OPTIMIZATION OF FERMENTATION CONDITIONS FOR THE PRODUCTION OF GLUCOSE OXIDASE BY *ASPERGILLUS NIGER*

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ABSTRACT: In this study conditions for the enhanced production of glucose oxidase by *Aspergillus niger* were optimized in shake flask. Biomass production, intra and extra cellular glucose oxidase production, $CaCO_3$, glucose and nitrogen source were optimized. Maximum glucose oxidase production was recorded after 12 hours of incubation. Glucose Oxidase was released at highest between 72-90 hours, intra-cellular enzyme production was highest at 36 hours. $CaCO_3$ and $NaNO_3$ were found as best inducer substrates and glucose was found as best carbon source. The extra cellular activity of enzyme was only 5μ/g while intracellular activity was 350 μ/g. At this time the fungal biomass was 42 g/l. However maximum fungal biomass was obtained at 90 hours of incubation. But the intra-cellular activity is lost till 90 hrs of incubation.

Key Words: Glucose Oxidase (GOX), Fermentation, *Aspergillus niger (A,niger)*, Intracellular, Extracellular

INTRODUCTION

Glucose oxidase (β-D-glucose: oxygen 1-oxidoredutase, 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. Glucose Oxidase is used in pharmaceutical and food industries (Underkofler, 1961). It is widely used as a diagnostic reagent in medicine (Coxon and Schaffer, 1971), in the measurement of glucose level in blood. Glucose Oxidase is also used as an antioxidant (Berg and Neibergall, 1992) and development of bioelectrochemical cell (Lannce *et al.*, 1984). Glucose Oxidase is mainly available from microbial sources and is generally produced by aerobic fermentation of *Aspergillus niger* (Fiedurak and Gormada, 1997 and Rando *et al.*, 1997) and *Penicillium species*. It has high specificity for beta D-glucose (Kuly and Cenas, 1983).

Glucose oxidase is produced by fermentation using different micro-organisms e.g. *Aspergillus niger*, *Penicillium amagasakiense* (Kusai, 1960), *Penicillium notatum*, *Penicillium purpurogem*, *Penicillium variables* and *Alternaria alternate* (Caridis *et al.*, 1991).

Kupletskaya and Karakov (1999) isolated 722 fungal strains capable of producing glucose oxidase. Most of the fungi produce intracellular glucose oxidase but about 76 strains of the genus *Penecillium* produce extracellular glucose oxidase.

The synthesis of glucose oxidase and catalase by *Aspergillus niger* was investigated by using resting culture system. $CaCO_3$ induced the synthesis of both enzyme and $CaCl_2$ inhibited it (Liu *et al* 1999. Liu and coworkers (2001) studied the effect of metal ions on simultaneous production of glucose oxidase. Clarke *et al* (2006) studied the location of glucose oxidase during production by *Aspergillus niger* .Enzyme location impacts significantly on enzyme recovery. Khattab and Bazaraa, (2005) reported the enhancement of extracellular glucose oxidase production by screening, mutagenesis and protoplast fusion of various strains of *Aspergillus niger*.Mu,Wang and their coworkers (2006) studied recombinant *Aspergillus niger* glucose oxidase expressed in *Trichroderma reesei.* This strain has capability to be a new recombinant host for *Aspergillus niger* producing Glucoe Oxidase. The present study includes optimization of fermentation conditions for the maximum production of GOX from *A.niger.*this will help for commercial production of GOX.

MATERIALS AND METHODS

Microorganisms: Strain of *A.niger* GCUC-10 was obtained from available stock cultures of

Biochemistry Laboratory of Department of Chemistry GCU Lahore. It was maintained on potato dextrose agar (PDA, 4.0%,w/v) medium and stored at 4°C (Fieduriek and Gromada, 1997).

Substrate (Carbon source): Glucose obtained from Biochemistry Laboratory of Department of Chemistry, GC University Lahore was used as substrate (carbon source) for the production of glucose oxidase.

Fermentation: This enzyme was produced by submerged fermentation of *A. niger* in 250 ml shake flask. The fermentation medium with the following composition was used.

Table 1: Composition of fermentation medium.

Compounds	Amount g/100 ml
$NaNO_3$	12.5
Urea	0.2
$MgSO_4.7H_2O$	1.25
$FeSO_4.7H_2O$	0.025
KH_2PO_4	2.5
Glucose	80 gm/L
$CaCO_3$	1.75/25 ml H_2O

(Petruccioli and Federici, 1993) with some modifications i.e. urea was used instead of peptone and KCl was not added in medium.

