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# Propagation of *Aspergillus niger* in Stirred Fermentor for the Production of Glucose Oxidase

**Irshad Ahmad[1]*, Zia Ul Islam[2], Zhisheng Yu[2] and Muhammad Mohsin Javed[3]**

[1]Department of Microbiology and Biotechnology, Sarhad University of Science and Information Technology, Peshawar, Khyber Pakhtunkhwa, Pakistan.
[2]College of Resources and Environment, University of the Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China.
[3]Institute of Industrial Biotechnology, Government College University, Lahore, Punjab, Pakistan.

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**The present research work was planned for enhanced glucose oxidase production in *Aspergillus niger* by optimizing the growth conditions in a stirred fermentor. The results revealed that medium M2 containing (g/L) glucose 80.0, peptone 3.0, $(NH_4)_2HPO_4$ 0.388, $KH_2PO_4$ 0.188 and $MgSO_4.7H_2O$ 0.156 was the best among four tested media. Glucose (8%) was the best carbon source for the maximum yield of glucose oxidase (118 U/g cell mass) followed by sucrose (103 U/g cell mass) and fructose (99 U/g cell mass). $(NH_4)_2HPO_4$ followed by peptone were the best nitrogen sources for the growth of *A. niger* and production of glucose oxidase. The maximum activity (118 U/g) of glucose oxidase was produced after 72 hours of fermentation at pH 5.0, temperature 30°C, agitation 200 rpm and aeration of 1 vvm.**

**Key words:** *Aspergillus Niger,* Glucose oxidase production, Stirred fermentor.

Fungi have the ability to produce extremely useful enzymes and therefore occupy a very key position in the realms of biotechnology and microbiology. A lot of fungal derived enzymes have been used for the rapid oxidation and breakdown of fats, carbohydrates and proteins. Enzyme producing fungi with useful characteristics continue to be a field for utilization (Gow and Gadd, 1995). The importance of *Aspergillus niger* in fermentation industries is not only limited to its more than 35 native products but also on the production and commercialization of new products which are derived by modern biotechnological techniques (Swoboda and Massey, 1965).

* To whom all correspondence should be addressed.
Tel.: +92937929124; Mob.: +923479789151;
Fax: +929379230619;
E-mail: adenzai_ia@yahoo.com

Enzyme production can be carried out either in solid state or submerged fermentation. In most cases enzymes are produced in submerged fermentation. Aeration and agitation can be supplied in submerged fermentation. Aeration is used to enhance oxygen supply while agitation increases the effect of aeration by forcing the separation of cells from mycelial aggregates and hence facilitates oxygen supply to every cell. Submerged fermentations have continuous shaking system and therefore more suitable for glucose oxidase production (Friedurek and Gromada, 1996). The production of enzymes by submerged fermentation has long been established (Papagianni *et al*., 2001). Submerged fermentation shows several advantages over other types of fermentation regarding automatation, handling, monitoring and control. Other positive factors of submerged fermentation are the addition of supplement nutrients, reagents and easy removal of heat generated duringmicrobial growth in a large

scale reactor (Jakubikova and Nemovic, 2006).

Glucose oxidase from *A. niger* is a dimer which is made up of two identical monomeric subunits having molecular weight of 150 to 180 kDa. It contains two tightly bound FAD molecules (Pazur and Kleppe, 1964). The two subunits dissociate only under denaturation conditions which results in the loss of cofactor FAD (Jones *et al.*, 1982). The enzyme exhibits a high specificity for β-D-glucose. It also oxidize other monosaccharides but at a much lower rate (Adams *et al.*, 1969). Many researchers have optimised the maximum production of glucose oxidase in different fungal strains by using various nutrients investigated the role of carbon and nitrogen sources on production of glucose oxidase (Tsuge *et al.*, 1965). Among natural carbon sources, beet molasses was noticed to be the best carbon source for the growth of *A. niger* and GOX production. Nitrate and urea were found to be the best nitrogen source as compared to ammonium salts. Glucose oxidase has a lot of industrial applications. Therefore in the present study cost effective production ofglucose oxidase was obtained in *A. niger* by using inexpensive substrates.

