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Purification, Characterization, Thermostability and Shelf Life Studies of Glucose Oxidase from *Aspergillus niger* PIL7.

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ABSTRACT

Purified glucose oxidase (GOx), from *Aspergillus niger* PIL7 was characterized, kinetics, thermodynamics and shelf life of enzyme was studied. The purified enzyme has 160 kDa molecular weight and monomer in nature. The purified GOx has more affinity towards glucose and less affinity to other glucose analogues. $CuCl_2$ and KCN enhanced the enzyme activity. The metals such as Ag^{+2}, Co^{+2} and Hg^{+2} shown strong inhibition activity on purified GOx. The GOx activity of *A. niger* PIL7 was influenced by the calcium ions.The enzyme displayed a temperature optimum between 30 to 40°C, and an optimum pH range of 5-7.The enzyme has good pH & thermostability. It was stable at 30°C for a minimum of 180 min. Kinetic characteristics of GOx from *A. niger* PIL7 displayed as V_{max} 11.82 U/µg, K_m 89.13 mM. The energy of activation (E_a) calculated from the Arrhenius plot was 3.9075 KJ/mol/K. Thermal inactivation and thermodynamic parameters such as Δ*H* (enthalpy), Δ*G* (free energy) and Δ*S* (entropy) of GOx of *A. niger* PIL7 were calculated from 20-70°C. Free energy values were observed from 98.03 to 109.13 K $J.mol^{-1}$. The purified GOx has half-life of 21.5 days without addition of additives. Thermostability data was used to calculate the shelf life of GOx enzyme.
Keywords: Glucose oxidase, Purification, Thermostability, Shelf life, Enzyme half-life and Enthalpy

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INTRODUCTION

Glucose oxidase (GOx) (β-D-glucose: oxygen-1-oxidoreductase) enzyme which converts the glucose to gluconic acid and hydrogen peroxide (H_2O_2) by utilizing molecular oxygen as an electron acceptor [1]. The oxidation property of GOx has several commercial applications including glucose removal from dried egg, improvement of color, flavor, and shelf life of food materials. GOx uses to remove the oxygen from fruit juices, canned beverages and from mayonnaise to prevent rancidity. In conjunction with catalase it has been using in an automatic glucose assay kit[1]. GOx is the chief component in glucose biosensors to estimation of in glucose in industrial solutions and in body fluids such as blood and urine [2]. GOD is reported to have the best antagonistic effect against different food-borne pathogens such as *Salmonella infantis*, *Staphylococcus aureus*, *Clostridium perfringens*, *Bacillus cereus*, *Campylobacter jejuni* and *Listeria monocytogens*[3]. GOD has also been used as an ingredient of toothpaste [2], for the production of gluconic acid, and as a food preservative [4]. Implantable glucose sensors have found applications for diabetes patients [5]. GOD in new formulations with useful properties for applications in biotechnology continues to be of considerable interest despite the abundant availability of commercial GOD [6].

Stability studies of enzyme helps to better understand the mechanisms of denaturation and stabilization of proteins from a fundamental view point, and also try to improve the stability of an enzyme that is used industrially. Many authors have studied the stabilization of enzymes, in view of mechanism study, either for their more practical application in solution or immobilized. It seems that environmental factors play a principal role in enzyme stability [7-11]. Thermodynamic parameters are also important for inactivation studies of enzymes [9, 10]. Enhancement of the properties of GOx is still receiving attention, presumably due to current and extensive applications of this enzyme.

At present GOx is commercially prepared from two fungal sources: *Aspergillus niger* [12] and Penicillium *amasakiense* [13]. In order to exploit new industrial potentials of glucose oxidase, it is necessary to investigate new microbial strains and to understand the structure stability relationship of this important enzyme. In a previous report, the enhanced production of GOx by *Aspergillus niger* PIL7 in submerged fermentation was described [14]. In this paper, the purification, kinetics, thermodynamics and shelf life of glucose oxidase from *Aspergillus niger* PIL7 are described.

MATERIALS AND METHODS

Microorganism, Production of glucose oxidase and Estimation of glucose oxidase enzyme activity

Potential strain *Aspergillus niger* PIL7, was used for GOx production. The fermentation media composition and estimation of GOx procedures were described in previous paper Jithendar et al [14].

Enzyme purification

All the purification steps were performed at 4°C. The supernatant was used as the cell-free extract. Solid ammonium sulphate was added to the supernatant up to 30% saturation. The precipitate was removed by centrifugation at 10 000 X g for 30 min. Further solid ammonium sulphate was added up to 80% saturation. After stirring for 1 h, the precipitate was collected by centrifugation at 10 000 X g for 30 min, dissolved in 20 mM sodium phosphate, pH 8.0, then dialyzed against the same buffer at 4°C overnight. The dialysed fraction was loaded on a DEAE-cellulose column (2 cm X 25 cm) previously equilibrated with 20 mM sodium phosphate, pH 8.0. Samples recovered from ion exchange chromatography having enzyme activity was allowed to penetrate through sephadex G-150 packed column (2 cm X 25 cm). A total of 60 fractions of 2 mL each were collected at a constant drop rate. GOx eluted as a single peak from gel filtration column, was used for kinetic and thermodynamic characterization.

Molecular-mass determination

The molecular mass of the purified enzyme was determined by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out according to the method of Laemmli [15] using a 10% cross linked polyacrylamide gel. A medium molecular weight marker was used. After electrophoresis, the gel wassilver-stained according to the method proposed by Davis [16].