Table 2: Composition of each 250 ml flask.

Ingredients	Amounts
$CaCO_3$	1.75 g/25 ml
$NaNO_3$	2 ml (from stock solution)
Urea	2 ml "
$MgSO_4.7H_2O$	2 ml "
$FeSO_4.7H_2O$	2 ml "
KH_2PO_4	2 ml "
Glucose	10 ml "
Spore suspension	5 ml "

Enzyme Extraction:

(I) For Intracellular Glucose oxidase: After 48 hrs (after optimizing) incubation in shaking incubator at 30 °C, the Mycelia were collected by filtration and washed with 0.1 % Saline water. The Mycelia collected and were weighed. The washed Mycelia were crushed in water in homogenizer at 1500 rpm for 30 minutes and centrifuged at 10,000 rpm for 20 minutes. Mycelium debris was separated and kept for dry mass estimation. The filtrate was precipitated with ethyl alcohol. The filtrate and ethyl alcohol were in 1:4 and kept at 4 °C over night.

The PPT was collected by centrifugation art 10,000 rpm for 15 minutes and pellets of each sample were dissolved in 2ml of distilled water and refrigerated till further use.

(ii) For Extra cellular Glucose oxidase: The process is same as described above only 72 hours (after optimizing) incubation was required and filterate used for extracellular Gox extraction instead of Mycelium which was used for intracellular
GOX extraction (as explained above)

Assay of Glucose oxidase: The glucose oxidase activity was determined by the fast spectrophotometric method by following the enzymatic reduction of benzoquinone to hydroquinone at 290 nm using glucose as substrate (Ciucu and Patroescu, 1984).

One unit (u) of enzyme activity was defined as the amount enzyme producing one micromole of hydrogen peroxide per minute at 30°C.

It was also defined as the amount of enzyme catalyzing the decomposition of one micromole of hydrogen-peroxide per minute at 30°C. or "One unit catalysis the oxidation of 1 µ mole glucose to gluconic acid per minute at 25°C pH 5 coupled with peroxide and benzoquinone. Glucose oxidase oxidize glucose to gluconic acid and H_2O_2 produced in above reaction reduces benzoquinone to hydroquinone. Both spectrometer cell (A and B) were filled up with 2 ml of 1 M glucose solution which was prepared a day before at room temperature so that and anomers formed at equilibrium.

In cell A, 1.0 ml of 0.1% benzoquinone solution (0.01 g in 10 ml distilled water) and 1.0 ml of buffer solution (sodium citrate buffer of 0.1 M, pH = 5) were added. This cell was taken as standard. In cell B, 1.0 ml of 0.1% benzoquinone solution and 0.9 ml of sodium citrate buffer were added. The mixtures of both cell (A and B) were allowed to equilibrate at 25°C for 5 minutes.

In cell B, at time zero 0.05 ml of glucose oxidase solution was added and mixture was stirred. An increase in absorbance at 290 nm was recorded for 1 – 2 minutes.

UV-Cecil CE 7200 Spectrophotometer was used

RESULTS AND DISCUSSION

The rate of growth and enzyme production by microorganisms is quite important in understanding their fermentation pattern.Figure (1) shows the rate of production of fungal biomass during GOX fermentation by *Aspergillus niger* at 30±1°C for120h of incubation.Maximum growth rate was observed between 80 and 90h of fermentation. At 90h 42g/l fungal mass was present. At 90h, there was slight decerease in mycelial mass. Figure 2 shows the extra cellular GOX activity during the fermentation. The rate of GOX secretion into fermentation broth was the highest between 80 to 90hrs and then the rate gradually decreased. The level of extra cellular GOX was the maximum till 90h i.e. 5µ/ml.Then its level slightly decreased due to denaturation of the formed enzyme. Figure3 shows the intracellular GOX activity during GOX fermentation .The intracellular GOX activity after 12 h of incubation was 0.8µ/g then its gradually reached to 350µ/g after 36h.The rate of GOX accumulation inside the mycelia was significantly higher between 24 to 36h.during this period the rate of GOX formation exceeded the rate of its secretion into the broth. After 36h the secretion determinated over its formation and the cell-bouned GOX activity fell down. Figure 4 shows the $CaCO_3$ consumption with time. The maximum $CaCO_3$ i.e.35g/l consumed during the first phase i.e. 12 to 36h and production of organic acid (gluconic Acid) is maximum at this phase.Figure 5 shows the glucose consumption with time. The maximum consumption of glucose i.e.80g/l waas observed between 12-36h.Figure 6 shows the consumption of nitrogen source i.e. $NaNO_3$ It was also between 12-36h (1.3g/l).Afterwards, consumption was slow down. The *production* of fungal biomass, intra and extra cellular Glucose Oxidase, consumption of glucose, $CaCO_3$ and nitrogen source were studied. The results have been represented in their respective graphs.