## MATERIALS AND METHODS

The chemical reagents used in this research work were of analytical grade and purchased from Sigma, Merck, Acros and Fisher scientific chemical companies.

**Microorganism and Culture Maintenance**

*Aspergillus niger* was used as an experimental organism which was obtained from the stock culture source of institute of industrial biotechnology, GC University, Lahore. The culture was maintained on potato dextrose agar slants and stored at 4°C in refrigerator. Sub-culturing of the mold was carried out after 15 days on potato dextrose agar slants. The slants were inoculated by transferring small amount of spores in asceptic conditions and incubated at 30°C for 5-7 days for maximum sporulation and then refrigerated.

**Inoculum Development**

The spore inoculum was prepared by addition of sterilized distilled water in the culture slants having perfused sporulation. The slants were vortexed at high speed to make an apparently homogenous suspension of spores. The suspension was eluted and used as spore inoculum. Inoculum for fermentor was developed in 250 ml sterilized Erlenmeyer flask having 75 ml growth medium. Each flask was then inoculated with 2 ml spore suspension from 48 hours old cultured slants. The flasks were then incubated in shaker at 28±2.0°C at 200 rpm.

**Fermentation Media**

Four fermentation media (g/l) were used for glucose oxidase fermentation.

M1: Glucose 80.0, Peptone 3.0, NaNO35.0, $KH_2PO_4$ 1.0, FeSO4.7H2O 0.01, $MgSO_4$. $7H_2O$0.5 and $CaCO_3$ 35.0(Petruccioli and Federici, 1993).

M2: Glucose 80.0, Peptone 3.0, (NH4)2HPO4 0.388, KH2PO4 0.188, MgSO4.7H2O 0.156 and $CaCO_3$ 35.0 (Fiedurek and Szczodrak, 1995).

M3: Glucose 60.0, $NH_4NO_3$ 0.3, $KH_2PO_4$ 0.25, $MgSO_4.7H_2O$ 0.25,Urea 2.0 and CSL8 ml(Li and Chen, 1994).

M4: Glucose 18.0, $NaNO_3$3.0, $K_2HPO_4$1.0, $MgSO_4.7H_2O$ 0.5, KCl, 0.5, $FeSO_4.7H_2O$0.01 and Glycerol 20.0 (Markwell *et al.*, 1989).

**Stirred Fermentor**

The fermentation was carried out in a 7.5 liter glass fermentor (Model; Bioflo 110, New Brunswick Scientific, USA). The fermentor glass vessels containing 5.0 L fermentation media was sterilized in a stainless steel autoclave (Model, KT-40 L, Japan) for 30 minutes at 15 lb/in$^2$ pressure and 121°C temperature and then cooled at room temperature. The inoculum was then transferred to the vessel through a hole at the top plate under aseptic conditions. The incubation temperature, speed of agitation and rate of aeration were maintained at 30°C, 200 rpm and 1.0 vvm respectively throughout fermentation. The sterilized 20% v/v synthetic antifoam was used to control foam formation during fermentation. Sample harvesting was done at various intervals. The suspension was filtered through a whatman filter paper and the separated mycelium was washed twice with distilled water and 1 g of mycelium was grinded in 10 ml of 0.1 M citrate phosphate buffer phosphate buffer (pH 7) for intracellular glucose oxidase determination.

**Enzyme Assay**

Three hundred microlitre glucose (18%), 2500 µl of O-Dianisidine solution and 100 µl

Peroxidase solution (10 Units/ml) was pipetted out in a cuvette. This substrate-chromogen solution was considered as blank solution. The spectrophotometer was set to zero reading at 25°C temperature and 450nm wavelength after inserting the cuvette with blank solution. An incubation of 3-5 minutes was given to achieve the temperature equilibrium. Then 100 µl of glucose oxidase containing solution was added in substrate-chromogen solution and increase in absorbance was recorded for 5 minutes at 450 nm wavelength with the interval of 60 sec (Worthingto, 1988). One unit of glucose oxidase was defined as the amount of enzyme required to oxidize one mmole of glucose per minute at 25°C. ΔA/min over the linear portion of the reaction was measured. One Unit of GOD catalyzed the oxidation of 1.0 micromole of glucose into gluconic acid. The amount of glucose utilized or that remained was also determined during the course study of optimized fermentation period. In case of soluble glucose, the remaining utilized glucose was determined by estimating the sugar by DNS (dinitro-salicylic acid) method using glucose as standard while protein was estimated according to the method as described by (Bradford, 1976).

