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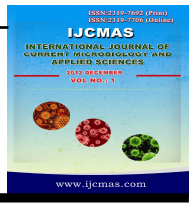

Original Research Article

Optimization of various parameters for the production of glucose oxidase using *Aspergillus niger*

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Isolation of *A. niger;*
Assay of GOD enzyme;
Characterization of GOD;
Optimization of GOD.

A B S T R A C T

In the present study industrially important fungi (*Aspergillus niger*) was isolated from soil samples using dilution and plating technique. *Aspergillus niger* was identified by LCB staining. The isolated strain of *A.niger* were screened for Glucose oxidase production by plate assay. The high yielding strain of *A.niger* was isolated and maintained in agar slant as wild type. The strain of *A.niger* was checked for the Glucose oxidase production under different conditions, like pH, temperature, different carbon sources, nitrogen sources and $CaCO_3$ concentration. Temperature (60) and pH stability (8) of enzyme were also studied. The strain of *A.niger* should increase the production of Glucose oxidase under most of the conditions. Glucose oxidase was characterized by TLC technique and confirmed as Glucose

Introduction

Enzymes are biocatalyst synthesized by living systems, which are important in synthetic as well as degradative processes. The study of enzymes is an important area, because it exists just on the borderline where the biological and physical sciences meet. Life depends on the complex network of chemical reactions carried out by specific enzymes may have far reaching consequences for the living organism. (Dixon and Web, 1964). The use of enzymes in food preservation and processing predates modern civilization. Fermentation of common substrates such as fruits, vegetables, meat and milk provide a diverse array of food in the human diet. Beer, wine, pickles, sausage, salami, yogurts, cheese and buttermilk are all fermented products. Irrespective of their origin, these fermented food products are, in fact, result of the enzymatic modification of constituents in the substrate. The use of enzymes in food industry also involves a range of effects including the production of food quality attributes such as flavors and fragrances and control of colour, texture, appearance besides affecting their nutritive value. Glucose oxidase (GOX) (b-D-glucose: oxygen 1-oxidoreductase, EC 1: 1:3:4) is an enzyme that oxidizes glucose to gluconic acid. It is present in all aerobic organisms and normally functions in conjunction with catalase. It is widely used as a diagnostic reagent in medicine, in the measurement of glucose level in blood. GOX is also used as an antioxidant (Berg *et al.,* 1992) and development of bioelectrochemical cell.

Glucose oxidase was first isolated from mycelia of *A. niger* and *Penicillium glaucum* by Müller (1928). Nowadays, the industrial production of GOx is carried out using both *A. niger* and *P. amagasakiense*. Beside these two fungi, many other microorganisms were recorded as GOx producers such as *Penicillium variabile* (Petruccioli and Federici, 1993), *Phanerochaete chrysosporium* (Kelly and Reedy, 1986), *Talaromyces flavus* (Kim *et al.,* 1990), *Penicillium expansum, P. italiacum,* and other *Penicillium spp.* (Petruccioli *et al.,* 1993), *Penicillium notatum* and *P. paxilli* (Fiedurek *et al.,* 1986),

Penicillium pinophilum (Rando, *et al.*, 1997) but in lower concentrations compared to the main industrial producer strains.

Materials and Methods

Isolation of fungi

The present study was carried out to isolate an industrially important fungi from soil sample. The soil sample collected from sampling spot was processed using serial dilutions and plating methods. The colonies observed on the PDA and RBA plates were transferred on to the growth medium amended with cellulose as sole carbon source. The growth medium used was as Basal fermentation medium. The clear zone observed around the colony was the indication Glucose oxidase production.

Fermentation

The fungi was inoculated into the Basel fermentation medium and incubated at room temperature for ten days and enzyme activity and protein production was determined.

Basal fermentation medium	**(g/l)**
Glucose	8.0
Urea	0.6
K_2HPO_4	1.2
CaCO3	0.08
Agar	20.0
Distilled water	200 ml

Preparation of Basel fermentation medium

Soil derived *A. niger* along with wild type was subjected to submerged fermentation for enhanced production of glucose oxidase. Basal fermentation media comprising (g/100mL) glucose 4, Urea 0.3, KH2PO4 0.6 and CaCO3 0.04, initial pH 5, temperature 30°C was inoculated by 5% inoculum and placed on an orbital shaking at 120 rpm for 36 h (Zia, 2007). Crude product was filtered using Whatman filter paper No. 1 and centrifuged for 15 min. at 10, 000 rpm and -10◦C in order to remove the biomass (Gromada and Fiedurek, 1997). Furthermore, different growth conditions (carbon and nitrogen sources, different salts and other conditions) for the wild strain were inspected to meet the higher yield of glucose oxidase.

