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Production, purification and characterization of glucose oxidase-an over view

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ABSTRACT

Glucose oxidase (β -D-glucose:oxygen 1-oxidoreductase; EC 1.1.3.4) is a flavoprotein which catalyses the oxidation of β -D-glucose by molecular oxygen to D-glucolactone and H_2O_2 . It has been reported from red algae, bacteria, citrus fruits, insects and moulds. Filamentous fungi synthesize GOx and it is intra- and extracellular where as Intracellular localization of GOx is a matter of discussion. GOx is a homo-dimer with molecular weight of 150-180 KDa and contains two tightly bound FAD molecules. K_m value of purified GOx lies around 20-38mM and 0.25mM with glucose and dioxygen, respectively. pH optima for the enzyme depends upon the source of GOx and It is in the range from pH 3.0-7.0. GOx from *Aspergillus* and *Penicillium spp.* requires β -D-glucose as a substrate where as D-mannose, D-galactose, 2-deoxy-D-glucose and D-xylose exhibit low activity as substrates. GOx has applications in oxidation reactions and it is used clinically and analytically in the determination of glucose in body fluid such as blood and urine and desugaring of egg products and in removing oxygen from food and beverages.

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KEYWORDS

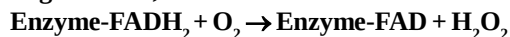
Glucose oxidase;
Microorganism;
Properties;
Purification.

INTRODUCTION

Glucose oxidase (β -D-glucose oxygen 1-oxidoreductase; EC 1.1.3.4) 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 glucose oxidase (GOx) is then re-oxidized by molecular oxygen and the hydrogen peroxide is produced:



glucono-1, 5-lactone

D-glucono-1, 5-lactone $\rightarrow$ Gluconic acid

The D-glucolactone hydrolyses spontaneously to gluconic acid, while H_2O_2 is usually depleted by catalase to water and oxygen^[30,20,4,18,14].

Production of GOx

Several microbial enzymes capable of oxidizing glucose are β -D-glucose: oxygen oxidoreductase (EC 1.1.3.4.), glucose dehydrogenase: β -D-glucose: NAD (P) oxidoreductase (EC 1.1.1.47), a glucose: 'accep-

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TABLE : 1.2 Medium Composition for production of GOx by different microorganism