Determination of Substrate specificity

Substrate specificity was scrutinized by means of the common procedure for determination of enzyme activity in which glucose was replaced by other sugars such as glucose derivatives xylose, mannose etc., Table 2 depicts the various sugars used for test the GOx specificity.

Effect of metal ions on GOx activity

The effects of metal ions on enzyme activity were studied at 1 mM concentration of different metal ions such as cobalt chloride, Copper acetate, Lead acetate, copper sulphate, silver sulphate, silver nitrate, zinc chloride, mercuric chloride and nickel chloride. The 100µl of enzyme with enzyme activity of 1U/ml was incubated along with 0.9 ml of 1 mM metal ions for 20 minutes. After incubation period the enzyme activity was analyzed by common procedure.

Effect of calcium ions on GOx activity

Enzyme (0.1 ml) with the activity of 1 U/ml was incubated with 0.9 ml of 1 mM calcium ions such as calcium chloride, calcium propionate, calcium lactate and calcium carbonate. The enzyme activity was measured after 20 minutes.

Determination of the pH optimum and pH stability

The pH optimum for purified GOx was assayed by analyzing its activity in the pH range of 2-9.pH stability study of the protein was analyzed by pre-incubating 5ml of purified enzyme in 3.5ml of selected pH buffer at 37°C for 0-180 min and subsequent analysis of residual activities under standard assay conditions.

Determination of optimum temperature and thermal stability

To study the temperature optimum of enzyme, the substrate and GOx reaction mixture was incubated at different temperatures ranging from in 20 °C to 70°C in citrate phosphate buffer and measured GOx activity. For determining thermal stability, the enzyme was pre-incubated at different temperatures ranging from 35-65 °C and the residual activity was measured at different time intervals under standard assay conditions.

Estimation of kinetic parameters

Determination of V_{max} and K_m values

The kinetic parameters, V_{max} and $K_{m,}$ of the purified GOx enzyme were determined by measuring enzyme activity at different concentrations of the substrate (100-1000mM) and constant enzyme concentration (20 µg). The kinetic parameters (K_m and V_{max}) values were determined using Michaelis-menton equation through non-linear regression analysis using the program enzyme kinetics module 1.3 in Sigma plot - 12.0.

Determination of activation energy (Ea), enthalpy (ΔH), free energy (ΔG) and Entropy (ΔS)

The activation energy of the purified enzyme was calculated using Arrhenius plot by plotting ln Vvs 1/T (K). The *ΔH* in kJ/mol, *ΔG* in kJ/mol and *ΔS* in J/mol/ K were calculated by using the following equations 1, 2 and 3, respectively.

$$\Delta H = Ea - RT \text{ ------(1)}$$

$$\Delta G = -RT \ln\left(\frac{k_{(a/d)}h}{kB*T}\right) \text{-----(2)}$$

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$$\Delta S = \frac{\Delta H - \Delta G}{T} \text{-------(3)}$$

Where;
Ea = the activation energy,
T (K) = corresponding absolute temperature,
R = the gas constant (8.314 J/mol/ K),
h = the Planck constant (11.04 × 10^{-36} J min),
*k*B = the Boltzmann constant (1.38 × 10^{-23} J/K),
$k_{(a/d)}$ = 'a' for activation and 'd' for deactivation.

First order reaction constant is the slope in the regression line which was obtained by plotting ln *v* versus time at different temperatures (kJ/mol).

Half-life measurement of GOx enzyme

The half- life of the (thermo inactivated) enzyme ($t_{1/2}$ 1/min) was calculated according to Cardoso and Emery [17] using following equation No. 4. The *D* (decimal reduction) value (in h) is defined as the time required to preincubate the enzyme at a given temperature to maintain 10% residual activity and was calculated using the equation No. 5.

$$t_{1/2} = \frac{A - 3.912}{k_d} \text{-----(4)}$$

$$D = \frac{A - 2.3026}{k_d} \text{-----(5)}$$

Where;

A = the intersect of the regression line, 3.912 and 2.3026 are the natural logarithms values of 50 (for $t_{1/2}$ min^{-1}) and 10 (for decimal reduction), respectively, given in the equations (4 and 5).

RESULTS AND DISCUSSION

Purification

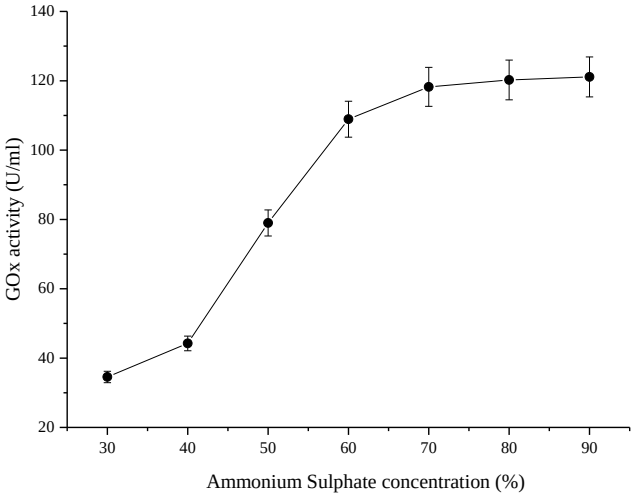

Figure 1:: Precipitation of protein by ammonium sulphate

After fermentation the cell free culture was analyzed for GOx activity and protein content. The obtained enzyme activity and protein content was denoted as a crude enzyme. The crude enzyme was further subjected to purification by precipitation and chromatographic methods. Table 1 shows the various steps in purification along with enzyme activity and protein recovery in each step.The crude enzyme was precipitated using ammonium sulphate fractions from 30–80% (w/v). The higher GOx activity was observed at 70–80% (w/v) of ammonium sulphate fractions. The activity was not observed at30-40% (w/v) salt concentration.Fig 1 shows the ammonium sulphate fractions corresponding to the enzyme activity. The proteins precipitated at 60-80% ammonium sulphate were pooled together and dialyzed. The dialyzed enzyme was further purified by chromatographic techniques.

