Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Pelagia Research Library

Advances in Applied Science Research, 2013, 4(3):250-257

Pelagia Research Library

ISSN: 0976-8610
CODEN (USA): AASRFC

Glucose oxidase from *Aspergillus niger*: Production, characterization and immobilization for glucose oxidation

Jagdish Singh* and Neelam Verma

Department of Biotechnology, Punjabi University, Patiala, Punjab, India

---

ABSTRACT

Glucose oxidase (GOx), a flavoenzyme, from Aspergillus niger was produced, purified and immobilized for glucose oxidation. Maximum activities of 0.6 and 0.31U mg^{-1} fungus dry weight were obtained as intracellular and extracellular respectively. Optimal production (4000 IU/L) was achieved when basal salt medium was supplemented with sucrose (7.5%), peptone (1.5%) phosphorus (0.2%) and $MgSO_4$ (0.2%) at pH 5.7 after 48 h at 250 rev. min^{-1}. Enzyme purified by ammonium sulphate precipitation (75%), gel filtration, Q-Sepharose and DEAE Sepharoses has 30.08, 63.3% and 22.3 fold purification, % recovery and specific activity respectively. Enzyme was dimeric consisting of two equal subunits with molecular weight of 80 kDa. and displayed temperature and pH optima 25-30°C and 5.5-6.0 respectively for the oxidation of-D-glucose. The enzyme was stable at 50 °C for 1 h without any prior stabilisation. V_{max}, and K_m value 17 Umg^{-1} and K_m, 7.1mM respectively. GOx was inhibited by Cu^{2+} (56.5%), and Ag^{2+} (48%) appreciably and to a lower extent by NaF (25%). Covalent immobilization of enzyme with oxirane beads was less effective and enzyme lost activity, but entrapment methods of sodium alginate and poly acrylamide was effective for the oxidation of glucose.

Keywords*:* GOx, *A. niger*, Production, Purification

INTRODUCTION

GOx (EC 1.1.3.4, β-D- glucose oxygen 1- oxidoreductase) is a flavoprotein which catalyses the oxidation of, β-D-glucose by molecular oxygen to D- glucolactone and H_2O_2. It removes hydrogen from glucose and gets reduced, the reduced form of the GOx is then reoxidised by molecular oxygen, and the produced hydrogen peroxide is decomposed by catalase to water and oxygen. The D-glucolactone hydrolyses spontaneously to gluconic acid [1,2,3,4].

β-D-Glucose + Enzyme-FAD →Enzyme-$FADH_2$+ D-glucono-1, 5-lactone

Enzyme-$FADH_2$ + O_2 → Enzyme-FAD+ H_2O_2

GOx is a commercially important and has application as a biosensor for determination of glucose [5,6,7], removal of glucose and/or oxygen and biofuel cells [8,9,10]. The mycelial fungi *Aspergillus* and *Penicillium* serve as industrial producers of GOx. Production and enhancement of the properties of GOx is still receiving attention, presumably due to current and extensive applications of this enzyme. In order to exploit new sources for industrial potentials of GOx production, it is necessary to investigate new microbial strains and to understand the structure-stability relationship of this important enzyme. This study describes the production, purification, characterisation and immobilization of a novel GOx from *A. niger*.

MATERIALS AND METHODS

Organism and maintenance

The fungus *A.niger* (MTCC 181) used in study was purchased from the Microbial type culture collection IMTech Chandigarh, Punjab (India). The microorganism was maintained on potato dextrose agar at 4-6^{0}C and sub cultured after every 20 days.

Pre culture of *A. niger*

Spores of fungus *A. niger* (7.5□10^5/ml) were grown in 250 ml Erlenmeyer flask containing 50 ml of medium the composition was (g/l): (NH4)2HPO4, 0.4; KH2PO4, 0.2;MgSO4, 0.2; peptone, 10; sucrose, 70 and pH 5.5. Culture was incubated for 24 h in rotary shaker at 200 rev. min^{-1} at 30^{0}C for germination of spore.