Microorganism	Medium composition (g/L)	Reference
<i>A.niger</i> 1026/5	Sucrose, 50-70; Ca(NO ₃) ₂ . 4H ₂ O 2.0; Citric acid, 7.5; KH ₂ PO ₄ , 0.25; KCl, 0.25; MgSO ₄ . 7H ₂ O, 0.25; FeCl ₃ . 6H ₂ O, 0.01; CSL, 20.0.	Zetelaky and Vas, 1968
<i>A.niger</i>	Sucrose, 50.0; Ca(NO ₃) ₂ , 2.0; Citric acid, 7.5; KH ₂ PO ₄ , 0.25; KCl, 0.25; MgSO ₄ . 7H ₂ O, 0.25; FeCl ₃ , 6H ₂ O, 0.01; CSL, 20.0.	Zetelaky , 1970
<i>A.niger</i> and <i>Penicillium spp.</i>	Glucose, 40.0; NaNO ₃ , 2.0; KCl, 0.5; KH ₂ PO ₄ , 1.0; MgSO ₄ .7H ₂ O, 0.5; FeSO ₄ .7H ₂ O, 0.01; YE, 2.0; pH 6.0.	Nakamatsu et al., 1975
<i>A.niger</i>	Glucose, 54; KH ₂ PO ₄ , 1.0; Methylamine Hydrochloride, 2.0; MgSO ₄ .7H ₂ O, 0.25; K ₂ SO ₄ , 1.0; ZnSO ₄ .7H ₂ O, 0.03; FeSO ₄ .7H ₂ O, 0.03; YE, 0.3; pH 5.6.	Van Dijken and Veenheus, 1980
<i>A.niger</i> G-13 Mutant	Glucose, 80; (NH ₄) ₂ HPO ₄ , 0.388; KH ₂ PO ₄ , 0.188; MgSO ₄ .7H ₂ O, 0.156; CaCO ₃ , 35; pH 5.6.	Rogalski et al., 1988
<i>A.niger</i> NRRL-3 and mutant	Glucose, 18.0; NaNO ₃ , 3.0; K ₂ HPO ₄ , 1.0; MgSO ₄ . 7H ₂ O, 0.5; KCl, 0.5; FeSO ₄ .7H ₂ O, 0.01; Glycerol, 20.0.	Markwell et al., 1989
<i>A.niger</i> N400 (CBS-120-49), mutant	Glucose, 100.8; Fructose, 100.8, Sodium acetate, 0.1 M; NaNO ₃ , 1.2; KH ₂ PO ₄ , 0.5; MgSO ₄ .7H ₂ O, 0.2; YE, 0.5; trace metal solution, 0.04 ml.; pH 5.5.	Witteveen et al., 1990
<i>A.niger</i>	Sucrose, 20.0; YE, 10.0; peptone, 20.0.	Frederick et al., 1990
<i>A.nige</i> (RRL 12-6/1-2)	Glucose –monohydrate, 100; Corn steep liquor (CSL), 20; KH ₂ PO ₄ , 1.0; MgSO ₄ .7H ₂ O, 0.25; pH 6.5.	Träger et al., 1991
<i>A.niger</i> GIV-10	Glucose, 80; Peptone, 3; (NH ₄) ₂ HPO ₄ , 0.388; MgSO ₄ .7H ₂ O, 0.156; CaCO ₃ , 35.0.	Fiedurek and Iieczuk, 1991
<i>A.niger</i> RRL-12-6/1-2	Glucose –monohydrate, 40.0; NH ₄ NO ₃ , 1.0; KH ₂ PO ₄ , 1.0; MgSO ₄ .7H ₂ O, 0.25; pH 6.5.	Trager et al., 1992
<i>A.niger</i> NRRL-3 and mutant	Glucose, 80.0; NaNO ₃ , 3.0; K ₂ HPO ₄ , 1.0; MgSO ₄ .7H ₂ O, 0.5; FeSO ₄ .7H ₂ O, 0.1; CaCO ₃ , 35.0; pH 7.0.	Sharif and Alaeddinoglu, 1992
<i>P.ostreatus</i>	Malt extract, 20; Peptone, 5; Yeast extract , 5; Glucose, 10. Starch hydrolysate, Dextrose basis, 200.0; (NH ₄) ₂ HPO ₄ , 0.2; CSL, 0.4; KH ₂ PO ₄ , 0.1; MgSO ₄ .7H ₂ O, 0.1; Urea, 0.4; Antifoam H-601, 0.5; pH 6.5.	Shin et al., 1993
<i>A.niger</i>	Glucose, 80.0; Peptone, 3.0; NaNO ₃ , 5.0; KH ₂ PO ₄ , 1.0; FeSO ₄ .7H ₂ O, MgSO ₄ . 7H ₂ O, 0.5; CaCO ₃ , 35.0.	Shah and Kothari, 1993
<i>P.variabile</i> P16	Glucose, 80.0; Peptone, 3.0; NaNO ₃ , 5.0; KCl, 0.5; H ₂ PO ₄ , 1.0; FeSO ₄ . 7H ₂ O, 0.01; CaCO ₃ , 35.0; pH 6.0.	Petrucchioli and Federici, 1993
<i>Penicillium spp.</i>	Glucose, 60.0; NH ₄ NO ₃ , 0.3; KH ₂ PO ₄ , 0.25; MgSO ₄ . 7H ₂ O, 0.25; Urea, 2.0; CSL, 8 ml; pH 6.0.	Petrucchioli et al., 1994
<i>P.variabile</i> P16	Glucose, 80.0; Peptone, 3.0; (NH ₄) ₂ HPO ₄ , 0.388; KH ₂ PO ₄ , 0.188; MgSO ₄ . 7H ₂ O, 0.156; CaCO ₃ , 35.0.	Petrucchioli et al., 1995
<i>A.niger</i> ATCC2029		Li and Chen, 1994
<i>A.niger</i> and other strains		Fiedurek and Szczodrak, 1995

tor' oxidoreductase(EC 1.1.9.10) and β -D-glucose: NADP⁺ oxidoreductase(EC 1.1.1.119) and each of these produces D-glucono- δ -lactone and a reduced acceptor^[11]. The GOx has been reported from a number of sources that includes red algae^[5], bacteria^[19], citrus fruits^[6], insects^[95] and moulds. The isolation of enzyme and enzymology of gluconic acid production with press juices from the mycelia of *Aspergillus niger* and *Penicillium glaucum* was first studied by Muller,^[64]. The common microbial source of GOx production are *A.niger*, *P.notatum*, *P.glacum*, *P.amagasakiense* and *P.purpurogenum*,

although majority of its production is obtained from *A.niger*(TABLE 1)^[3,31,66,97,11,92,60,125,126,98,9]. Gox is also produced from *P.chrysosporium*^[11,45] *Talaromyces flavus*^[11,47], *P.ostreatus* GOx^[106], *P.expansum*, *P.italium*, *P.oxalicum*, *P.variable*, *P.paxilii*^[80], *P.variabile*(P16)^[29], *P.pinophilum*^[90], *Penicillium funiculosum*^[100] and *Penicillium adametzii* LF F-2044^[62].