Purification by chromate graphic techniques

The dialysed enzyme was further purified by ion exchange (DEAE-cellulose) and gel (sephadex G-150) chromate graphic methods. The desalted enzyme was first loaded on the DEAE-cellulose column previously equilibrated with sodium phosphate buffer. The samples eluted from the ion exchange column having GOx activity was loaded on the sephadex column. Results of ion exchange chromatography showed that increase in specific activity from 10.14 to 22.42 Units/mg protein (Table 1). In the final step purification Gel-filtration increased the specific activity from 22.42 to 28.81Units/mg protein indicates the removal of non GOx proteins. However ion exchange chromatography has removed the major no GOx proteins. With the help of sequential steps the GOx was purified 9.5 folds relative to the cure enzyme with a recovery of 27 %. The purity of the enzyme was confirmed by an electrophoretic method. The preparation of the purified enzyme gave a single band with enzyme activity after conventional PAGE.

Table 1: Purification of GOx from *A. niger* PIL7

Step	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg protein)	Yield	Fold purification
Crude enzyme	174000	57600	3.02	100	1
Ammonium Sulphate precipitate	123921	14321	8.65	71.21	2.86
Dialysis	105840	10428	10.14	60.82	3.35
DEAE-Cellulose column	76859	3428	22.42	44.17	7.42
Sephacryl S-200 column	47345	1643	28.81	27.20	9.53

Molecular weight determination by SDS-PAGE

The purity of GOx was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). On SDS-PAGE, the purified GOx showed a single band indicating electrophoretically homogenous (Fig 2). The molecular weight of GOx was found to be 160 kDa by comparing with relative mobility ofthe molecular weight of standard protein marker. The obtained GOx molecular weight is in accordance with standard literature [18]. The molecular weight of GOx is varying along with the microorganism produced [19-20].

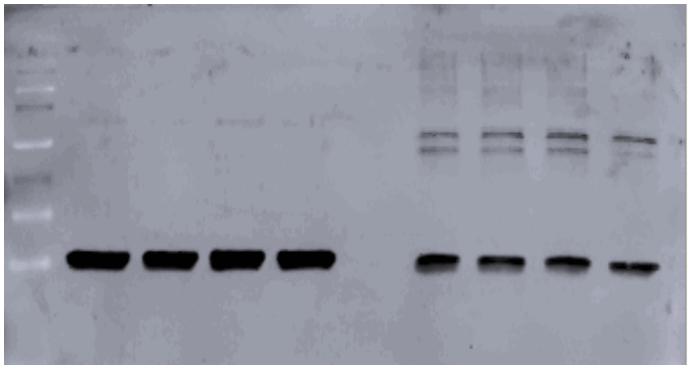

Figure 2: Molecular weight determination on SDS-PAGE of GOx obtained from *A. niger* PIL7

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Substrate specificity

Substrate specificity of GOx plays a vital role using it in biosensors. In the present study the purified enzyme was subjected to hydrolyze the various glucose analogs and other carbohydrates. Table 2 shows the substrates and their relative activity with glucose. The activity of GOx toward a series of sugars such as Mannose, L-sorbose, 2-deoxy-d-glucose, 4-Deoxy-d-glucose, 6-Deoxy-d-glucose, 4-O-methyl-d-glucose, 3,6-methyl-d-glucose were inferior than D-glucose (Table 2) and no activity on other tested sugars and their derivatives. The purified GOx substrate specificity is similar to that of GOx of *P.chrysosporium* [19-21].

Table 2: Substrate specificity of GOx from *A.niger* PIL7.

S.No	Substrate	Relative activity *	S.No	Substrate	Relative activity *
1	D-Glucose	100	13	D-Gulono- γ-lactone	0
2	2-Deoxy-d-glucose	10	14	D-Glucuronolactone	0
3	4-O-methyl-d-glucose	8	15	Mannose	9
4	6-Deoxy-d-glucose	12	16	Altrose	0
5	4-Deoxy-d-glucose	7	17	Galactose	0
6	2-Deoxy-6-fluoro-d-glucose	0	18	Xylose	0
7	3,6-Methyl-d-glucose	10	19	Idose	0
8	4,6-Dimethyl-d-glucose	0	20	L-Sorbose	15
9	3-Deoxy-d-glucose	0	21	Cellobiose	0
10	6-O-methyl-d-glucose	0	22	D-kabinose	0
11	D-Glucono-δ-lactone	0	23	L- Arabinose	0
12	L-Gulono- γ-lactone	0	24	D-Fructose	0

Effect of activity enhancers on GOx activity

From Fig 3 it was observed that among studied compounds $CuCl_2$ shown the marginal enhancement of GOx activity followed by KCN and NaF. It indicates the divalent ions has a significant enzyme activity. With the addition of $CuCl_2$ 26 % enzyme activity was enhanced compared with the control.