Optimization of culture conditions for maximum GOx production

For the optimization of GOx production basal salt medium (BSM) with slight modification [11] containing (g/l): $(NH_4)_2HPO_4$ 0.4, KH_2PO_4 0.2, $NaNO_3$ 2.0, KCl 0.5, $MgSO_4$ 0.2 and pH 5.5, was used for the optimization process parameters for maximum GOx production. All the growth experiments were carried out in 250 ml Erlenmeyer flask containing 50 ml of the medium. The sterilized medium (15 min at 15 psi/cm^2) was inoculated with the germinated pre culture spores of 24 h age and incubated in orbital shaker (250 rev. min^{-1}) at 30^{0}C. Different physical and chemical process parameters for regulation the enzyme production was optimized by classical methods of medium optimization. The mycelia mass was harvested by centrifugation at 7000x *g* for 20 min in a centrifuge fitted with fixed angle rotor. The supernatant constituted the extracellular GOx fraction and for the intracellular fraction 50 gm mycelial biomass (wet weight) was suspended in 10 ml sodium citrate buffer pH 5.75(50mM). Liquid nitrogen was added on the fungal biomass and after evaporation of nitrogen, biomass was crushed to powder in buffer. The clear supernatant of the broken cell suspension constituted the intracellular GOx fraction.

Enzyme and protein assays

GOx activity was determined spectrophotometrically by Ciucu and Petroescu [12], as modified by Markwell *et al*. **[13] method** by the reduction of benzoquinone to hydroquinone. The hydroquinone estimation was carried out at 290 nm (ε=2.31mM^{-1}cm^{-1}) on UV-VIS spectrophotometer. One unit (IU) of GOx activity was defined as amount of enzyme, which reduces 1.0µM of benzoquinone ml^{-1} min^{-1}. Protein concentration was determined using absorption method [14,15].

$$\text{Protein (mg/ml)} = 1.55\ A_{280} - 0.76\ A_{260}$$

Purification of GOx

The enzyme fraction was centrifuged at 9000x*g* for 10 min and protein was subsequently precipitated with ammonium sulphate (75% fractionation) at 4 ^{0}C. Protein precipitate was centrifuged for 45 min (10,000 xg at 4 ^{0}C) and pellet was suspended in 10 ml buffer and Gel filtration (Sephadex G-100) was carried for salting out and extraction of GOx on the basis of molecular mass. Fraction with maximum enzyme activity was pooled and further purified with Q-Sepharose (strong anion exchanger) and DEAE–Sepharose (weak anion exchanger) by ion exchange chromatography. Bound protein was eluted with a 500 ml linear gradient from 50 to 500 mM NaCl in 20mM Tris buffer (pH 7.2) at a flow rate of 2 ml min^{-1}, with collection of 05 ml fractions. Protein and GOx activity determined for each fraction with the above mentioned assay method.

Characterization of GOx

Molecular weight determination

GOx molecular weight was determined by SDS–PAGE with 12% acrylamide gels using a Mini-Gel electrophoresis cell (Genei). Purified GOx was loaded and the proteins were visualized with Coomassie Brilliant Blue R-250. The approximate subunit molecular weight was calculated

Optimum temperature and pH

GOx assay buffer was equilibrated for 10 min at the different temperatures of interest (15 to 40°C) in the temperature-controlled water bath, before initiating the reactions with the addition of the GOx (0.15Uml^{-1}). The pH profile for GOx was performed in different buffer systems containing 50 mM conc. adjusted to pH values of interest ranging from 4.0-8.5.

Thermal Stability testing

The stability of purified GOx was determined at 30-60 °C, since these temperatures correlated to potential applications of enzyme. Purified GOx at a concentration of 1.25 $IUml^{-1}$ was prepared and incubated in water bath pre equilibrated at different temperature range, 30-60 °C. Samples (100µl) were removed after different time intervals for GOx activity analysis and compared to an initial sample taken at the onset of the experiment.