Localization of GOx in fungus

Filamentous fungi synthesize GOx and it is intra- and extracellular. Intracellular localization of GOx is a matter of discussion. GOx from *Penicillium spp.*

was reported extracellular^[53,80] and from *A.niger* an intracellular associated with mycelium^[78,79,58]. In the past there have been inconsistent reports that GOx in wild type *A.niger* is intra- as well as extracellular^[79,65,118,61,113,125,119,33] and involved in energy producing mechanism of the microorganism^[97]. Subcellular localization of GOx in *A.niger* was investigated using cytochemical staining techniques and enzyme was located in peroxisome^[118]. In *A. niger* GOx has been found to be intracellular but under Mn²⁺ deficient conditions and pH shift experiments it was released in broth. Mn²⁺ deficient conditions cause alteration in cell wall composition^[61]. In fermentation process oxygen enriched air was passed and it was reported that intra- and extracellular GOx contributed almost equally to gluconic acid production. The presence of enzyme in broth was due to a controlled excretion^[113]. Subcellular localization of GOx in *A.niger* N400(CB 120.49) was investigated by immunocytochemical methods, the bulk of enzyme was found to be localized in the cell wall^[125]. GOx from *P.ostreatus* was located in periplasmic space^[106].

GOx production by recombinant microorganisms

However, the natural host organisms have not proved to be ideal for GOx over-expression due to many reasons. First, purified *Aspergillus* GOx is often contaminated with host cell enzymatic impurities such as catalase which interfere with its applications. Second, GOx is usually cell associated, thus making purification more difficult. Third, GOx production leads to gluconic acid and hydrogen peroxide production which complicates the cell culture techniques. To solve these problems gene for GOx from *A.niger* was cloned from both c-DNA and genomic libraries into yeast *Saccharomyces cerevisiae* with the help of plasmid and yeast derived enzyme comparable specific activity and have more extensive N-linked glycosylation than the *A.niger* protein^[16]. Cloning of *A.niger* GOx gene and its use to elevate GOx productivity in *A.niger* was done by increasing the gene dosage in the *A.nidulans* where it provided the novel capacity to produce Gox^[122]. The GOx gene from *A.niger* was expressed in *Hansenula polymorpha* using the methanol oxidase promoter and transcription termination region and

MF-alpha leader sequence from *S.cerevisiae* to direct secretion. The expression cassette was cloned into *S.cerevisiae* vector Yep13 and used to transform *H.polymorpha* strain A16^[37]. Wild type *A.niger* NRRL-3 was transformed with multiple copies of the GOx structural gene(god). The gene was placed under the control of gpdS promoter of *A.nidulans*. The recombinant strain NRRL-3 (GOD3-18) produced upto four times more extracellular GOx under identical culture conditions^[21]. The gene coding for *P.amagasakiense* GOx has been cloned by Polymerase Chain Reaction (PCR) amplification with genomic DNA as template with oligonucleotide probes derived from amino acid sequence of N- and C-terminals peptide fragments of the enzyme. Recombinant *Escherchia coli* expression plasmid have been constructed from pCYTEXP1 expression vector containing the mature GOx coding sequence. The purified GOx exhibits kinetics of glucose oxidation similar to those of, but have lower pH and thermal stability than native GOx from *P.amagasakiense*^[124]. The GOx gene of *A.niger* was cloned into the yeast shuttle vector Yep352 with combination of various promoters and terminators and then used to transform *S. cerevisiae*. The expressed GOx was successfully secreted into the culture due to the presence of intrinsic signal peptide of GOx^[75]. The gpdA-promotor controlled extracellular production of GOx by recombinant *A.niger* NRRL-3(GOD 3-18) during growth on glucose and non glucose carbon sources^[21].