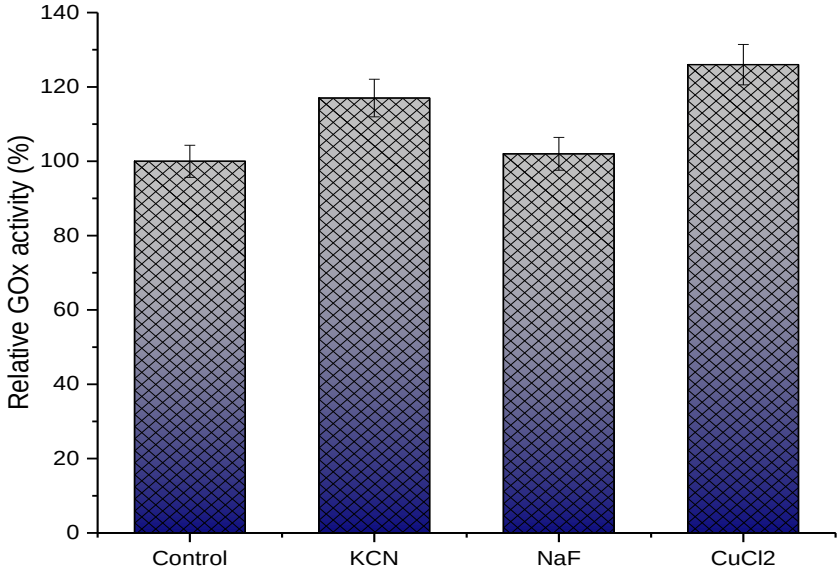

Figure 3: Effect of enzyme activity enhancers on GOx from *A. niger*PIL7

Effect of metal ions on GOx activity

Fig 4 depicts the effect of metal ions on the GOx activity. From this figure it was noticed that Ag^{+2}, Co^{+2} and Hg^{+2} has remarkably inhibition activity on purified GOx. Ni^{+2} and Zn^{+2} also has good inhibition activity on the enzyme activity. Pb^{+2} has moderate inhibition activity. The copper sulphate mixed enzyme showed 63 %

activity however the copper acetate added GOx showed 18 % activity compared with untreated enzyme, indicates the level of inhibition by Cu^{+2} is depends on the conjugated anion. Literature reports depicts that the reaction catalyzed by GOx from *A niger* was markedly inhibited by Ag^{++}, Hg++ and Cu^{++} [19-21]. The inhibition sequence was in the order: silver Nitrate>silver sulphate> cobalt chloride> mercuric chloride> copper acetate>nickel chloride> zinc Chloride> copper sulphate> lead acetate at same concentration.

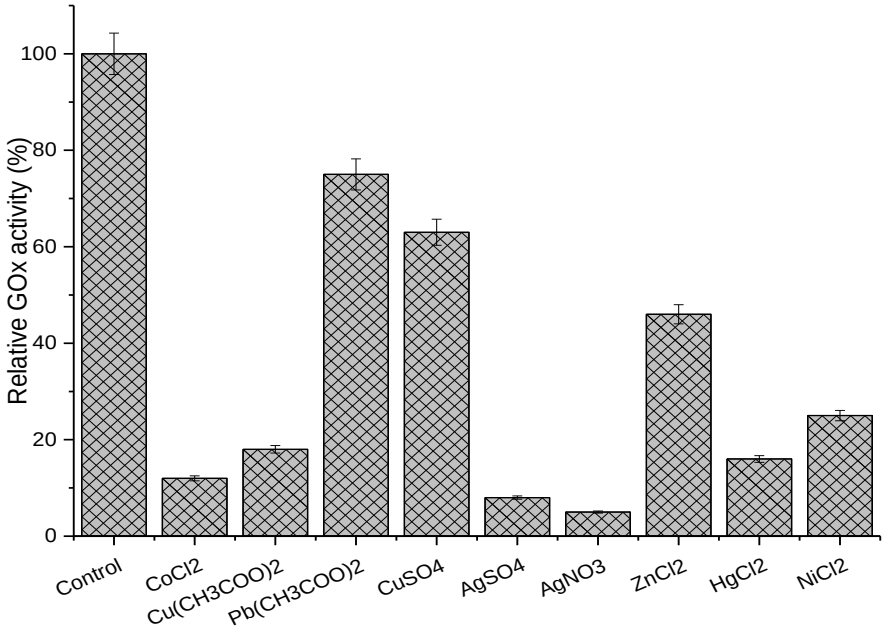

Figure 4: Effect of various metal ions on activity of GOx from *A. niger*PIL7

The reason for the activation and inhibitions is that lower concentrations of metal ions can stabilize the conformation of GOx and cause change in conformation to a more active form, so GOx is activated by metal ions. GOx having interactions of the FAD molecule at the active site of GOx are 23 potential hydrogen bonds mostly involving the ribose and pyrophosphate groups. At higher metal ion concentration, the great number of ions will compete with FAD for the binding sites on the pyrophosphate or ribose groups, which causes the interactions of the hydrogen bonds between the FAD molecule and pyrophosphate or ribose groups to become weakened. They will also compete with the substrate for the binding sites on the enzyme, so GOx activity was partially inhibited. Divalent metal ions bind to pyrophosphate and ribose more strongly than monovalent ions, so the inhibition of divalent ions is stronger than that of monovalent ions.