Substrate specificity and kinetic constants determination

The substrate specificity of purified GOx was tested on D-glucose, maltose, fructose, galactose and arabinose at different conc. of 1-40 mmol l^{-1} for each sugar. The enzyme assay was performed under conditions of optimum pH and temperature. A Lineweaver-Burke plot was plotted between 1/(v) (enzyme activity) *Vs* 1/(S) (substrate conc.) and from the plot K_m and V_{max} were calculated. K_m value is ½ of the substrate concentration at which enzyme show its maximum activity.

Immobilization of purified enzyme

The covalent immobilization of GOx was carried using Eupergit C via the oxirane reactive groups of the polymer [16]. Enzyme becomes inactivated in covalent immobilization process so entrapment methods of sodium alginate [17] and polyacrylmide method [18] with modifications was used for the entrapment of GOx. After the immobilization, parameters like optimum pH, temperature, kinetic characterization and thermal stability were studied for the glucose oxidation.

RESULTS AND DISCUSSION

Pre culture of germinated spores of *A. niger* after 24 h (7 x 10^5 spores/ml) was inoculated in BSM at various rates (5, 10, 15, and 20%) and maximum GOx production (240 IU/L) with spores of 24 h age was achieved as compared to 30 h when inoculums size was varied from 5-15% (Fig. 1a). Different carbon sources glucose, fructose, galactose, lactose, sucrose and starch was used to observe their effect on GOx production and growth of *A. niger*. Microorganism was able to grow on every carbon source but maximum GOx activity was with sucrose. Different concentrations of sucrose (4.0-10.0%) were supplemented in BSM. GOx production enhanced as concentration of sucrose was increased from 4.0-7.5% (w/v), but above that no relevant increase in GOx production was observed. There was maximum production at 7.5% sucrose concentration. (Table.1) Zetelaky and Vas[19] used 5-7% sucrose concentration for GOx production which was less as compared to the optimum concentration. Hatzinikolaou and Macris [20] used 8% of sucrose for GOx production, which was more as compared to results mentioned above (7.5%) Peptone, yeast extract and beef extract were the best nitrogen sources, but peptone as a nitrogen source was used previously for the production of GOx [21,20, 22], so different concentration of peptone was used to optimize best peptone concentration. There was increase in GOx and FDW production with increased concentration of peptone from 0.5 to 2.0%, having maximum production (3200 IU/L) at 2.0% concentration (Fig.1b). This concentration was less as compared to the concentration used for the production of GOx [23] which was 3%, and more as compared to the concentration used by Hatzinikolaou and Macris, [20] i.e. 1% for GOx production from *A. niger*. $(NH_4)_2HPO_4$ was the best phosphorus source as compared to $(NH_4)_2H_2PO_4$ and KH_2PO_4, There was increase in GOx production when $(NH_4)_2HPO_4$ concentration was increased from 0.1-0.2% and above that enzyme production was constant (Fig.1b). $CaCO_3$ enhanced GOx production at the concentration of 0.5-2.0% conc (4000 IU/L), but above this concentration there was decrease in biomass and GOx production (Fig 2a). Increase was due to direct effect of solid salt phase on the mechanism of enzyme action [20]. Various pH levels (4.5, 5.0, 5.5, 5.75, 6.0, 6.5 and 7.0) effected enzyme production and optimum production was in pH range 5.5-6.5 having optima pH 5.75(Fig. 2b) that is comparable to pH used for GOx production [23,24,25]. Different temperatures (25, 30, 35 and 40 ^{0}C) effected GOx production with optimum production was at 30 ^{0}C (Fig. 2c) which is relevant to growth temperature used for the GOx production. [13,26]. Different agitation rate (50, 100, 200 and 250 rev. min^{-1}) was applied for the GOx production and there was maximum GOx production at 250 rev. min^{-1} .There was maximum GOx (4000 IU/L) production after 48 h of fermentation and remains constant up to 60 h followed by decline in enzyme production (Fig. 2d).