## RESULTS

### Screening of media

Four different media were used for the cultivation of *Aspergillus niger* for glucose oxidase production. Figure 4shows the comparative yield of glucose oxidase (U/g) of cell mass, biomass content (g/l) and intracellular protein content (mg/g). Maximum yield (Glucose oxidase 104 U/g, Intracellular protein 19.3 mg/g and biomass Content 33 g/l) was obtained on medium M2. Therefore M2 medium was used in subsequent studies and optimized.

### Effect of time of fermentation

The effect of fermentation time in hours (12, 24, 36,48,60,72 and 80) on the production of glucose oxidase by *A. niger* in stirred fermentor is shown in Figure 2. An increasing trend in glucose oxidase activity was observed with the passage of

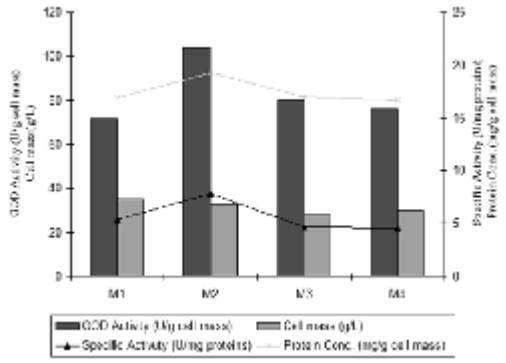


**Fig. 1.** Screening of media

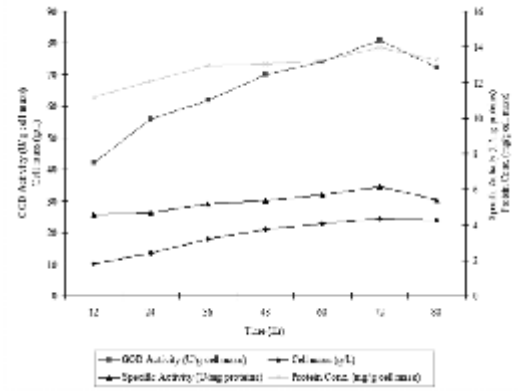

**Fig. 2.** Effect of fermentation time

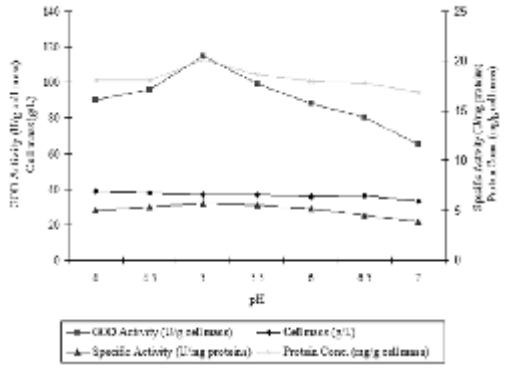


**Fig. 3.** Effect of pH

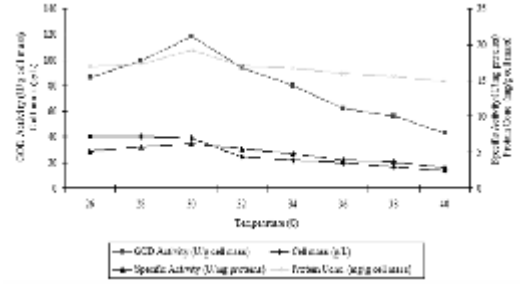

**Fig. 4.** Effect of temperature

fermentation time. The maximum activity of glucose oxidase (80 U/g), total protein content (13.96 mg/g of cell mass), specific activity of glucose oxidase (6.153 U/mg of protein) and total cell mass (24.49 g/l) was obtained after 72 hrs of fermentation. After 72 hrs no significant increase was observed in enzyme activity. Therefore 72 hours of fermentation was optimized for maximum activity of glucose oxidase in stirred fermentor.

