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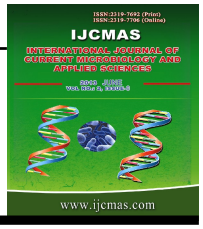

Original Research Article

Isolation and charecterization of Glucose Oxidase (GOD) from *Aspergillus flavus* and *Penicillium* sp.

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A B S T R A C T

The present investigation was aimed to isolate soil and endophytic fungi from various sources and GOD production from them was studied. Glucose oxidase is FAD dependent glycoprotein catalysing the oxidation of β-D-glucose to glucono-1, 5- lactone. It means they reduce the glucose level in blood as well as it can be used as glucose detector. Totally, two soil *Aspergillus flavus, Aspergillus niger* and two endophytes *Penicillium* sp, and *Fusarium* sp were isolated. They were mass cultured on potato dextrose broth media. All four fungal species were subjected to titration method and Diastrix method for identification of GOD activity. *Aspergillus flavus* and *Penicillium* sp showed high GOD activity compared to *Aspergillus niger* and *Fusarium* sp. Enzyme activity assay was carried out by spectrophotometric method for *Aspergillus flavus* and *Penicillium* sp, which showed the highest presence of GOD enzyme. The maximum precipitation occurred at 10% saturation from Penicillium species and 20% saturation from Aspergillus flavus species and it was subjected to protein estimation by Lowry's method. Ammonium sulphate fractionation was done for the precipitation of crude extracellular GOD. The maximum precipitation occurred at 20% giving 36.94% with enzyme recovery of 99.8 from *Aspergillus flavus* and 36.54% with enzyme recovery of 99.6 from *Penicillium* sp. at 10%. The other two fungal species showed less recovery of protein and less glucose oxidase activity.

Keywords

Glucose oxidase; *Aspergillus flavus;* *Penicillium* sp.; characterisation; partial purification

Introduction

Fungi have an important place in the realms of microbiology and biochemistry due to their ability to produce useful enzymes. Fungal enzymes have been employed for the rapid oxidation and decomposition of proteins, carbohydrates and fats. Glucose oxidase (GOD) has been purified from a range of different fungal sources, mainly from the genus *Aspergillus* and *Penicillium* (Kusai, 1960; Eryomin *et al*., 2004; Sukhacheva *et al*., 2004; Rando *et al*., 1997). Despite the fact that GOD has been produced by a variety of filamentous fungi, *A. niger* is the most

common fungus utilized for the production of GOD (Pluschkell *et al*., 1996). The *Penicillium* species GOD has been shown to exhibit more advantageous kinetics for glucose oxidation than that of *A. niger* GOD (Kusai, 1960; Witt *et al*., 1998). GOD has been used in large scale technological applications since the early 1950s (Fiedurek and Gromada, 1997). Many fungal species such as *Penicillium notatum*, *P. chrysosporium*, *Aspergillus niger* and *Botrytis cinerea* have the ability to produce GOD (Lium *et al*., 1998; Hafiz *et al*., 2003).

GOD (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is a glycoprotein which catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone and hydrogen peroxide using molecular oxygen as the electron acceptor. It removes hydrogen from glucose and reduces itself. The reaction can be divided into, a reductive and an oxidative step. In the reductive half reaction GOD catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone which is non-enzymatically hydrolyzed to gluconic acid. Subsequently the flavine adenine dinucleotide (FAD) ring of GOD is reduced to FADH2 (Witt *et al*., 2000). In the oxidative half reaction the reduced GOD is reoxidised by oxygen to yield hydrogen peroxide. The hydrogen peroxide is cleaved by catalase (EC 1.11.1.6) (CAT) to produce water and oxygen (Beltrame *et al*., 2004l; Witteveen *et al*., 1992) stated that in *A. niger*, the enzyme lactonase (EC 3.1.1.17) was responsible for catalyzing the hydrolysis of D-glucono-1,5-lactone to gluconic acid, although the presence of lactonase was not necessary since the hydrolysis step does occur spontaneously but at a lower rate.

The molecular weight of GOD ranges from approximately 130kDa (Kalisz *et al*., 1997) to 175kDa (Eriksson *et al*., 1987). The GOD enzyme is highly specific for the β-anomer of D-glucose, while the α-anomer does not appear to be a suitable substrate (Kusai, 1960). Low GOD activities are exhibited when utilizing 2-deoxy- D-glucose, D-mannose and D-galactose as substrates. Inhibitors of GOD include p-chloromecuribenzoate, Ag+, Hg2+, Cu2+, hydroxylamine, hydrazine, phenylhydrazine, dimedone and sodium bisulphate. On average, the isoelectric point of GOD has been shown to fall between pH 4 and pH 5 (Eriksson *et al*., 1987; Kusai, 1960; Kalisz *et al*., 1997).