Purification and molecular weight of GOx

GOx from *A.niger* was purified by four steps: ammonium sulphate precipitation (75%) and desalted using gel filtration column. After gel filtration with Q-Sepharose and DEAE Sepharose there was 30.08 fold purification

63.3% recovery and 22.3 specific activity (Table 2). The purity of the enzyme after DEAE cellulose chromatography was more than 30 fold, when compared to the crude extract of enzyme supernatant. Purified GOx to homogeneity was confirmed by SDS-PAGE. Molecular weight of native GOx from *A .niger* was approximately 160 kDa while denaturing SDS–PAGE indicated a two band with a molecular weight of approximately 80kDa, indicating that the GOx was likely a homo-dimeric protein consisting of two equal subunits.

Optimum temperature and pH GOx activity

The GOx from *A. niger* was optimally active at 25 °C and exhibited more than 90% of the maximum activity between 20-35 °C, but above 45°C activity decreased rapidly (Fig. 3a). The GOx maintained high activity of 90% at 37°C, when compared to optimal activity of this enzyme between 25 and 30°C. This is indicative that enzyme is suitable for operation at this temperature after stabilisation. GOx was optimally active at pH 5 and displayed a narrow pH profile between pH 5.5-6.0. Below pH 5.0 activity decreased sharply, maintaining only 20% activity at pH 4 (Fig. 3b).

Temperature Stability of purified enzyme

The residual activity of purified GOx remained relatively unchanged over 10 h at 25 °C, whereas exhibiting a half life of approximately 30 min at 50 °C. The enzyme was stable up to 40 ^{0}C and stability decreased at higher temperatures. Where as GOx from *P. ostreatus* has stability at 70 ^{0}C for 120 min [27].The shelf-life of the purified GOx without the addition of costly stabilisers was an attractive feature for business related applications of enzyme

Substrate specificity and kinetic characterization:

The activity of purified GOx enzyme was tested on D-glucose, maltose, fructose, galactose and arabinose and enzyme was highly specific for D-glucose. The other sugars were oxidized at lower rate (maltose and fructose) or not (galactose and arabinose) (Table 3). Similar results have been reported for GOx production from *A. niger*[28] and *P. ostreatus* [27]. Relationship between the enzyme activity and substrate concentration is of Michaelis-Menten type. K_m and V_{max} determined from the Lineweaver-Burk plot were 7.1 mM and 1.7U, respectively (Table3). K_m values for glucose (7.1mM) was lower than that for the other sugars, according to the literature value for other fungal GOx (9.6-110 mM) [29,30,31,32,33]. The turnover number (*k*cat) of the GOx was determined to be 641 s^{-1}. The specificity constant (*k*cat/*K*m) for GOx was determined to be 90 $s^{-1}mM^{-1}$.

Effect of enzyme inhibitors on the activity of purified

Percentage inhibition of purified GOx was 56.5 and 48% by Cu^{2+} and Ag^{2+} respectively as was reported for the enzyme from *Aspergillus* and *Penicillium spp*. [34,35](Fig. 4). The inhibition of GOx by Ag^{2+} ions is due to reaction of Ag^{2+} with thiol group of enzyme essential for enzymatic activity which is close to FAD binding region of protein [34]. GOx from *P. chrysosporium* was also inhibited by Ag^{2+}, similar to the GOx from *A. niger* [35].

Immobilization of purified GOx

Purified enzyme was immobilized with covalent and gel entrapment methods. Former was carried out with the oxirane beads, while latter was carried in sodium alginate and polyacrylamide gel. Covalent immobilization very less effective for glucose removal as compare to the entrapment methods. Immobilized enzyme by entrapment methods was stable up to 50-55 ^{0}C for an hrs and above this temperature there was fall in enzyme activity. Thermal stability of enzyme immobilized in polyacrylamide gel was more as compared to free and sodium alginate immobilized system. Glucose oxidation rate was 600 and 500mg/h by acrylamide and sodium alginate immobilized enzyme (20 IU) at optimum pH and temperature 6 and 30^{0}C respectively. For the kinetic characterization of immobilized enzyme, various concentrations of glucose (1-40 mM) were used kinetic constants K_m and V_{max} was calculated with Linewever-Burk plot. K_m of immobilized enzyme was 12.0 and 8.0 mM was respectively for the sodium alginate and acryl amide (Table 4).