Estimation of Proteins

Total proteins in plant samples were estimated by the methods of Lowry's *et al*.(1951). 5ml of the sample centrifuged at 2000 rpm for 10 minutes.The supernatant was collected for protein estimation. For test 0.2 ml of diluted sample was taken and made up to 1 ml with distilled water. Add 5 ml of reagent C to each tube including blank and it was allowed to stand for 10 minutes. Exactly 0.5 ml of diluted Folin's reagent was added to all tubes with continuous shaking and allowed to stand for 30 minutes. The colour developed was read at 645 nm using reagent blank. Then O.D value was obtained and compared with the standard graph which was plotted using BSA as standard and the concentration of unknown protein was calculated.

Measurement of Glucose oxidase assay

The GOD activity was determined by titration method under the following conditions. 1 ml of enzyme solution was added to 25 ml of 60 Mm sodium acetate buffer pH 5.6 containing 2% glucose. The mixture was shaken for 1 hr in air at 30ºC in a rotatary shaker at 200 rpm. Sodium hydroxide solution was added to stop the reaction. The resulting mixture was titrated to red end point by 0.1 M standard HCL solution using phenolphthalein as an indicator. The volume of added standard HCl solution was V ml. the blank assay was performed under the same conditions**.**

Effect of varoius parameters on Glucose oxidase production

Influence of pH on Glucose oxidase production

One set of 5 test tubes were taken for wild type. 10ml of sterile Basel fermentation broth in 5 test tubes. The first test tube was maintained as blank. Tubes from 4 to 8 were adjusted for pH 4, 5, 6, 7 and 8. The test tubes were inoculated with wild strain. The tubes were incubated at 30ºC for 96 hours. After the incubation GOD assay was carried out.

Influence of different temperature on Glucose oxidase production

One set of 5 test tubes were taken for wild. 10ml of sterile modified nutrient broth in 5 test tubes. The tubes from 1 to 5 were marked as 25, 30, 35, 40 and 45ºC respectively. The first sets of test tubes were inoculated wild strain. All the tubes were incubated at 30ºC for 96 hours. After the incubation Glucose oxidase assay was carried out.

Influence of carbon source on Glucose oxidase production

The BFM was altered with various carbon sources to

find out the effect of different carbon sources on Glucose oxidase production. The carbon sources used for the alteration were maltose, lactose, sucrose, fructose and starch. To find out optimum concentration carbon substrates required for the production of Glucose oxidase. The test tubes were inoculated with wild strain. The corresponding tubes were incubated at 30ºC for 96 hours. After the incubation Glucose oxidase assay was carried out.

Influence of nitrogen source on Glucose oxidase production

The BFM was altered with various nitrogen sources from its usual ingredients. The nitrogen sources used for the alteration were peptone, yeast extract, $NaNo_3$, potassium nitrate, and nutrient broth the altered inoculation medium was prepared. The first sets of test tubes were inoculated wild strain. The second sets of test tubes were inoculated with mutant strain. The corresponding tubes were incubated at 30ºC for 96 hours. After the incubation Glucose oxidase assay was carried out.

Influence of CaCO3 concentrations on Glucose oxidase production

A.niger taken into test tube containing inoculum medium was grown in BFM supplemented with various concentrations of CaCO3 (0.5, 1.0, 1.5, 2.0., 2.5%). All the inoculated test tubes were incubated at 30ºC for 96 hrs. Then enzyme assay was performed after incubation period.

Influence of different incubation periods on Glucose oxidase production

A.niger taken into test tube containing inoculum medium was grown in BFM supplemented with various incubation periods (24, 48, 72, 96, 120 hrs). All the inoculated test tubes were incubated at 30ºC for 96 hrs. Then enzyme assay was performed after incubation period.

Stability of Glucose oxidase enzyme pH stability of the enzyme

The optimum pH of the enzyme was determined was using different pH buffer (i. e) using acetete (pH 5-9), The purified buffers was incubated in these 30 minutes at 30ºC and then assayed for the residual activity for determining its pH stability.

