme.[39]

The D-value, the decimal reduction time is defined as the time needed for a 90% reduction of the original activity. D-value is also, one of the parameters used in the estimation of enzyme stability. The D-value for *Aspergillus fumigatus* AFS4 GOx decreased with an increase in temperature. The D-value was minimum (209.4 min) at 60°C and maximum (1151.5 min) at 37°C. The increase in D-values suggests a rise in the stability of *Aspergillus fumigatus ASF*4 GOx. The Z-value was obtained from Figure 11. The low Z-value suggests that *Aspergillus fumigatus* AFS4 GOx was stable and more sensitive to a rise in temperature than the period of heat treatment.[40] High activation energy value (70 KJ/mol/K) (obtained from Figure 12) suggests a greater sensitivity of GOx to change in temperature.

Thermodynamic parameters including Gibb's free energy, enthalpy, and entropy are the tools used to analyze the heat stability of enzymes. They give information on the secondary stabilization and destabilization effects that may not be captured by the kinetic parameters. These parameters provide precise proof of the unfolding of protein during heat inactivation.[41] The results showed that enthalpy of inactivation at 37, 40, 45 50, and 60 °C were 68.06, 68.03, 67.99, 67.94, and 67.86 KJ/mol, respectively (Table 2). A slight decrease in enthalpy from 68.06 to 67.86 KJ/mol, was observed with a temperature increase from 37 to 60 °C.

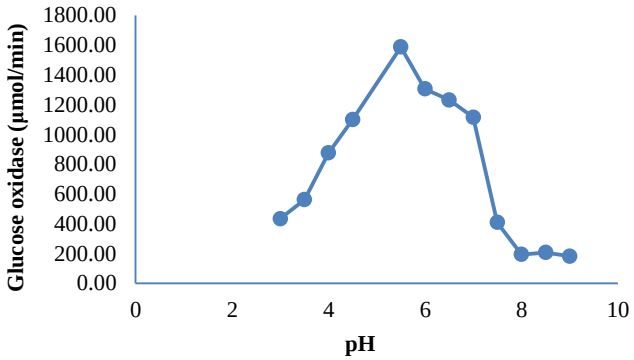

Figure 5: Effect of pH on *Aspergillus fumigatus ASF*4 GOx activity

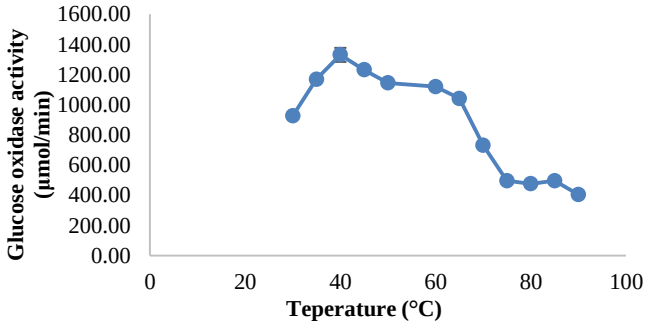

Figure 6: Effect of temperature on *Aspergillus fumigatus ASF*4 GOx activity

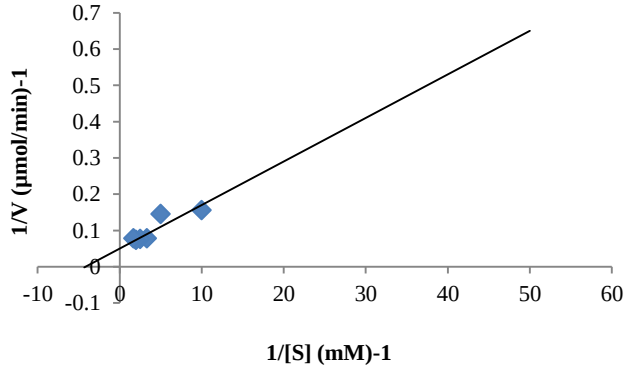

Figure 7: Lineweaver-Burk plot of initial velocity data at various glucose concentrations