Effect of calcium ions on GOx activity

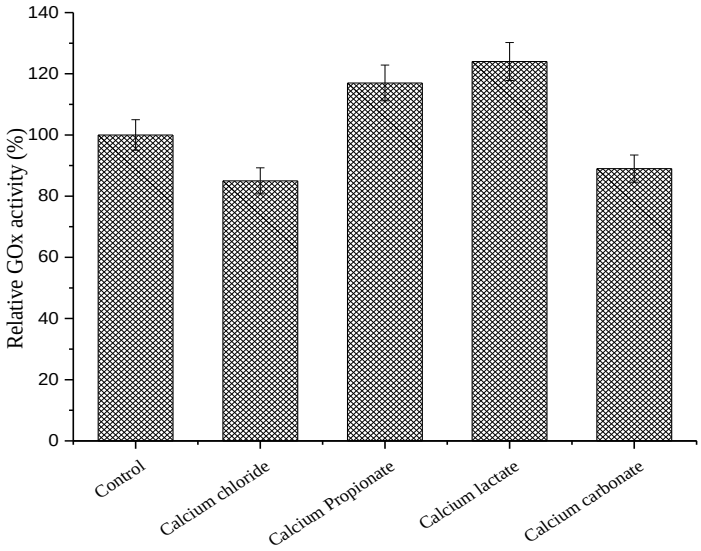

Fig 5: Effect of calcium salts on activity of GOx from *A. niger* PIL7

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Addition of calcium ions has changed the GOx activity. The shift of enzyme activity is depends on the conjugated anion. It was found that relative activity of calcium lactate was more i.e., 124 % when compared to control. Calcium lactate &calcium propionate enhanced the enzyme activity however calcium carbonate and calcium chloride shows a mild inhibition on purified GOx (Fig 5).

Effect of pH on GOx activity and pH stability

The highest enzyme activity was observed at pH 6 indicates this pH is optimum for purified GOx. Above and below the optimum pH decreased activity was observed. Neutral and alkaline pH is not favorable for isolated GOx (Fig 6). At pH 7 it has 51 % relative activity. Compared to the neural pH at low pH the loss of enzyme activity is moderate at pH 5 the enzyme retain the 85 % of enzyme activity.

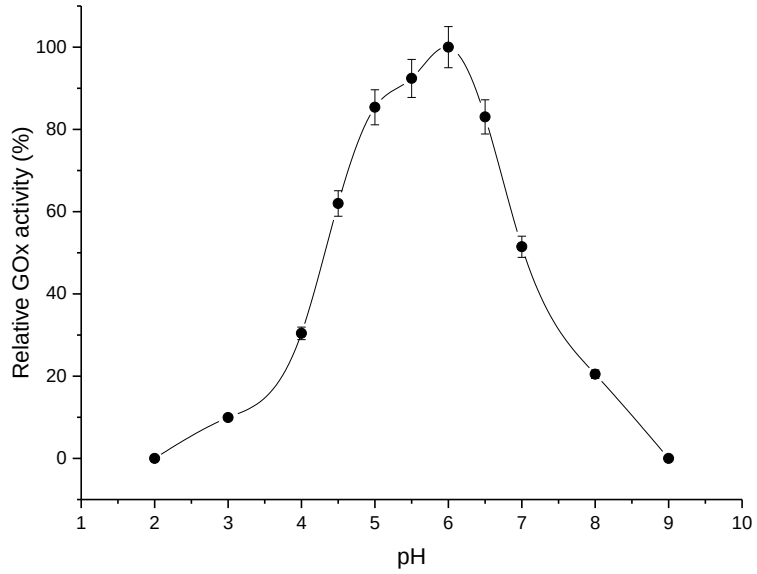

Figure 6: Effect of pH on activity of GOx from *A. niger*PIL7

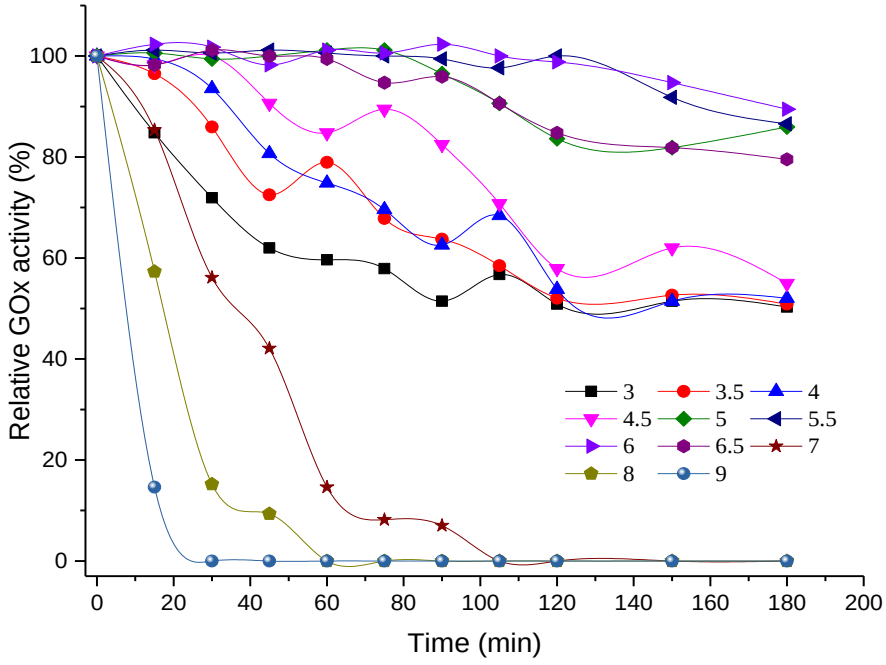

Figure 7: pH stability of GOxfrom *A. niger*PIL7at various time intervals

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The incubated pH and time has shown the influence on the stability of GOx activity. At pH 6 the purified enzymehas shown upright stability,it lost only 11 % activity at 180 min incubation time (Fig 7). At low pH the enzyme has a good stability. pH 5 & 5.5 the enzyme has 86 % and 87 % relative activity after incubation at 180 min. The stability study results indicates that the structural changes of the enzyme at low pH is minimal and the changes are reversal at optimum pH. Adams [22] and Bentley [23] reported that *A. niger* were reported to have pH optima of 5.5 and 5.6, respectively.