**Effect of pH**

The effect of different pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) on the production of glucose oxidase is shown in Figure 3.It was found that glucose oxidase activity increased with increasing the pH value. The maximum activity of glucose oxidase (115 U/g), total protein content (20.1 mg/g of cell mass) and specific activity of glucose oxidase (5.75 U/mg of protein) was obtained at pH 5.0. While the total cell mass was higher (39 g/l) at low pH 4.0 and lower (33 g/l) at higher pH 7.0. Further increase in pH showed a decline in enzyme activity and total protein content. Therefore pH level of 5.0 was optimized for optimum activity of glucose oxidase in stirred fermentor.

**Effect of temperature**

Figure 4 illustrates the effect of different temperatures (26, 28, 30, 32, 34, 36, 38 and 40 °C on glucose oxidase production. It was found that the activity of glucose oxidase (118 U/g of cell mass) with total protein content (19.16 mg/g of cell mass) and specific activity (6.178 U/mg of protein) was maximum at 30ÚC. Further increase in temperature resulted in decreased activity of glucose oxiadse. The total biomass content was higher (40.2 g/l) at lower temperature (26°C) and lower (13.39 g/l) at high temperature (40°C).Therefore an incubation temperature of 30°C was optimized for the maximum production of glucose oxidase.

**Effect of various carbon sources**

Effect of various carbon sources (glucose, sucrose, fructose, maltose and molasses) on the production of glucose oxidase were investigated in stirred fermentor as shown in Figure 5.Maximum glucose oxidase activity (115 U/g of cell mass) with specific activity (6.284 U/mg of protein) and protein content (18.39 mg/g of cell mass) was obtained when glucose was used as carbon source. Low

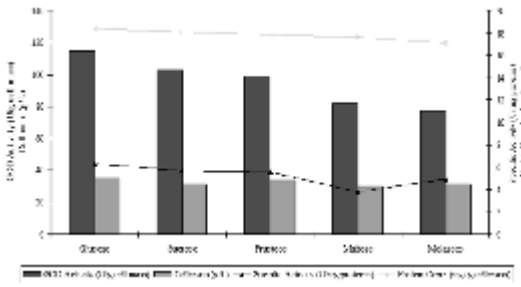

**Fig. 5.** Effect of various carbon sources

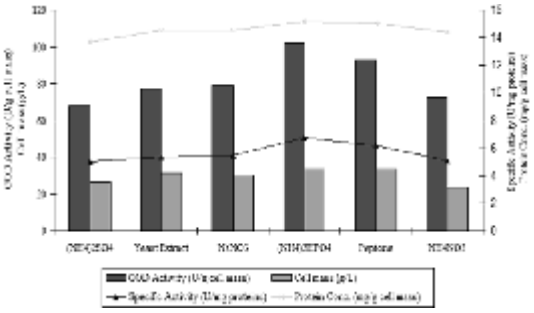

**Fig. 6.** Effect of different nitrogen sources

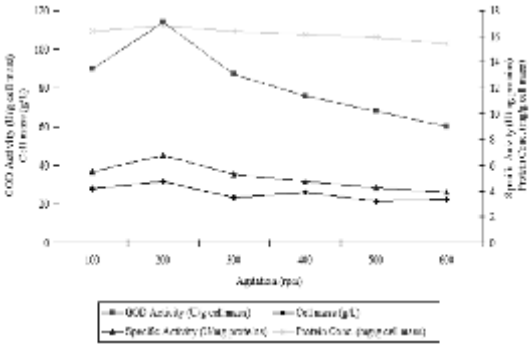

**Fig. 7.** Effect agitation rate

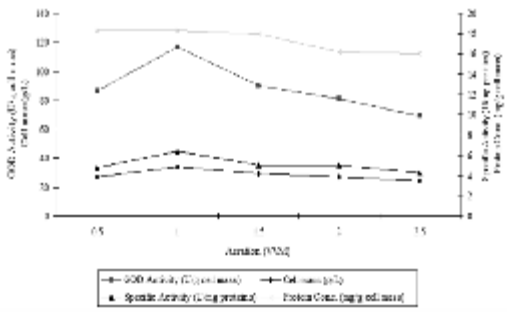

**Fig. 8.** Effect of aeration rate

activity was obtained with maltose and molasses. Hence glucose was used as the sole carbon source in further studies.