GOD has been used in large scale technological applications since the early 1950s (Fiedurek and Gromada, 1997). Implantable glucose sensors have found application in treating diabetic patients. GOD, usually in combination with CAT, is used to stabilize colour and flavour in beer, fish, tinned foods and soft drinks, by the removal of oxygen (Crueger and Crueger, 1990). GOD is also used to remove glucose during the manufacture of egg powder, preventing browning during dehydration caused by the Maillard reaction (Crueger and Crueger, 1990). GOD has also found application in the baking industry providing slight improvements to the crumb properties in bread and croissants (Rasiah *et al*., 2005). GOD is also widely used to produce gluconic acid, which is used as a mild acidulant in the metal, leather and as a food preservative in food industries (Pluschkell *et al*., 1996; Crueger and Crueger, 1990; Nakao *et al*., 1997; Klein *et al*., 2002). The most important application for GOD is for the diagnostic determination of glucose using biosensor technology (Wilson and Turner, 1992; Chudobova *et al*., 1996). Commercial

diagnostic kits for the determination of glucose in blood, serum and plasma are supplied commercially in colorimetric diagnostic kits (Wilson and Turner, 1992). The use of GOD has found application in the textile industry as a method for producing hydrogen peroxide for bleaching (Tzanov *et al*., 2002). GOD in new forms with useful properties for applications in biotechnology continues to be of considerable interest despite the abundant availability of commercial GOD (Rando *et al*., 1997).

The literature survey clearly indicates that, there are no reports available in India related to isolation, characterization and kinetics of GOD. The present investigation was aimed to identify GOD producing fungal species which were isolated from soil and plants. The fungal extracts were subjected to know possible GOD activity.

Materials and Methods

Collection of soil samples

The soil samples were collected randomly from Agricultural Experimental Station, Hirehalli, Tumkur, Karnataka, India and plant materials (Amaranthus caudatus) were collected from Shridevi Institute of Engineering and Technology campus, Tumkur during February, 2011. Collected soil and samples were named and serial dilution method was done to get the fungal species whereas the plant parts were used for siolation of endophytes.

Isolation, Identification and mass cultivation of soil microbes

Potato Dextrose Agar (PDA) medium was prepared and sterilized. The serially diluted soil samples and surface sterilized plant parts (with 0.1% sodium hypochlorite) were incubated individually on PDA containing Petri plates. All Petri plates were incubated for seven days at room temperature (24 ± 2^0C) for fungal development. After incubation each fungal species were identified based on morphology and conidia using standard manuals (Ellis, 1971; Barnett and Hunter, 1972). Each fungal species were mass cultured on potato dextrose broth medium and after seven days each fungal mass and extra cellular matrix was used to determine GOD activity by various standard methods.

Determination of GOD activity

Titration method

The GOD activity was determined by the titration method (Underkofler, 1958). One ml of enzyme solution was added to 25 ml of 60 mM sodium acetate buffer pH 5.6 containing 2% β-D-glucose. The mixture was shaken well for 1 h in air at 30^0C in rotary shaker at 200 rpm. Sodium hydroxide solution (20 ml of 0.1 M) was added to stop the reaction. The resulting mixture was titrated to a dark pink endpoint by 0.1M standard HCl solution using phenolphthalein as an indicator. The volume of HCl added was V ml. The blank assay (enzyme absent) was performed under the same experimental conditions. The volume of standard HCl added was V_0. The GOD activity could be calculated by the formula:

$\{(V_0\text{-}V) \times N \times 1000\}/60$

Here N is the concentration of standard HCl solution (M). One unit of enzyme was defined as the amount that could oxidize 1.0 µmol of β-D-glucose to gluconic acid and H_20_2 min^{-1} at pH 5.6 at 30^0C.