Table:1. Effect of different sucrose and $(NH_4)_2HPO_4$ conc. on GOx production and growth of *A. niger*

Sucrose Conc. (%)	Enzyme activity (IU/L)	FDW (g/L)	$(NH_4)_2HPO_4$ Conc. (%)	Enzyme activity (IU/L)	FDW (g/L)
4.0	1290	3.5	0.1	3280	4.2
5.0	1500	3.6	0.2	3310	**4.2**
6.0	1750	3.7	0.3	3310	**4.2**
7.0	2400	4.0	0.4	3312	**4.2**
7.5	2600	4.1	0.5	3310	**4.2**
8.0	2640	4.1	0.6	3300	**4.2**

Table: 2. Purification of GOx from *A. niger*

Methods	Units (IU)	Protein (mg)	Specific activity (IU/mg of protein)	Purification fold	Recovery (%)
Culture filtrate	18	33	0.73	----	100
Ammonium sulphate	17	22.0	0.75	0.1	95
Gel Permeation chromatography	14.4	2.38	6.05	8.6	84.6
Q-Sepharose strong anion exchange chromatography	12.2	0.78	15.6	22.2	71.6
DEAE-Sepharose as weak anion exchange chromatography	11.4	0.51	22.3	30.08	63.3

Table: 3. Substrate specificity of purified GOx from *A. niger*

Substrate	Vmax(IU/ml)	Km(mM)	Relative enzyme activity (%)
Glucose	0.7 ± 0.1	7.1 ± 0.5	100
Maltose	0.11 ± 0.03	32	15.7
Fructose	0.09 ± 0.05	35	12.8
Galactose	--	--	--
Arabinose	--	--	--

Table:4. Comparison of the optimum parameters of free and immobilized enzyme GOx for glucose oxidation

Parameters	Free System	Immobilized enzyme	
		Sodium alginate	Acrylamide system
pH	5.75	6.0	6.0
Temp. (^{0}C)	30	30	30
Thermal stability (^{0}C)	50	50	55
K_m (mM)	7.1	12.5	8.4
V_{max} (U/ml)	1.7	1.8	1.7

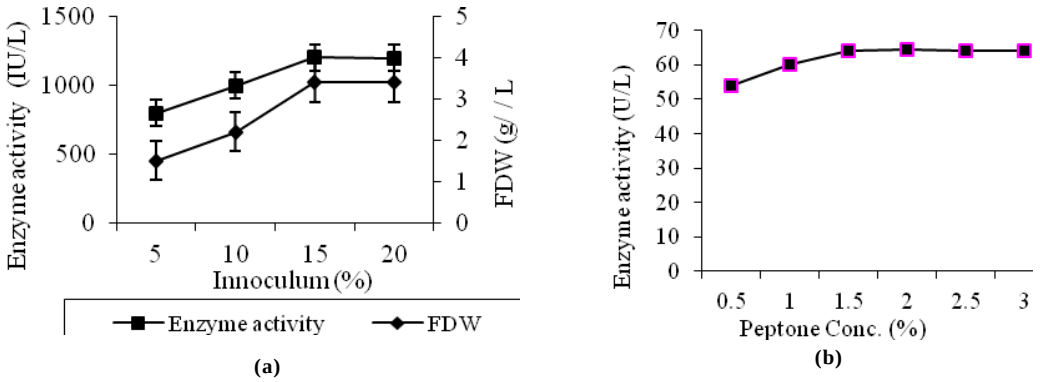

Fig. 1. Effect of different % of inoculum (a) and peptone (b) Conc. on GOx production from *A. niger*