Biomass Production: Microscopic examination of the fermentation flasks showed that spores did not germinate till 8 hours of incubation. Germinating of microbes from the spores was seen after 8 hours, and was completed till 10 hours. An average of 0.8 grams biomass per liter was found after 12 hours of incubation. Results showed that the culture remained in experimental phase till 24 hours with specific growth rate of 2.5 gh^{-1}. After 90 hours pellets began to form in the flasks, as growth started to decrease.

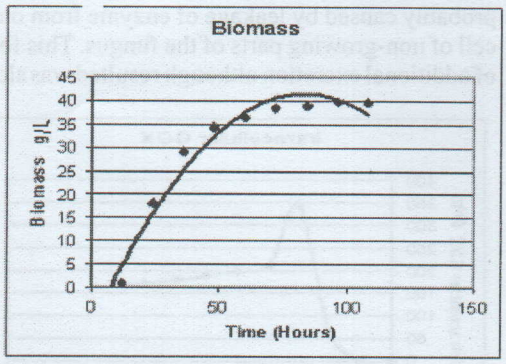

Fig: 1 Biomass production rate with respect to time (hrs).

Extra-cellular Glucose Oxidase: Studies on the extra-cellular production of Glucose Oxidase by A. *niger* revealed that the excretion of Glucose Oxidase was highest between 70-85 h.

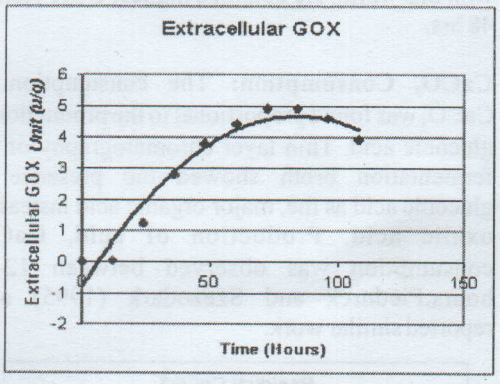

Fig. 2 Extra-cellular glucose oxidase production.

The high excretion during this phase of cultivation was probably growth (Mycelial mass) associated. Although secretion of Glucose Oxidase decreased afterwards but the maximum amount of enzyme was noted after 72 hours cultivation. After 85 hours denaturation of the enzyme dominated over its production and the enzyme titre decreased.Fiedurek (1998) also found highest GOX activity at 72 hrs.

Intra-cellular Glucose Oxidase: The formation of Glucose Oxidase started after 12 hours cultivation. After 24 hours the production of the enzyme exceeded. The highest activity per gram mycelium at 36 hours shows the maximum Glucose Oxidase production at this time. The excretion of enzyme into the culture medium during the second phase of cultivation was

probably caused by leakage of enzyme from older cell of non-growing parts of the fungus. This form of additional excretion although resulted was slow.

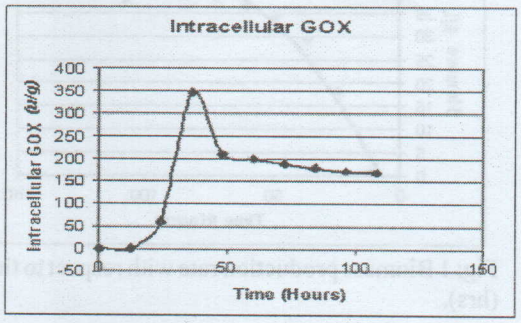

Fig: 3 Intracellular enzyme production with respect to time (hrs).

The results of Willis (1966) are somewhat accord with this work.. He obtained highest GOX yield at 48 hrs.

$CaCO_3$ Consumption: The consumption of $CaCO_3$ was found proportional to the production of gluconic acid. Thin layer chromatography of the fermentation broth showed the presence of gluconic acid as the, major organic acid instead of oxalic acid. Production of acid, $CaCO_3$ consumption was observed between 12-36 hours.Fiedurek and Szezodark (1995) also reported similar work.