Diastix method

Diastix Reagent Strips method was also followed for the detection of GOD from

extracellular or intracellular fraction of fungal species. Diastix Reagent Strips are used for in vitro diagnostic use**.** Diastix Reagent Strips are highly specific test strips for detecting glucose in urine or blood and are commonly used in doctor's surgeries and in home. Diastix strips contains glucose oxidase (Microbial 1.3 IU) 2.2% W/W, peroxidase (Horseradish 3300 IU) 1.0% W/W; potassium iodide 8.1% W/W; buffer 69.8% W/W; nonreactive ingredients 18.9% W/W. The enzymes glucose oxidase and peroxidase are immobilized on a paper pad at the tip of the strip. The pad is covered with a thin cellulose membrane which is permeable only to small molecules such as glucose.These strips, when dipped in extracellular or intracellular fraction of fungal species shows a colour change if Glucose is reduced as per the reaction given below. The intensity of the colour developed indicates the glucose concentration.

How diastix test strip works

Glucose oxidase acts specifically on glucose to give hydrogen peroxide and gluconic acid:

$$\beta\text{-D-glucose} + \text{oxygen} + \text{water} \xrightarrow[\text{Oxidase}]{\text{Glucose}} \text{hydrogen peroxide} + (\alpha\text{-Gluconolactone}) \xrightarrow[\text{hydrolysis}]{\text{Spontaneous}} \text{Gluconic acid}$$

Horse radish peroxidase then catalyses the reaction of hydrogen peroxide with potassium iodide. The colourless iodide is oxidised to brown iodine.

$$\text{Hydrogen peroxidase} + \text{Potassium iodide} \xrightarrow[\text{peroxidase}]{\text{Horse radish}} \text{Iodine} + \text{water}$$

Enzyme assay

Glucose oxidase activity in different samples were determined by spectrophotometric method at 460 nm wavelength using glucose as a substrate and o-dianisidine buffer as coupling reagent (Worthington, 1988). The assay is based on the estimation of residual sugar and was carried out using the method of Moneral and Reese (1969). The amount of reducing sugar released in the supernatant was determined by Shindia *et al*., (2001) and El-Sherbeny *et al*., (2005).

Estimation of protein: Protein content was determined using Lowry *et al*., (1951), Ohnishi and Barra (1978) and El-Sherbeny e*t al.,* (2005), using bovine serum albumin as standard.

Purification of intracellular GOD: The crude enzyme extract was subjected to different purification steps as shown in Table 4. All purification steps were carried out at 4^0C as follows: The precipitation of crude intracellular glucose oxidase enzyme extract was carried out by adding different amounts of ammonium sulphate to give saturation from 20 to 100%. The solution was left overnight at 4^0C until the complete precipitation occurred, and then centrifuged at 15000 rpm for 15 minutes to remove the undissolved particles. Each fraction precipitate was dissolved immediately in a known volume (20 ml) of 0.1 citrate phosphate buffer (pH 5.6). The dissolved fractional precipitates were tested for both glucose oxidase activity and protein content.

Result and Discussion

Four different fungal species; *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* sp, *Fusarium* sp. were identified based on morphology, spore/conidia using standard manual (Table 1) from both soil and endophytes of *Amaranthus caudatus*. *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* sp, obtained from soil source and *Penicillium* sp, *Fusarium* sp. obtained from plant parts were mass cultured on potato dextrose agar and broth medium. Soil fungus, *Aspergillus niger*, *Aspergillus flavus* and endophytes, *Penicillium* sp. and *Fusarium* sp. were selected for further enzyme study.

Table.1 Fungal species found in different samples

Fungi	Soil	Plant
Aspergillus niger	+	-
Aspergillus flavus	+	-
Penicillium sp.	+	+
Fusarium sp.	-	+

+: Presence, - : absences. Above experiment was repeated thrice

Table.2, clearly indicates the glucose oxidase activity by titration method, in the fraction of all soil and endophytic fungal by forming dark pink end point.

In diastrix strip method, *Aspergillus flavus* and *Penicillium* sp. have changed the strip colour from colourless to brown and more activity was noticed in these fungal species. *Aspergillus niger* has also showed positive results. The result clearly indicates that, all fungal species which showed +ve results have the ability to produce GOD in varying concentration as discussed above; and the results are shown in Table 3. Above experiment was repeated thrice.

Table.2 GOD activity of fungal species from titration method

Fungi	Activity
Aspergillus niger	26.2 U/min ml
Aspergillus flavus	66.3 U/min ml
Fusarium sp.	22.3 U/min ml
Penicillium sp.	66.6 U/min ml

Table.3 Bayer diastix strips test showing the activity and concentrations of GOD

Fungi	Activity	Conc. of glucose
Aspergillus niger	+	2+++
Aspergillus flavus	+	2+++
Fusarium sp.	-	-
Penicillium sp.	+	2+++

+ : presence , - : absence. 2+++: more concentration.