The DNA fragment encoding *A.niger* GOx was amplified by PCR using *A. niger* genomic DNA as template and was cloned into vector of pPIC9 for expression in *Pichia pastoris*. When transformed into methylotrophic yeast *P.pastoris* GS115, the constructed plasmid pPICGOD1 directed the synthesis and secretion of functionally active GOx. The recombinant yeast GOx has very high catalytic activity, showed about 1.6 fold increase in specific activity over the commercial *A.niger* GOx. Kinetic analysis showed similar substrate affinity for glucose to *A.niger* GOx but the turnover number of the GOx from the yeast was determined to be much higher than that of *A.niger* GOx^[133]. The gene encoding GOx from *A.niger* was expressed as a secretory

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product in the yeast *S.cerevisiae*. Six constitutive histidine residues were fused to the c-terminus of GOx to facilitate purification^[49]. The GOX-encoding gene of *P.variabile* P16 was isolated and characterized to identify the molecular bases of its high level of expression and in view of improving enzyme production by developing a process based on heterologous expression^[89].

Methods for the estimation of enzyme activity

GOx enzyme activity was determined at 25°C using coupled reaction with dianisidine and Horseradish peroxide^[17]. GOx activity was determined at 30°C according to the methods proposed by Pick and Keisari^[87]. GOx activity was assayed at 420nm, using 2, 2 azino-di-(3-ethylbenzthiazoline-6-sulphonic acids) (ABTS) as dye^[21]. GOx activity was measured by Spectrophotometric methods of Ciucu and Patroescu^[13], as modified by^[60] by following the enzymatic reduction of bezoquinone to hydroquinone at 290nm. GOx activity was determined spectrophotometrically at 420nm by coupled chromogenic assay^[15]. Titration method was used by the Lu et al.^[58] for the determination of GOx activity.

Enhancement of GOx

Several reports have been published describing the over production of GOx from *A.niger* through the selection of mutants^[130,18,52,25,60,126]. CaCO_3 was used as an enhancer in *A.niger* G-13^[92] and *P.variabile*^[33]. Gene for GOx from *A.niger* was cloned in GOx deficient *A.niger* NRRL-3. The use of this gene for the elevation of GOx in parental strain and further improvement of GOx production was carried by subjecting the transformants to nitrous acid mutagenesis. In *A.niger* the increased GOx activity was due to increase in the copy no. of GOx gene^[102]. Hydrocarbon (n-dodecane, n-hexadecane and Soyabean oil) has positive effect on the formation of both intra- and extracellular GOx from *A.niger*^[38]. Sodium alginates and blocks of sodium alginate (oligomanuronate and oligoguluronate) was used to enhance the GOx production from *P.variabile* and there was 69%, 32% and 12.7% increase as compared to control respectively^[81].

Purification and characterization of GOx

For the commercial production of GOx, the mycelium at the end of the calcium or sodium gluconate fermentation was disrupted by grinding or sonication for the recovery of the enzyme. Enzyme was purified from suspended solids by filtration and precipitated using cold ethanol or acetone^[57,108]. Modification of the carbohydrate moiety of GOx results in separation from the contaminating enzyme with a Sepharose 4B column^[46] and diethylaminohydroxypropyl-cellulose^[51]. In another technical-scale of purification the crude enzyme solution at pH 3.5 was mixed with polyacrylonitrile powder and eluted with phosphate buffer (0.2M, pH 7.5). The final product has a yield 54%, with specific activity of 108 units/mg protein^[103]. Using DEAE-Sephadex, DEAE sepharose, Sephacryls, GOx from *P.chrysogenum* was purified and there was 89.3 fold purification with 9% recovery^[45]. GOx from *T.flavus* was purified using Acetone precipitation, TSK-3000SW and DEAE -SPW and obtained 58% yield^[47]. Ammonium sulphate precipitation, Sephadex, Sepharose, techniques were used for the purification of GOx from *P.ostreatus* and achieved 23.8% yield and 27.8 fold purification^[106]. GOx from *P.variabile* P16 was purified using DEAE-sepharose and sephacryl-S-300 and achieved 30 fold purification with 87% recovery^[29]. S-sepharose and Sephacryl S-300 were used for the purification of GOx from the *P.pinophilium* and there was 74% recovery with 18.0 fold purification^[90].