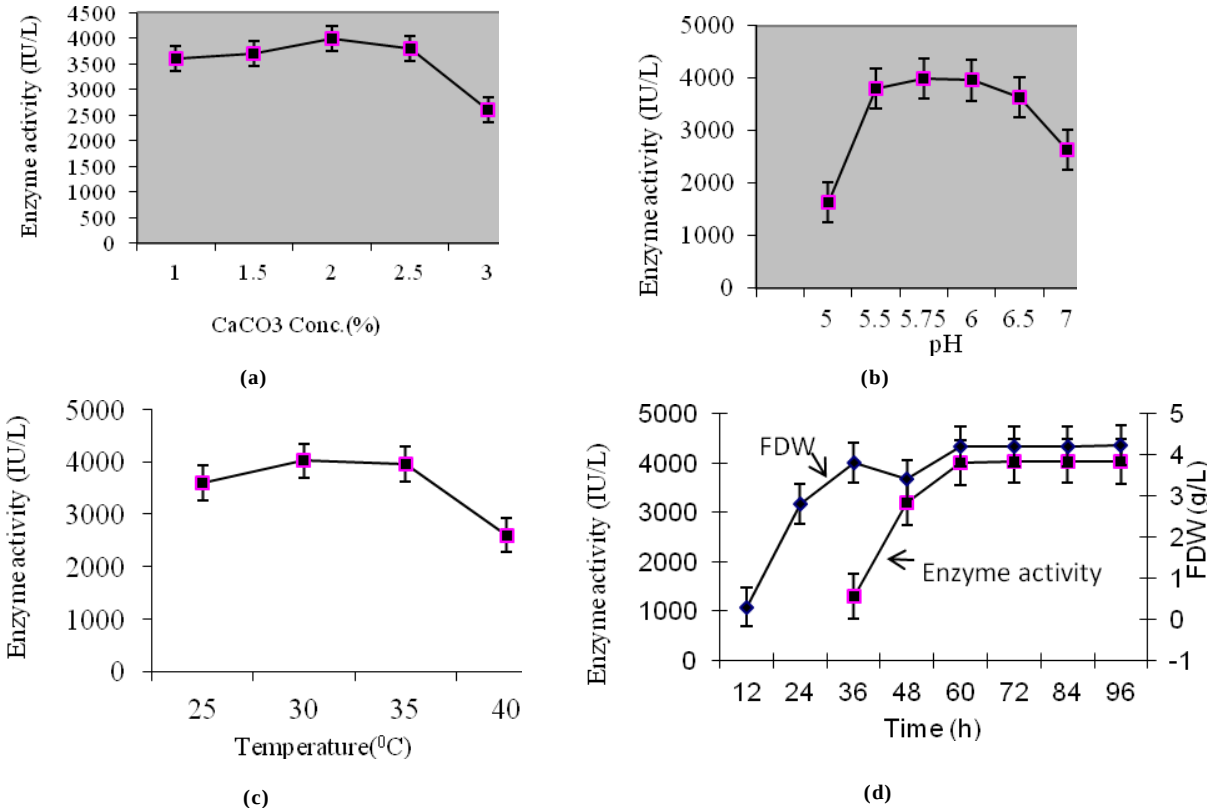

Fig. 2. Effect of (a)pH (b) $CaCO_3$ (c) temperature) and (d) time on GOx production from *A. niger*