Inoculated medium was centrifuged at 5000rpm and supernatant enzyme fluid were only taken. To the enzyme 1ml and 2 ml of buffer were added in a series of test tubes and kept at 30ºC for 30 minutes the Glucose oxidase producing strain were tested.

Thermal stability of the enzyme

The medium was centrifuged at 5000rpm and supernatant enzyme fluid was only taken. To the enzyme 1ml and 2ml of Tris acetate buffer was added to make the pH 7. These preparations were taken in a series of test tubes and incubated at different temperature, from 40ºC to 80ºC and the incubation temperature various from 30minutes. This test was done in Glucose oxidase producing strain and the thermal stability of the enzyme was noted.

Separation of Glucose oxidase by thin layer chromatography

Dry clean glass plates were placed over a plain surface. The silica slurry was prepared and poured on the glass slide with thickness of 0.25mm. The Plates were heated and allowed to dry at room temperature for 5-30 minutes. The plates were heated in an oven a 100ºC to 120ºC for 1-2 hrs.

The moisture was removed and the absorbent were activated on the plate. The plates were dried and can be stored in a dessicator to prevent moisture adsorption. The enzyme samples and standard were applied by means of a micropipette or syringe as a small spots. All the spots should be placed at equal distance from one end of the plate. The samples were allowed to dry so that spotting can be done repeadly for more concentrated sample spot. The plates were sprayed with 5% Aniline-diphenylamine.The plates were kept in a oven at 100ºC. The brown colour spots observed in wild of *A.niger* and Rf values were measured.

Results and Discussion

Screening of Gluco oxidase producing organisms

Soil samples collected from rhizosphere soils were analysed for Glucose oxidase producing fungi. Colonies observed with zone of utilization on Basel fermentation medium were isolated. (Table.1). The above results was accepted with previous studies done by Hamilton *et al* (1999) Cordeiro *et al* (2002).

Table -1 Glucose oxidase fermentation behaviour of *A.niger*

Sl. No	Time (hours)	GOD* Activity	Soluble sugar*	Soluble protein
1	24	1.65	08.05	2.70
2	48	2.66	09.53	4.00
3	72	2.00	10.40	1.60

*mg/ml

Influence of carbon sources on Gluco oxidase production

To evaluate the influence of different carbon sources of GOD production using *A.niger* culture was inoculated in production media containing lactose, starch, maltose, sucrose and fructose. Among this five carbon sources more amount of GOD was assayed in maltose containing media. Maximum GOD activity was observed in maltose containing medium with 2.66 IU/ml(Table.2). This was agreed with earlier reports by (Rajanakanth and Ravi, 1998). *Myceliophora thermophilia* D 14 (Sadhukhan *et al.*, 1990), *Aspergillus fumigatus* (Goto *et al*., 1998), *Rhizopus* sp. (Cruz *et al.*, 1997).

Table 2 Effect of various carbon sources on GOD production by *A.niger*

Sl.No	Carbon sources	Enzyme activity (IU/ml)	Protein (mg/ml)
1	Maltose	2.6	1.94
2	Lactose	7.0	1.74
3	Sucrose	0.66	1.96
4	Starch	1.30	3.16
5	Fructose	1.16	2.74

Influence of nitrogen sources on Gluco oxidase production

Another set of experiment was carried out to find out which nitrogen source influence the glucose oxidase production by *A.niger*. Different sets of media were prepared containing nitrogen sources such as peptone, yeast extract, $NaNO_3$, Potassium nitrate and nutrient broth. Each nitrogen source had individual influence glucose oxidase production in wild type. Potassium nitrate containing media showed higher glucose oxidase production with 3.5 IU/ml (Table.3).

Table 3 Effect of various Nitrogen sources on GOD production by *A.niger*

Sl.No	Carbon sources	Enzyme activity (IU/ml)	Protein (mg/ml)
1	Peptone	2.83	3.92
2	Yeast extract	2.66	2.60
3	Sodium Nitrate	0.82	2.36
4	Potassium nitrate	3.50	2.34
5	Nutrient Broth	1.66	2.54

This was agreed with earlier reports by (Rajanakanth and Ravi, 1998). *Myceliophora thermophilia* D 14 (Sadhukhan *et al.*, 1990), *Aspergillus fumigatus* (Goto *et al.*, 1998), *Rhizopus* sp (Cruz *et al.*, 1997).