Molecular properties of GOx

GOx is a homo-dimer with molecular weight of 150-180 KDa and contains two tightly bound FAD molecules^[76]. Molecular weight of GOx from *A.niger* is 160 KDa^[110,73,114]. Based on gel filtration chromatography on a sephaacryl S-300, the molecular weight of purified enzyme GOx from *P.chrysosporium* was estimated to be 180 Kda and denatured molecular weight determined by SDS-PAGE was 80 KDa^[45]. Molecular weight of purified GOx from *T.flavus* is 164±4 KDa^[47]. GOx from *P.amagasakiense* has molecular weight 130 KDa and 150 KDa, for aglyco and glyco respectively with TSK G-3000 chromatography and PAGE. Molecular weight of the aglyco subunits was 60 KDa while that of glyco subunits was 70 KDa. The reduced molecular weight of aglyco GOx corresponds to a 13-14% by the loss of the

glyco component of the enzyme, the aglyco and glycol GOx content is estimated about at 2.9 and 20.4% respectively^[40]. By gel filtration methods and SDS / PAGE it was revealed that enzyme GOx purified from *P.ostreatus* has molecular weight of 290 KDa and consists of four sub units with a molecular weight of 70KDa^[106]. The molecular weight of native enzyme purified from *P.variable* is 126 KDa, estimated by gel filtration method^[29], it contains two polypeptides chains of identical mass that is lower than other *Penicillium spp*^[68,22]. Molecular weight of enzyme purified from *P.pinophilium* was observed to be 154.7 KDa $\pm$ 4.95 KDa. In gradient SDS page there was a single peptide band of molecular weight 77.7 KDa that indicates that GOx is homogenous and composed of two sub units of identical size^[90]. GOX from *Penicillium sp.* was shown to be dimeric with a molecular weight of 148kDa, consisting of two equal subunits with molecular weight of 70k Da^[99].

Kinetic properties of GOx

Activity of GOx in pure oxygen was about two and half times high as in air^[44]. The production of gluconic acid by mould mycelia was strongly dependent on dissolved oxygen tension. This phenomenon reflects one of the peculiar properties of GOx shared by other H₂O₂ producing oxidases^[30]. Literature values for K_m of GOx lies around 20mM and 0.25mM with glucose and dioxygen, respectively^[30,110,70]. GOx from *P.chrysosporium* has K_m values for glucose and oxygen 38 and 0.95mM, respectively^[45]. GOx from *T.flavus* has high affinity for glucose with K_m 10.9mM^[47], this is in lower part of range 9.6-11.0mM reported for other fungal GOx^[30,110]. In *P.amagasa kiense* there was no significant difference in catalytic properties of glyco and aglyco GOx. For glyco and aglyco GOx, K_m values are 3.4 and 2.7mM, respectively. The V_{max} values of glyco and aglyco GOx were 320 and 279U/mg of proteins, respectively at pH 7.0. These results support the point that carbohydrate moiety plays no essential role in enzyme activity^[40]. GOx from *P.ostreatus* has Michaelis type relationship between enzyme activity and substrate concentration. The K_m and V_{max} values determined from Lineweaver- Burke plot were 1.34mM and 53U, respectively^[106]. K_m value of GOx from

P.variable was 6 mM, when glucose was substrate and this was five to twenty folds lower than for other sugars^[29]. GOx from *P.pinophilium* has K_m values of 6.2mM for glucose and for other carbon sources there was no enzyme activity or very less^[90]. GOx from *Penicillium adametzii* LF F-2044.1 was characterized by KM values of 1.56 $\times$ 10⁽⁻²⁾ and 2.19 $\times$ 10⁽⁻²⁾M, respectively; the corresponding values of kcat equaled 235.1 and 318.2 s⁽⁻¹⁾^[23]. GOX from *Penicillium sp.* CBS 120262 has kinetic characteristics: Vmax, 240.5U mg⁽⁻¹⁾; Km, 18.4mM; kcat, 741 s⁽⁻¹⁾ and kcat/Km, 40 s⁽⁻¹⁾mM⁽⁻¹⁾^[99].