Effect of temperature on GOx activity and thermo stability

Enzymes are known to be sensitive to changes in temperature. The relationship between reaction rate of an enzyme and temperature is exponential. For every 10 °C rise in temperature, the rate of an enzyme reaction doubles. Enzymes are known to display maximal activity at a temperature known as the optimum temperature of the enzyme [24].

The optimum temperature of purified GOx was found at 30 ºC and about 90 % of relative activity was observed at 20^{o}C. The obtained GOx has a broad range of thermo stability, at 55 ^{o}C it has 90% relative activity. Above 55^{o}C incubation the enzyme was denaturized fast. Gouda et al. [25] reported that the dissociation of FDA from free holoenzyme in aqueous medium was at 59ºC and concluded that dissociation of FDA from the holoenzyme was responsible for the thermal inactivation of GOx. Ozyilmaz et al [26] also observed that, temperature affected the activity of the GOx sharply and maximum activity at 35ºC. At 60ºC the relative activity of GOx were found as 33% (Fig 8).

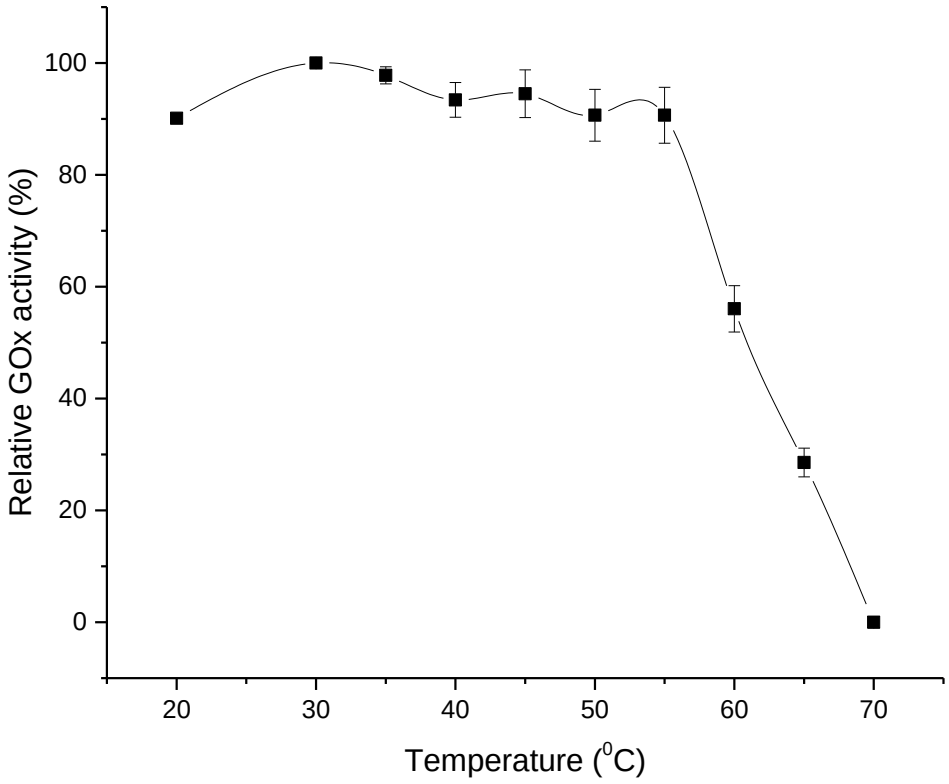

Figure 8: Effect of temperature on the activity of GOx from *A.niger*PIL7

At temperature range between 40°C and 70°C most enzymes get denaturized and lost its activity. The thermo stability studies shows that GOx obtained from *A.niger*PIL7 has a good stability at higher temperatures for a long incubation times. At optimum temperature 180 min incubation the enzyme shown 100 % relative activity (Fig. 9). After 180 min incubation at 55^{o}C the purified GOx retain 57 % activity. The half-life of enzyme at 55^{o}C& 60^{o}Cis 534 and 83 min respectively. Indicates that the enzyme has good thermo stability at higher temperatures. However, at 65°C it was less stable and displayed a half-life of 27 min. For shelf-life studies the lyophilized GOx stored at -20 ^{o}C for 6 months without addition of any stabilizing agents. After 6 months of

incubation the enzyme remained 100 % activity. The shelf-life of the purified GOX without the addition of potentially costly stabilizers is thus an attractive feature of this enzyme for commercial applications.