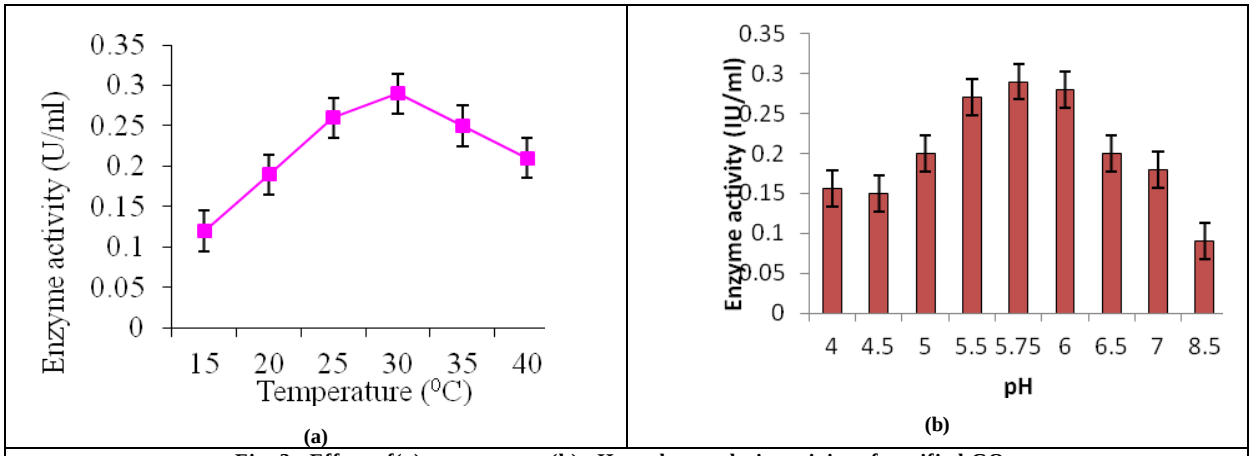

Fig. 3. Effect of(a) temperature(b) pH on the catalytic activity of purified GOx

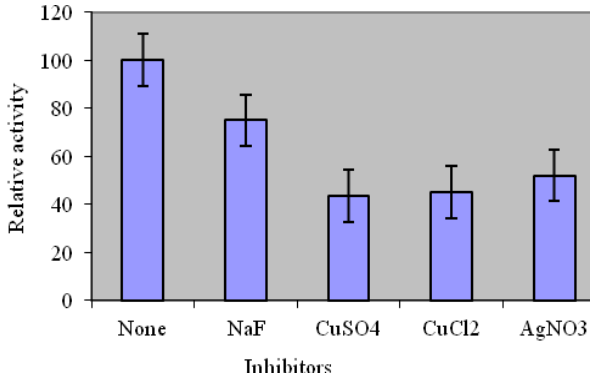

Fig. 4 Effect of ions on the GOx activity from *A. niger*

CONCLUSION

Optimal production of the GOx was achieved when basal salt medium was supplemented with sucrose, peptone phosphorus, and $MgSO_4$ at pH 5.7. Purified enzyme was dimeric consisting of two equal subunits having thermal stability at 50 °C for a maximum of 1 h. V_{max}, and K_m value was compatible to literature. Cu^{2+} and Ag^{2+} inhibited the enzyme activity appreciably. In comparative study for different immobilization methods on the performance of glucose oxidation ccovalent immobilization with Oxirane beads was less effective and enzyme lost activity in comparison to sodium alginate and poly acryl amides methods.