Carbohydrate and flavin content in the GOx enzyme

GOx is a flavo enzyme and contains FAD (flavin adenine dinucleotide)^[44]. FAD content of GOx was removed by treatment with 80% saturated ammonium sulphate at pH <2.8 at 0°C, after the removal there was no activity but complete reactivation (93%) was achieved by the addition of an amount of FAD equal to that was liberated. FMN did not substitute for FAD in this reaction^[53]. GOx from *A.niger* contains two moles of FAD per mole of protein^[8,110,121,115]. The carbohydrate content of the enzyme consists mainly of D-mannose(about 14% of the enzyme biomass) but it also contains D-glucoamine(2.3%) and D-galactose (0.3%)^[77]. It has been suggested that glucose is also present^[70], but other workers failed to confirm this^[78,114]. GOx is highly glycosylated protein, carbohydrate content is ranged from 10-24% of its molecular weight^[78,34]. Some workers have proposed that there are six different forms of GOx each with different carbohydrate content. It was proposed that carbohydrate content range from 8-12% and these differences account for variation in the isoelectric point between 3.9 and 4.3, elsewhere isoelectric point has been quoted as 4.2^[110,114]. GOx is composed of two identical polypeptide chain subunits covalently linked by disulfide bonds^[73]. Two molecules of FAD are tightly bound, but not covalently attached to the apoenzyme and can be removed without permanently denaturation^[72,63,71]. Staining of purified GOx from *P. chrysosporium* for protein bound carbohydrate by dansyl hydrazine method showed no detectable car-

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bohydrate, whereas an equal amount of commercially prepared GOx from *A.niger* stained positive. Flavin analysis indicated that the purified enzyme from *P.chrysosporium* contained 1.5mole of flavin per mole of protein, but *A.niger* GOx has 1.6mole of flavin per mole of protein^[45]. The purified enzyme GOx from *T.flavus* is a glycoprotein^[47]. The absorption spectrum of GOx purified from *P.ostreatus* reveals a typical flavoprotein spectrum with the absorption maxima at 460, 360 and 280nm. The GOx has flavin as a prosthetic group and enzyme contains 4mole flavin/mole enzyme^[106]. Homogenous GOx from *P.pinophilium* exhibited the spectral characteristics of flavoprotein with absorption maxima at 275, 380 and 460nm. The glycoprotein nature of GOx was qualitatively assessed by staining with periodate/Schiff's reagent and by precipitation with concanvalin A^[90].

Amino acid composition of GOx

Amino acid composition of GOx from *T.flavus* was compared with the amino acid composition of GOx from *P.amagasakiense* and *A.niger* and it was found that aspartic acid, glutamic acid, glycine, alanine, and leucine were the most abundant residues in all of the enzymes, while cystine was least abundant^[78,34,114]. With few exceptions, amino acid composition was same for the enzyme from the *Penicillium spp.* and *A.niger*. There was more lysine and phenylalanine and less histidine and arginine in *Penicillium* enzyme as compared to the *A.niger* enzyme^[34]. Amino acid sequence for the 583 acid residue proteins has been derived from DNA sequence independently by Kriechbaum et al.,^[50,16]. The GOx from *T.flavus* was more basic than that from *A.niger* due to higher content of lysine in the *T.flavus* enzyme^[47]. The contents of glutamate/glutamine and aspartate/asparagine were significantly higher than other amino acids in GOx from *P.ostreatus*^[106]. In three-dimensional structure of GOx, it was found that the Asp 548 forms part of bottom of catalytic active site, Trp 515, and His 516, which are close to the front of isoalloxanine moiety, allowing them to be involved in catalytic reaction of the substrate binding. Phe 414, Try 426 and Asn 514 are found in position where they form additional contact to the substrate.

Crystal structure of GOx from *A.niger* was refined at 2.3Å resolution. Final crystallographic R-value is 18.1% for reflection between 10.0-2.3Å. The refined model contains 580 amino acid residues, FAD co-factor, six N-acetylglucosamine residue, three mannose and 152 solvent residues. The substrate-binding domain is formed from non-continuous segment of sequence and is characterized by deep pocket^[35].