**Effect of different nitrogen sources**

Figure 6 represents the effect of various nitrogen sources (ammonium sulphate, ammonium nitrate, yeast extract, peptone, diammonium hydrogen and sodium nitrate). Among the tested nitrogen sources, diammonium hydrogen phosphate gave the highest activity of glucose oxidase (102 U/g of cell mass) with specific activity (6.75 U/mg of protein) and total protein content (15.18 mg/g) while the other nitrogen sources showed less effect on glucose oxidase activity.

**Effect of rate of agitation**

The influence of different agitation speeds (100,200,300,400,500 and 600 rpm) was also studied for the production of glucose oxidase in stirred fermentoras shown in Figure 7. It was observed that maximum glucose oxidase activity (114 U/g of cell mass) with specific activity (6.785 U/mg of protein) was obtained when the culture was agitated at 200 rpm. Further increase in agitation speed did not show any significant increase in enzyme activity. Therefore 200 rpm was optimized for glucose oxidase production in stirred fermentor.

**Effect of rate of aeration**

The effect of aeration on glucose oxidase production in stirred fermentor is shown in Figure 8. It was noticed that the activity of glucose oxidase (117 U/g of cell mass) with total protein content (18.28 mg/g of cell mass) and specific activity (6.43 U/mg of protein) was maximum at 1.0 vvm as compared to the other aeration conditions. Further increase in aeration did not show significant increase in glucose oxidase activity.

## DISCUSSION

Glucose oxidase is an important enzyme in food industry, pharmaceutical industry and glucose biosensors. Initially glucose oxidase was isolated from mycelium of *Penicilium glaucum* and *Aspergillus niger* (Muller, 1928). Many other fungal species belonging to genus *Penicillium* and *Aspergillus* have been reported to produce glucose oxidase but currently *A. niger* is mostly used for industrial production of glucose oxidase. Therefore the present study was carried out to optimize cultural conditions for the glucose oxidase production in stirred fermentor having 5.0 L working volume.

A successful fermentation depends on an appropriate strain and optimization of cultural conditions (Gupta *et al*., 2003; Saxena *et al*., 2007).Selection of a suitable fermentation medium is very essential for the optimum production of glucose oxidase. Four previously optimized media were tested for glucose oxidase production by *A. niger*. Among the tested media, M2 was found to be the best medium for glucose oxiadse production. It might be due to the fact that other three medium lack some nutrients essential for the productivity of glucose oxidase.Various carbon sources were used to improve the growth and total activity of glucose oxidase using *A. niger* (Hatzinikolaou and Macris, 1995).

Fermentation time for the production of the GOX was studied from 12-80 h. It was noticed from the results that glucose oxidase production was enhanced with increase in fermentation duration and optimum production (80 U/g) was achieved after 72 h of fermentation which is in accordance with the results of (Bodade *et al*., 2010) who also obtained maximum production of glucose oxidase after 72 h of fermentation. Maximum glucose oxidase activity was obtained after 36 hours of fermentation (Zubair *et al*., 2002). Highest glucose oxidase activity was obtained after 48 hours and 60 hours of fermentation (Kona *et al*., 2001; Friedurek and Gromada, 2000). These earlier reports are different to our results due to differences in conditions. After 72 h of fermentation, glucose oxidase activity decreased, which can be due to the decrease in concentration of nutrients or excretion of some toxic waste products by the organisms in fermentation media.