REFERENCES

[1] Gibson, Q.H., Swoboda, B. E. P. and Massey, V, *Biol.Chem.,* . **1964**, 239, 3927.
[2] Duke, F.R., Weibel, M., Phage, D.S., Bulgrin, V.C. and Luthy, J, *J. Amer. Chem. Soci.*,**1969**, 91,3904.
[3] Barker, S.A. and Shirley, J.A., Glucose oxidase, Glucose dehydrogenase, Glucose isomerase, β-Galactosidase and Invertase. In Microbial Enzymes and Bioconversion, A. H. Rose (eds.). San Francisco, London New York, Toronto and Sydney: Academic Press. pp. 173- 226. **1980**
[4] Doppner, T. and Hartmeir, W, *Starch/Strake.*, **1984**, 36, 283.
[5] Kalisz, H.M., Hecht, H.J., Schomburg, D. and Schmid, R.D, *Biochem. Biophys. Acta.*, **1991**, 1080 (2), 138.
[6] Hatzinikolaou, D.G., Hansen, O.C., Macris, B.J., Tingey, A., Kekos, D., Goodenough, P. and Stougaard, P, *Appl. Microbiol. Biotechnol.*, **1996**, 46, 371.
[7] Kusai, K., Sekuzu, I., Hagihara, B., Okunuki, K., Yamauchi, S. and Nakai, M, *Biochem. Biophys. Acta.*, **1960**, 40, 555.
[8] Eryomin, A.N., Droshdenyuk, A.P., Zhavnerko, G.K., Semashko, T.V. and Mikhailova , R.V, *Appl. Biochem. Microbiol.*,**2004**, 40 (2), 178.
[9] Sukhacheva, M.V., Davydova, M.E. and Netrusov, A.I, *Appl. Biochem. Microbiol.*,**2004**, 40 (1), 25.
[10] Eriksson, K. O., Kurteva, J., Yao, K., Liao, J., Kilar, F., Hjerten, S. and Chaga, G, *J. Chromatogr.*, **1987**,397,239.
[11] Rogalski, J., Fiedurek, J., Szczordrak, J., Kapusta, K. and Leonowicz, A, *Enz. Microb. Technol.*, **1988**, 10, 508.
[12] Ciucu,A. and Patroescu, C, *Anal. Lett.*, **1984**, 17,1417.
[13] Markwell,J., Frakes, L., G., Brott, E.C., Osterman, J. and Wagner, F.W, *Microb. Biotechnol.*, **1989**, 30,166.
[14] Kirschenbaum, D. M, *Anal. Biochem.*, **1975**, 68, 465.
[15] Kalb, V.F. and Bernlohr, R. W, *Anal. Biochem.*, **1977**, 82, 362.
[16] Solomon, B., Koppel, R., Pines, G. and Katzir, E.K, *Biotechnol. Bioeng.*, **1986**, 28,1213.
[17] Palmieri, G., Giardina, P., Desidero, B., Morzullo, L., Giamberini, M. and Sannia, G, *Enzy. Technol.*, **1994**, 16, 151.
[18] Skryabin, G.K. and Koshcheenko, K. A, *Methods. Enzymol.*, **1987**, 135, 198.
[19] Zetelaky, K.Z, *Biotechnol. Bioeng.*, **1970**, 12, 379.
[20] Hatzinikolaou, D.G. and Macris, B.J, *Enzyme. Microb. Technol.*, **1995**,17,530.

[21] Fiedruek, J.and IIeczuk, Z, *World. J. Microbiol and Biotechnol.*, **1991**, 7, 379.
[22] Fiedurek, J. and Szczodrak, J, *Acta. Biotechnol*., **1995**, 15, 107.
[23] Nakamatsu, T., Akamatsu, T., Miyajima. R.and Shiio, I, *Agric. Biol. Chem.*, **1975**, 39, 1803.
[24] Van, Dijken., J.P. and Veenheus, M, *Eur. J. Appl. Microb. Biotechnol*., **1980**, 9,275.
[25] Witteveen, C.F.B., van da Vondervort, P., Swart, K. and Visser, J, *Appl. Microb. Biotechnol*., **1990**, 33, 683.
[26] Träger, M., Qazi, GN., Buse, R.and Onken, U, *J. Fer. Bioeng*., **1992**, 74,274.
[27] Shin, K.S., Youn, H.D., Han, Y.O. and Kang, S.O, *J. Biochem*., **1993**, 215, 747.
[28] Eriksson, K.O., Kourteva, I., Yao, K., Liao, J.L., Kilár, F. and Hjertén, S, *J. Chromatogr*., **1987**,397, 239.
[29] Swoboda, B. E. P. and Massey, V, *J. Biol. Chem.*, **1965**, 240, 2209.
[30] Kim, K. K., Fravel, D. R. and Papavizas, G. C, *Can. J. Microbial.*, **1990**, 36, 760.
[31] Jong, M. K. and Rolf, D. S, *FEMS. Microb. Lett.*, **1991**, 78, 221.
[32] Garzillo, A. M.V. and Paolo, S.D, Fenice, M.; Petruccioli, M.; Buonocore, V. and Federici, F, *Biotechnol. Appl. Biochem.*, **1995**, 22, 179.
[33] Rando, D., Kohring, G. W. and Giffhorn, F, *Appl. Microb. Biotechnol*., **1997**, 48, 34.
[34] Scott, D, Oxidoreductase. In Enzymes in food processing, 2nd ed, D. Scott (eds.). New York: Academic Press. pp. 219-254 (**1975**).
[35] Kelley, R.L.and Reddy, C.A, *J. Bacteriol.*, **1986**, 166(1), 269.