Thermal stability and optimum temperature for the activity of GOx

Thermal stability and temperature for maximum activity of GOx various with respect to the producing microorganism. GOx as a solid was stable for two years at 0°C and for 8 years at -15°C and aqua's solution (0.1-0.2%) was stable for a week at 5°C. The crystalline enzyme was stable in ammonium sulphate for long time at room temperature. The enzyme activity was not affected by incubation for one hour at 39 °C at pH 5.6^[44]. The enzyme was unstable at temperature over 40°C^[53]. GOx from *A.niger* has optimum temperature 35-40°C^[97]. Maximum activity of GOx was at temperature 35 °C in *P.purpurogenum*^[66] and 45°C from *A.niger* G13 mutant^[38]. Ethyl alcohol and glycerol did not affect the thermal stability of GOx from *A.niger*, but erythritol, xylitol, sorbitol has an important positive effect. BaCl₂, CaCl₂, MgCl₂, LiCl₂, NaCl, and KCl also affect the thermal stability of GOx from *A.niger*. KCl or NaCl have positive effect, while negative effect was observed when divalent cations. It was reported that Half-life of GOx increased in the presence of 4M xylitol and polyethylene glycol of different molecular weight. It was observed that GOx solution was more resistant to thermal denaturation in D₂O solution (97%) than in water and it also affect Half-life of enzyme, which was increased by a factor of 2.6 when solvent was changed. This shows the important effect of the solvent organization on enzyme stability^[128]. Aglyco and glycol preparation has stability up to 35°C for 30 minutes, both were considerably inactivated after 30 minutes at 45°C^[40]. Optimum temperature for GOx from *P.ostreatus* under the standard condition was 50°C and loses 70% of its activity at 73°C in one hrs^[106]. The activity of GOx in *P.variabile* (P16) reached maximum at 55°C, then rapidly decreased at higher temperature, the enzyme was completely inactive at 65°C^[29]. GOx

from *P.pinophilium* has optimum temperature in the range of 30-50°C in 100mM phosphate buffer (pH 5.0)^[90].

pH tolerance and Isoelectric point for GOx

pH optima for the enzyme depends upon the source of GOx. It was in the range from pH 3.0-7.0. Optimum pH of enzyme GOx was 5.6 and variation of phosphate and acetate buffer concentration between 0.03-0.25M does not influence the rate of oxygen consumption. The enzyme was unstable at pH values greater than 8.0 and at pH 8.1 only about 10% enzyme activity remains after 10 minutes, the inactivation process was even more rapid at pH 9.1^[44]. The crystalline GOx was stable between pH 3.5 and 7.0 at 40°C^[53]. GOx from *A. niger* has pH 5.6^[7]. The enzyme has optimum pH 5.5 with a broad range from 4.0 -7.0^[73]. GOx purified from *P.chrysosporium* has pH optima between 4.6-5.0^[45], *A.niger* G13 mutant, pH 5.6, *P.purpureogenum*, pH 5.6^[28], *T.flavus* has optimum pH 5.0, but it was more stable at much more broader range of 3.0-7.0 when incubated at 40°C for two hrs^[47]. Glyco and aglyco GOx purified from *P.purpureogenum* has pH optima at 5.5 and 6.0, respectively^[40]. GOx from *P.ostreatus* has highest activity at pH 5.5-6.0, but it was almost insensitive to pH change from pH 4.5 to 6.5^[106]. Enzyme purified from *P. variable*(P16) has maximum activity at pH 6.0^[29], this value was slightly higher than the enzyme obtained from *P.amagasakiense*, pH 5.5^[68], *P.notatum*, pH 5.5^[1] and *P.chrysogenum*, pH 5.6^[22]. GOx of *P.pinophilium* exhibited a relatively broad pH range between 4.0-4.6 and activity reduces to 50% at pH 3.0 and 7.5^[90].

Isoelectric point of GOx depends upon the producing microorganism. GOx from *A.niger*^[34] has one major band at pI 4.3 and minor band at pI 4.2 and GOx from *T.flavus* has pI in the range 4.45-4.55^[47]. GOx from *P.amagasakiense* has same pI for aglyco and glyco GOx with major band at pI 4.2 and two minor bands between pI values 4.3 and 4.5^[40]. GOx from *P.variable* (P16) has pI 4.8-4.9^[29]. Isoelectric focussing revealed two isoenzymes present in both intra- and extracellular fractions, having pI's of 4.30 and 4.67 from *Penicillium* sp. CBS 120262^[99].