Table 4 shows that, all the isolated fungal species showed GOD activity but more activity was observed in *A. flavus* (6.9 u/ml) and *Penicilium* sp. (5.7 u/ml) followed by *A. niger* (4.3 u/ml) and *Fusarium* sp. (4.7 u/ml).

Table.4 GOD activity in different fungal species

Fungi	GOD activity
Aspergillus niger	4.3 u/ml
Aspergillus flavus	6.9 u/ml
Fusarium sp.	4.7 u/ml
Penicillium sp.	5.7 u/ml

Table.5 A precipitation summary pattern of fungal species glucose oxidase by ammonium sulphate

Ammonium sulphate conc. %	Volume	Total glucose oxidase activity (U/100 ml)	Total protein content (mg/100 ml)	Specific activity (U/mg protein)	Enzyme yield percentage
Penicillium sp.					
10	100	99.6	2.8	36.54	86
20	100	86.6	2.6	34.16	79
30	100	78.6	2.5	33.36	77.8
40	100	59.1	2.4	26.89	58
50	100	35.6	1.2	21.08	37
Aspergillus flavus					
10	100	36.9	1.4	22.62	38
20	100	99.8	2.8	36.94	86
30	100	87.1	2.7	34.87	80
40	100	78.9	2.7	34.14	77.9
50	100	31.2	1.1	17.98	59
Aspergillus niger					
10	100	24.2	1.1	17.41	31
20	100	41.2	1.2	18.32	53
30	100	53.4	1.3	19.64	61
40	100	61.8	1.4	20.17	69
50	100	66.7	1.5	21.62	54
Fusarium sp.					
10	100	21.6	1.1	16.63	27
20	100	38.4	1.2	17.86	48
30	100	42.1	1.3	17.94	57
40	100	57.7	1.4	18.01	63
50	100	60.4	1.5	18.26	47
Control*	100	99.2	6.0	17.64	100

* Control: without ammonium precipitation. Repeated the each replicate at three times

Table 2, 3 and 4 clearly explains that *Aspergillus flavus* and *Penicillium* sp. have the ability to produce GOD. The specific activity, recovery, total protein content and specific activity of GOD were carried out for all isolated fungal species (Table.5). The maximum precipitation occurred at 100% in ammonium sulphate fraction method giving 36.94% with enzyme recovery of 99.8 from *Aspergillus flavus* and 36.54% with enzyme recovery of 99.6 from *Penicillium* sp. The other two

fungal species showed less recovery of protein and less glucose oxidase activity.

In the present study, four different fungal species were isolated from soil and plant source (endophyte). In GOD identification tests, *Aspergillus flavus* and *Penicillium* sp. showed highest activity compared to other two fungal species. Lium *et al*., (1998) and Hafiz *et al*., (2003) have reported glucose oxidase producing fungal species (*Penicillium notatum, P. chrysosporium, Aspergillus niger* and *Botrytis cinerea*). The fungal species isolated in present investigation is also capable of producing GOD and it was confirmed by various methods such as titration, diastrix and enzyme assay. GOD is intracellular enzyme present in the mycelium of the organism (Hafiz *et al*., 2003). El-Sherbeny *et al*., (2005) have optimized the intracellular GOD production from *Aspergillus niger*.

Results of present investigation are confirmatory with the findings of El-Sherbeny *et al*., (2005), Lin *et al*., (2001) and Kona *et al*., (2001), but they worked on different fungal species (*Aspergillus niger*). Many authors have reported the use ammonium sulphate fractionation and other organic solvents for the precipitation of intracellular GOD enzyme and other microbial enzymes (Rando *et al*., 1997; Sherif, 1998; Tohamy and Shindia, 2001).

The preliminary investigation gives positive hopes on isolation and usage of GOD from other fungal species. The two fungal species (soil *Aspergillus flavus* and endophyte *Penicillium* sp.) produce higher levels of GOD. The intracellular GOD activity was more in above mentioned fungal species. No work has been reported on these two species National and International. This is the first report in India on fungal species isolated from endophytes. In ammonium sulphate fraction, these two fungal species yielded more enzyme recovery. Further work has to done on purification and optimization of various parameters for the isolated fungal species.

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