Optimal pH is very essential for the growth of *A. niger* and maximal glucose oxidase activity. Different pH (4.0-7.0) was used for glucose oxidase production in stirred fermentor. It was noted that the activity of glucose oxidase was maximum at pH 5.0 which is in accordance with the results of (Swoboda and Massey, 1965) who also obtained highest production of glucose oxidase at 5.0.Maximum activity of GOX was reported at pH 6.0 (Liu and Curry, 2004). Further increase in pH showed a decrease in enzyme activity.

Temperature is another important

parameter for the production of glucose oxidase. Different incubation temperatures (26-40°C) were used for optimal production of glucose oxidase in shake flask. Maximum activity of glucose oxidase was obtained at 30°C which is in accordance with the results of (Kona *et al*., 2001). Maximum production of glucose oxidase was reported at 30°C using *A. niger* (Friedurek and Gromada, 2000). Highest glucose oxidase activity was obtained at 28°C (Petruccioli and Federici, 1993). The activity slowly decreased above 30°C. The use of different carbon sources is very critical for the improvement and cost effective production of glucose oxidase. Carbon sources are not only used as an energy source but also a major part of mycelial mass. Most studies have shown that among different carbon substrates, glucose and sucrose show higher activity (Friedurek and Gromada, 1996; Kona *et al*., 2001).

In the present study *A. niger* showed growth on all the carbon substrates.Maximum level of glucose oxidase was achieved using glucose followed by sucrose and fructose. Glucose was the principal inducer for the transcription of glucose oxidase gene (Hatzinikolaou and Macris, 1995). High level of glucose oxidasewas produced using glucose and mannose (Petruccioli *et al*., 1995). Among different concentrations of glucose tested, 8% was found to be optimal for glucose oxidase using *A. niger* which is in accordance with the results of (Rogalski *et al*., 1988;0Petruccioli and Federici, 1993). Glucose level of 15% showed optimalglucose oxidase production (Ray and Banik, 1999). Among tested nitrogen sources, diammonium hydrogen phosphate (0.0360% w/v) was found to be optimal for the growth and total activity of glucose oxidase. In contrast, ((Hatzinikolaou and Macris, 1995) reported that using sucrose as carbon source, peptone (1-2%) and molasses (0.2-0.3%) was found to be optimal for glucose oxidase activity.

Aeration and agitation both are very important factors for the growth and enzyme production by *A. niger*. Agitation is used to increase the efficiency of aeration by breaking the air bubbles into smaller ones. Different agitation speeds (100-600 rpm) were used in stirred fermentor. Maximum glucose oxidase was obtained at 200 rpm. Similar results were obtained in *A. wentii*and and *P. chrysogenum* (Chander *et al*., 1980) and it was found that a shaking speed of 200 rpm was the best for glucose oxidase production. An increase in glucose oxidase activity was observed when the stir speed was raised from 150 to 300 rpm (Jafari *et al*., 2007). 300 rpm was the best for maximum growth and higher glucose oxidase activity (Fiedurek, 1998). Highest amount of glucose oxidase was produced at 700 rpm (Zetelaki and Vas, 1968). Further increase in agitation speed has resulted in decrease enzyme activity and growth possibly due to increase in shear stress.

Different volumes of air supply were studied for the production of glucose oxidase in stirred fermentor. Aeration not only provides the oxygen required for the growth of microorganism but also facilitate the removal of gaseous waste products. Oxygen for growth and production in fungal cultures can be ensured by the aeration and agitation of the mycelial culture (Zetelaki and Vas, 1968). It was noted that 1.0 vvm of air supply was optimum for glucose oxidase production using *A. niger* in stirred fermentor. Maximum glucose oxidase activity was obtained at 1.5 vvm while the best aeration rate for growth was 2 vvm (Jafari *et al*., 2007). Maximum activity of glucose oxidase was obtained at 0.9 vvm (Liu *et al*., 2003).

## CONCLUSION

In this work, glucose oxidase production was studied in *A. niger* by optimizing the growth conditions in a stirred fermentor. Among tested media, M2 was the best for the production of glucose oxidase. The maximum activity (118 U/g) of glucose oxidase was produced after 72 hours of fermentation at pH 5.0, temperature 30°C, agitation 200 rpm and aeration of 1 vvm.

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