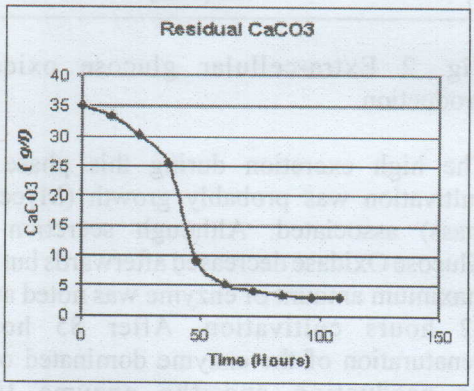

Fig: 4 Calcium carbonate consumption with time (hrs).

Glucose consumption: The consumption of carbon source is an important fermentation parameter as it gives the idea about the growth rate. The carbon source used in the present study was glucose with initial concentration of 80 g/L. The maximum consumption rate was observed between 12-36 hours. After 36 hours the rate of glucose consumption decreased significantly and approach to zero at the end of fermentation.The results are in agreement with the results of Michak *et al*(1985),Petruccioli and Federici(1993), and Rogalski *et al*(1988).

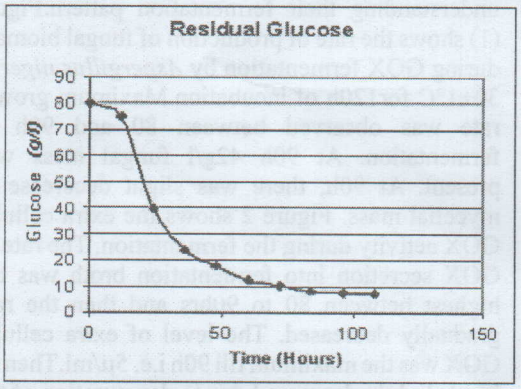

Fig: 5 Glucose consumption by determination residual glucose in the fermentor

Consumption of nitrogen sources: The fungus utilized both organic(Peptone) and inorganic(NaNO3) nitrogen compounds for its metabolic activities, cellular proteins synthesis and for the production of enzyme. Our studies on the consumption of nitrogen sources by A. *Niger* revealed the consumption of nitrogen was highest between 12-36 hours. Afterwards consumption was slowed down our results are in accordance with the findings of Willis (1966) and Ray and Banik (1999).

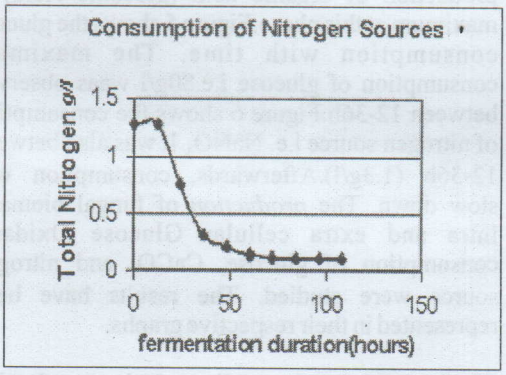

Fig: 6 Nitogen source consumption determination by residual substrate with time (hrs).

Conclusion: The production of Glucose Oxidase by *A. niger* was investigated using enzymatic activity measurement. The majority of Glucose Oxidase was produced during rapid growth in first phase cultivation the high excretion rate during this phase did not prevent the intracellular accumulation of Glucose Oxidase up to 40% of the total soluble cell proteins

demonstrating that the production rate exceeded the excretion rate of the enzyme into the culture medium. During the secondary phase of cultivation of Glucose Oxidase occurred at the slow rate although the majority of Glucose Oxidase produced during the first phase and was excreted during the second phase of cultivation at the end about 90% of the total Glucose Oxidase produced was recovered from culture medium . The result demonstrate the initial steps of the secretary pathway are fast and that the excretion of the enzyme into the culture fluid was most likely delayed due to retention by cell wall. We can have following conclusions from the whole work:

- Biomass production was at peak between 80-90 hours.
- Maximum production of extra-cellular enzyme was observed at 80-90 hours.
- At 36 hours maximum enzyme accumulation was present intra-cellularly.
- As the enzyme was being secreted intra-cellularly (36-50 hours) the $CaCO_3$ and nitrogen source consumption rate was at highest.
- Glucose consumption rate was also highest at time intra-cellular enzyme secretion.

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