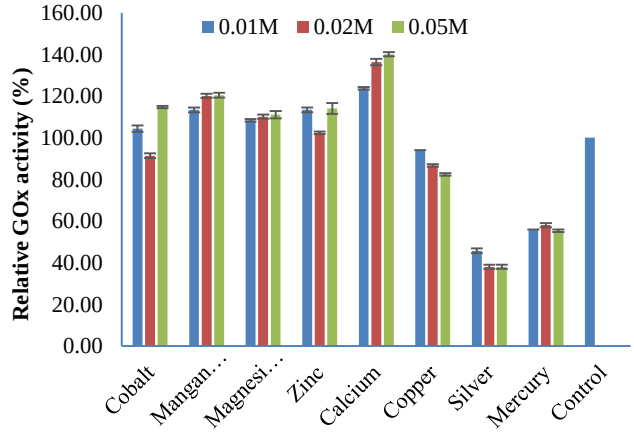

Figure 8: Effect of divalent metal ions on GOx activity

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The enthalpy of denaturation is the quantity of energy needed to denature the enzyme. Change in enthalpy is a measure of the number of bonds broken during inactivation and indicates whether the inactivation process is endothermic or exothermic.[42]
Also, the relatively low enthalpy value reflects the enzymes' resistance nature, while increased values represent a response to protein denaturation.[41] The positive values of ΔH obtained in this research suggest that the oxidation of glucose by *Aspergillus fumigatus ASF*4 *GOx* was an endothermic reaction. Positive and large enthalpy values suggest high enzyme thermal stability.[39] Therefore, for *Aspergillus fumigatus ASF*4 *GOx* to catalyze endothermic reaction suggests that it was able to withstand the heat energy supplied into the reaction system. Gibb's free energies were 90.04, 89.91, 90.64, 91.53, and 92.31 KJ/mol at the respective temperatures studied (Table 2). There was an increase in ΔG with a rise in temperature, which was maximum at 60 °C (92.31) $KJmol^{-1}$. The ΔG increased with a rise in temperature indicating that *Aspergillus fumigatus ASF*4 glucose oxidase has the resistance against thermal unfolding at a higher temperature.[41] The Gibbs free energy (ΔG) tells about how spontaneity a reaction is. The positive values of Gibb's free energy obtained in this research suggest that glucose oxidation to gluconolactone and H_2O_2 is a non-spontaneous process as high energy is required for the process to occur. The ability of *Aspergillus fumigatus ASF*4 glucose oxidase to catalyze the oxidation process suggests that it was able to withstand the high energy needed for the process, indicating that it is thermally stable.[39]
Entropy (ΔS) shows the net enzyme and solvent disorder. Entropies obtained at all temperatures were negative. At 60 °C, the entropy of the system was found to be -0.071$Jmol^{-1}K^{-1}$ (Table 2). A negative ΔS° value indicates a decrease in the disorderliness of the enzyme solution during denaturation. The low level of disorderliness suggests that *Aspergillus fumigatus ASF*4 glucose oxidase was stable at all temperature tested.[40] The negative values of ΔS obtained at all temperatures suggest a rise in the order of the system via (a) protein aggregation that involves the formation of few intra-/inter molecular bonds (b) compaction of enzymes around enzyme molecules leading to the formation of charged particles around the enzyme molecule and the ordering of solvent molecules.[43,44,45,46] Hydrophobic interactions are the stabilizing factor that allows enzymes to retain their structures at high temperatures. These interactions reduce the disorderliness (entropy) of the system.[13,25]