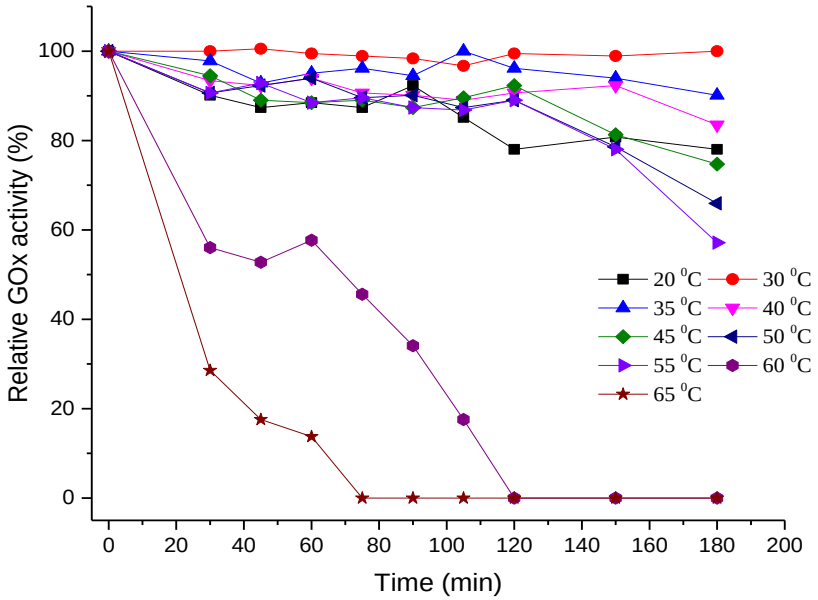

Figure 9: Thermo stability of GOx at various temperatures and time intervals

The lowest optimum temperature for GOD is reported to be 25–30 °C from *P. funiculosum* 433 [27] and the highest of 40–60 °Cfrom *A. niger* and *P. amagasakiense*ATCC 28686 [28,29].

Kinetic characterization

The Line weaver's Burk plot was used to determine the maximal limiting rate velocity (V_{max}) and Michaelis constant (K_m) values. The GOx from *A. niger*PIL7 displayed the kinetic characteristics with *Vmax* 11.82 U/µg, K_m 89.13 mM. The obtained kinetic characteristics are accordance with the literature reports. The K_m value of the GOx from *T. favus*is 10.9 mM[30] and is 33 mM from *A. niger*[31].*P. amagasakiense* ATCC 28686 and *P. funiculosum*433 shows lower K_m value of 5.7 mM and 3.3 mM respectively [27,32].*A. niger* shows V_{max} value of 458 U/mg while *P.amagasakiense* ATCC 28686 exhibited 925 U/mg [29,32].

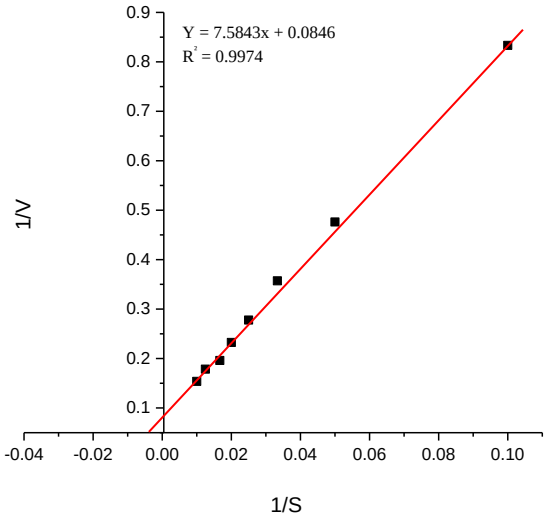

Figure 10: Kinetic parameters for purified GOx of *A. niger*PIL7 by Lineweaver's Burk plot

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Determination of activation energy (Ea), enthalpy (ΔH), free energy (ΔG) and Entropy (ΔS)

The energy of activation (Ea) was calculated by using the Arrhenius plot by taking the value of Ln *v* against the reciprocal of absolute temperature (Fig. 11). As seen, the temperature dependence of the inactivation constant followed the Arrhenius equation. The E_a calculated from the slope of Arrhenius plot was 3.9075 KJ/mol/K.

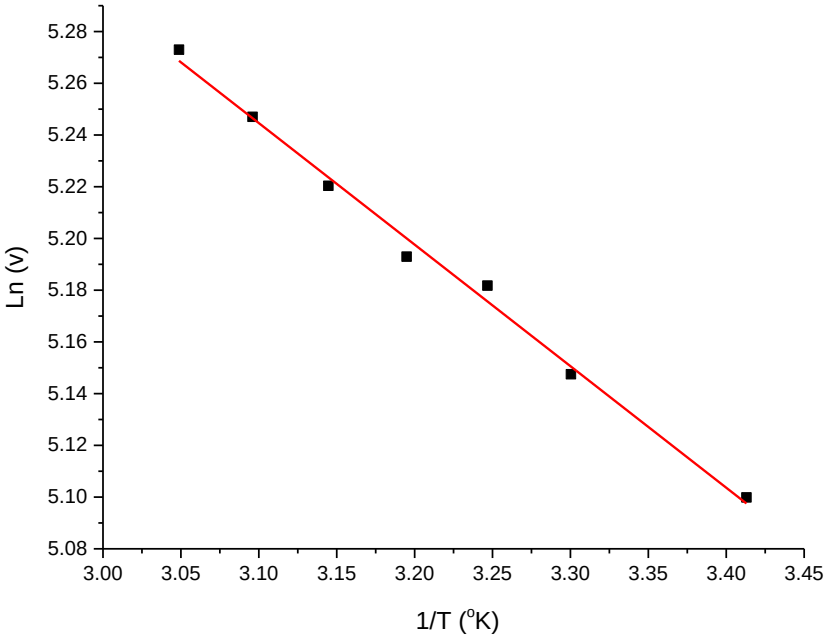

Figure 12: Arrhenius plot of purified GOx obtained from *A. niger*PIL7

Since the enzyme activity decreased beyond 55°C, the thermal inactivation studies were carried from 55-70 °C by plotting the relative activity in natural logarithm (ln *v*) versus the pre-incubation time after fitting the data in linear regression (Fig. 12).