Inhibition of GOx activity

Activity of GOx was inhibited by 0.1M concentration of hydroxyquinoline, sodium nitrate and semicarbazide and the percentage of inhibition was 11%, 13%, 20%, respectively^[44]. The crystalline enzyme was completely inhibited by 10⁻³M p-mercuribenzoate and partially by 10⁻³M solution of dimendon, phenylhydrazine, hydrazine, hydroxylamine and sodium bisulfate^[53]. GOx activity was inhibited by micromolar amount of heavy metals such as Ag²⁺, Hg²⁺, Cu²⁺^[70,111]. Millimolar amounts of hydrazine, hydroxylamine, and phenylhydrazine partially inhibit the enzyme activity^[7]. Binding of FAD residues to protein was inhibited by several nucleotides^[109]. The oxidative activity of GOx from *A. niger* was inhibited by D-arabinose^[1]. Halide ions inhibited GOx activity at low pH^[93], for example, at pH 3.0 enzyme activity was completely inhibited by 0.1M potassium chloride^[20]. Inhibitory effect of Ag²⁺ was common among fungal GOx and it was explained by the high reactivity of this ion for thiol group essential for enzymatic activity^[97]. GOx from *P.variable* P16, *A.niger* and *Penicillium spp.* was severely inhibited by Cu²⁺^[97,45]. Activity of GOx from *P.chrysosporium* was inhibited by Ag²⁺ similar to GOx of *A.niger*, but was not inhibited by Cu²⁺, KCN or NaF. It was also severely inhibited by o-phthalate, where as commercial GOx from *A.niger* showed only limited inhibition of activity^[45]. GOx from *P.ostreatus* was severely inhibited by HgCl₂, silver sulphate, sodium azide and phenylmercuric acetate. Mercury and silver ions inhibit oxidation of the reduced FAD moiety competing with molecular oxygen as a hydrogen acceptor^[106]. GOx from *P. variable* was also appreciably inhibited by the NaF but to a lower extent by AgNO₃^[29]. At higher concentration all the metals inhibit oxidase activity from *A. niger*, the sequence of inhibition is Mg²⁺>Ca²⁺>K⁺>Na⁺ at the same salt concentration. Inhibition of monovalent ions was less than that of divalent metal ions^[58]. Addition of MgSO₄.7H₂O at the rate 0-0.5g/l⁻¹ resulted significantly lowering activity of GOx from *P.variable* M-80-10 strain^[83].

Substrate specificity of GOx

GOx from *A.niger* and *Penicillium spp.* specifically requires β-D-glucose as a substrate. D-man-

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nose, D-galactose, 2-deoxy-D-glucose and D-xylose exhibit low activity as substrates^[1,7,30,68]. Glucose was primary substrate for the GOx from *P. chrysosporium* with specific activity of 12.27, whereas other substrates like cellobiose, glycolate, mannose, ethanol, acetate, lactate and β -D-gluconate has less than 1% specific activity^[45]. Activity of GOx from *P. ostreatus* toward series of sugars such as L-sorbose, D-gluconolactone, 2-deoxy D-glucose were lower than glucose^[106], this was similar to that of glucose-2-oxidase of *P. chrysogenum*^[22]. GOx from *A. niger* G-13 mutant was highly specific for glucose and it also attacks 2-Deoxy D-glucose, other sugars with much lower yields as compared to glucose^[92]. GOx from *P. variable*, *A. niger* and *P. ostreatus* has high specificity for glucose^[4,106,29]. GOx from the *P. pinophilum* has also specificity for D-glucose^[90].

Applications of GOx

GOx has applications in oxidation reactions. GOx was used clinically and analytically in the determination of glucose in body fluid such as blood and urine^[94,127]. Commercially it is used in desugaring of egg products and in removing oxygen from food and beverage^[120,4,91,88,42]. On the industrial scale it is used for production of gluconic acid and works as a food preservative^[123,14]. GOx has been attached to the antibodies for use as a label in immunoassay^[59,129,32] and for staining tissues^[54]. GOx is used in the production of hydroquinone from benzoquinone^[2]. Sofue and Takamoto^[107], used GOx for the detection of glucose released from liposomes in liposomal immunoassay. It is used to produce the polymer like propylene bromohydrin^[43]. It has been reported that H_2O_2 produced by GOx plays an important role in lignin degradation by *P. chrysosporium*, a white rot basidiomycetes^[45]. It is used for the quantitative determination of D-glucose in samples such as blood, food, agriculture, and fermentation products^[116,96]. GOx has antifungal properties^[48]. GOx has been used in production of tablet grade calcium gluconate^[101]. Serum glucose was analyzed using GOx from *A. niger* immobilized to alkaline glass by the glutaraldehyde coupling method^[41]. Cheese whey was used as a substrate for the production of gluconic acid by

the enzyme GOx^[12]. GOx covalently immobilized on commercially available alumina and glass support was successively used for textile bleaching^[117]. Glucose oxidase from fungus *Penicillium chrysogenum* has cytotoxic potential for a series of bacteria, yeasts and filamentous fungi^[56]. The use of a GOX based glucometer at moderately high altitude may be useful in detecting hypoglycaemia at these conditions, since significantly higher blood glucose levels were measured with a GDH based glucometer compared to reference readings^[10].

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