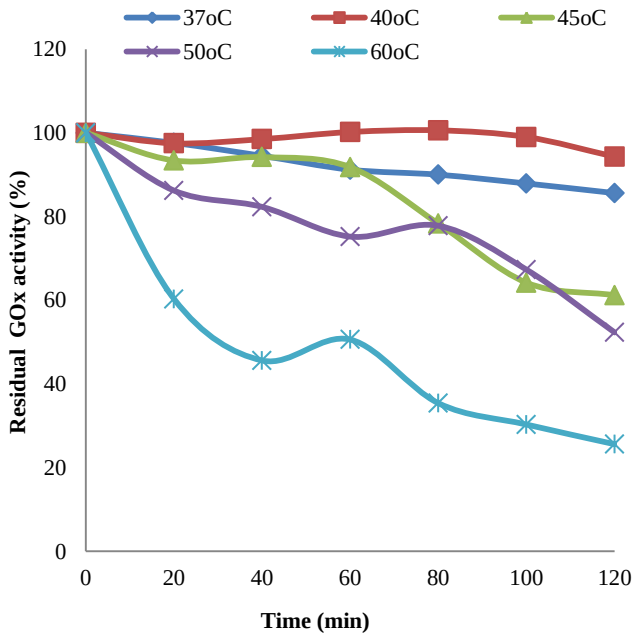

Figure 9: Plot of percentage residual glucose oxidase activity against time

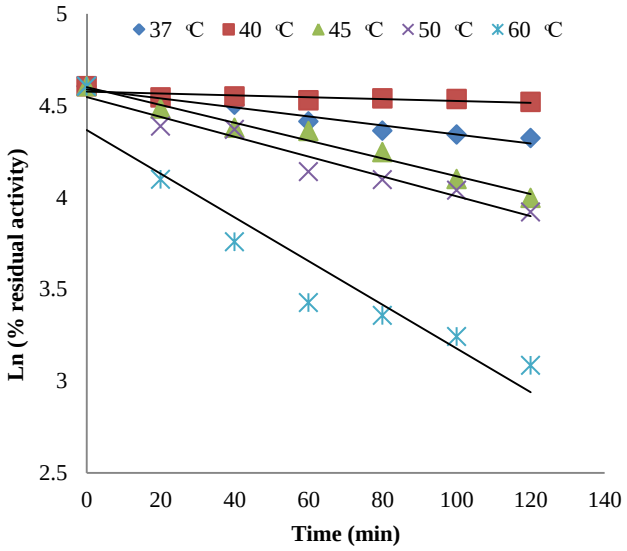

Figure 10: Plot of ln(%residual glucose oxidase activity) against time.

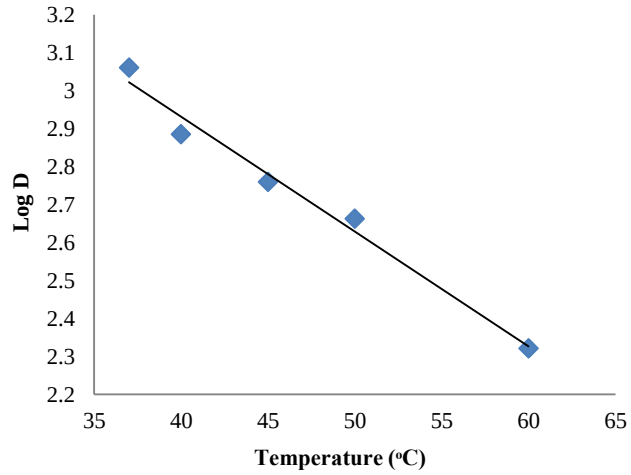

Figure 11: Temperature dependence of the decimal reduction time

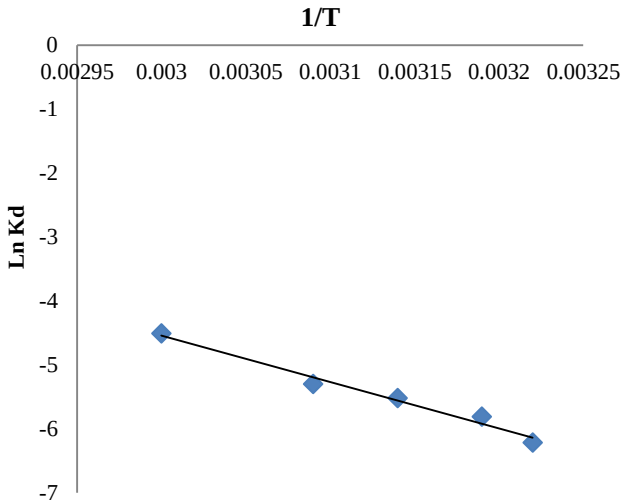

Figure 12: Arrhenius plot for thermal inactivation of *Aspergillus fumigatus* AFS GOx

Table 2: Kinetics and thermodynamic parameters of thermal inactivation of *Aspergillus fumigatus AFS* GOx

Temperature (°C)	K_d(min^{-1})	$t_{1/2}$ (min)	D-value (min)	$\Delta H^o_{(D)}$ KJ/mol	$\Delta G^o_{(D)}$ KJ/mol	$\Delta S^o_{(D)}$ KJ/mol
37	0.002	346.55	1151.5	68.06	90.04	-0.071
40	0.003	231.03	767.7	68.03	89.91	-0.070
45	0.004	173.28	575.8	67.99	90.64	-0.071
50	0.005	138.62	460.6	67.94	91.53	-0.073
60	0.011	63.01	209.4	67.86	92.31	-0.076
Z-value (°C)	0.300					
Ea KJ/mol/K	70.64					

Conclusion

The optimal pH and temperature for oxidation of glucose into gluconic acid and hydrogen peroxide by GOx locally isolated from *Aspergillus fumigatus ASF*4 were 5.5 and 40°C in the presence of Ca^{2+}, Mg^{2+}, and Mn^{2+}. Thermal stability studies showed that glucose oxidation by *Aspergillus fumigatus ASF*4 GOx was reversible ($\Delta S<0$), endothermic ($\Delta H>0$), and non-spontaneous ($\Delta G>0$) at all temperatures tested. Also, This paper presents for the first time, the kinetic properties of a heat-stable glucose oxidase obtained from *Aspergillus fumigatus*, with a high level of activity suitable for industrial production of gluconic acid and hydrogen peroxide. These results would help in determining the heat-stability and possibly economic sustainability of *Aspergillus fumigatus ASF*4 glucose oxidase in the production of gluconic acid, bio-preservation of food, and non-food systems.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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