The thermal inactivation thermodynamic parameters of GOx: Δ*H* (enthalpy), Δ*G* (free energy) and Δ*S* (entropy) were calculated from20-70 °C. Enthalpy values were changed from 20-70 °C the change of enthalpy is not rapid at studied temperatures indicates that at all enzyme was stable in its structure as there is a minor change in enzyme heat capacity. Free energyvalues were observed from 98.03 to 109.13 K J.mol^{-1}in the studied temperatures (Table 3). Highest free energy was observed at optimum temperature, up to the 55°C the change in the energy was minimal. After 55°C the free energy was steadily decreased. Entropy (*ΔS*) value was highest at 30°C (-355.58) afteroptimum temperature rise of temperature decrease in entropy was observed.

Table 3: Deactivation kinetics parameters enthalpy (ΔH), free energy (ΔG), Entropy (ΔS) and half-life $t_{1/2}$ of the purified GOx obtained from *A. niger*PIL7

Temperature (°C)	K	ΔG (KJ/mol)	ΔH	ΔS	$t_{1/2}$ (min)	$t_{1/2}$ (hrs)
20	293	98.03484	1.471498	-329.568	1778.458	29.64
30	303	109.1315	1.388358	-355.588	30970.770	516.17
35	308	106.3995	1.346788	-341.080	4708.868	78.48
40	313	106.4202	1.305218	-335.831	2185.546	36.42
45	318	106.5610	1.263648	-331.124	1109.005	18.48
50	323	107.2255	1.222078	-328.184	712.863	11.88
55	328	108.3062	1.180508	-326.603	533.437	8.89
60	333	105.0899	1.138938	-312.165	83.009	1.38
65	338	104.1383	1.097368	-304.855	27.446	0.45

Table 3 depicts the half-life of GOx enzyme at studied temperatures. It was noticed that the obtained GOx from *A. niger*PIL7 has half-life of 516.17 hrs (21.5 days) which is very high. While increase or decrease temperature than optimum temperature, reduction in half-life was noticed. The higher $t_{1/2}$ suggests the thermo stable nature of enzyme.

Shelf life analysis

The thermostability data was used to calculate the shelf life of GOx enzyme. These calculations were made with the help of shelf life analysis module in Sigma plot 12.0 software. Fig 13 shows the shelf life of purified GOx from *A. niger*PIL7. Based on the software predictions the purified GOx from *A. niger*PIL7lost its 10 % activity and retains the 90 % (t_{90}) at 2797.49 min.

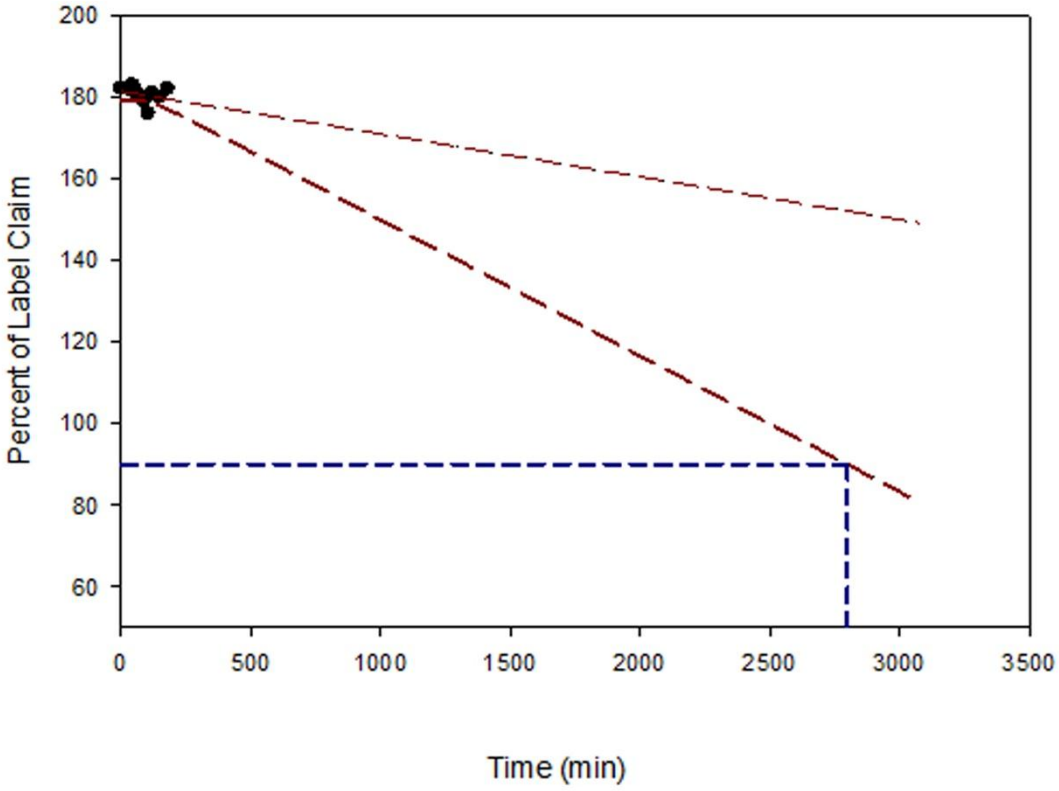

Figure 13: Shelf life determination of purified GOx from *A. niger*PIL7

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