Effect of CaCO3 on a Gluco oxidase production

In this, maximum glucose oxidase production was observed in 1% with 4.0 IU/ml. The low level of glucose oxidase production was observed in 2.5% with 0.16 IU/ml (Table.4).

Table-4 Effect of various Different Conc.CaCO3on GOD production by *A.niger*

Sl.No	Different Conc. CaCO3	Enzyme activity (IU/ml)	Protein (mg/ml)
1	0.5%	2.33	3.42
2	1.0%	4.0	3.52
3	1.5%	1.83	3.04
4	2.0%	3.50	3.62
5	2.5%	0.16	3.74

Influence of temperature on Gluco oxidase production

To observe the influence of temperature of GOD production for wild strain of *A.niger* was maintained at 25,30,35 40 and 45˚C. Low level of glucose oxidase was assessed at 40˚C with 13.1 IU/ml. The production was high at temperatures 30˚C with 19.83 IU/ml (Table.5). This results was correlated with previous works by Lui and Xu (2007); Konsula and Kyriakides (2004); Rajanikanth and Ravi (1998).

Table-5 Effect of various Different temperature on GOD production by *A.niger*

Sl.No	Different Temperature ranges(ºC)	Enzyme activity (IU/ml)	Protein (mg/ml)
1	25	17.0	3.54
2	30	19.83	3.32
3	35	18.1	3.56
4	40	13.1	3.64
5	45	14.0	3.62

Influence of pH on production Gluco oxidase

An experiment was carried out to find out the effect of pH GOD production for wild strain of *A.niger*. The range of pH taken for this experiment was 4,5,6,7 and 8. The maximum production was observed at pH 8 with 3.5 IU/ml (Table.6). This was agreed with earlier reports by Rajanikanth and Ravi (1998); (2001); Liu and Xu, (2007); Mitsuiki *et al* (2005).

Table-6 Effect of various Different pH on GOD production by *A.niger*

Sl. No	Different pH Ranges	Enzyme activity (IU/ml)	Protein (mg/ml)
1	4	0.16	3.54
2	5	1.33	3.10
3	6	1.0	3.38
4	7	0.66	3.76
5	8	3.5	3.52

Effect of incubation periods on Gluco oxidase production

In this, maximum glucose oxidase production was observed in 96% with 21.5 IU/ml. The low level of glucose oxidase production was observed in 2.5% with 13.83 IU/ml (Table.7). .

Table-7 Effect of various Different Incubation periods on GOD production by *A.niger*

Sl. No	Different incubation periods (hrs)	Enzyme activity (IU/ml)	Protein (mg/ml)
1	24	17.83	2.84
2	48	13.83	3.52
3	72	17.33	2.30
4	96	21.50	2.52
5	120	18.66	2.60

Enzyme characterization
Thermal stability

Thermal stability of GOD enzyme isolated from A.*niger* was examined at various temperatures, (40-80ºC) along with various incubation periods, (30 minutes). The enzyme was stable at 60 ºC with 4.68 (Table.8). Thermostability of GOD was correlated with earlier studies of Liu and Xu (2007); Mitsuiki *et al* (2005).

Table-8 Thermostability of GOD activity

Sl.No	Temperature	Enzyme Activity
1	40	3.54
2	50	3.87
3	60	4.68
4	70	4.05
5	80	3.64

pH stability

The optimum pH of the enzyme was determined using Tris HCl (pH 5-9.0). The purified enzyme was incubated in these buffers for 30minutes at 30ºC and the residual activity was assayed for determining its pH activity. The enzyme was stable for 30 minutes at pH 8 with 6.31 (Table.9). Similar results have been reported by Liu and Xu (2007); Mitsuiki *et al* (2005).

Table-9 pH stability of GOD activity

Sl.No	Different pH	Enzyme activity (IU/ml)
1	5	4.34
2	6	5.67
3	7	5.03
4	8	6.31
5	9	5.99

Separation of Gluco oxidase by Thin Layer Chromatography

GOD was characterized by TLC technique. In this study, Rf value was found to be maximum in 0.81. It was compared with standard and confirmed as glucose. This chromatography studies was compared with previous work done by Dhanasekaran *et al* (2006); Najafi *et al* (2005). Therefore under optimum fermentation conditions involved in maltose and potassium nitrate as supplements and pH of 8 at 30ºC enhanced the production of Glucose oxidase. The strain of *A.niger* showed increased production under most of the conditions.

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