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Full Length Research Paper

Optimization of glucose oxidase production by *Aspergillus niger*

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The glucose oxidase was produced by fermentation using *Aspergillus niger* isolated from potato as producer organism and glucose as the carbon source (substrate). Maximum production of the enzyme (1.59 µmoleHQMin^{-1}ml^{-1}) was achieved at 10% glucose concentration after 48 h of submerged fermentation. The pH for the optimal production of enzyme was found to be 5.5 (enzyme activity 1.56 µmoleHQMin^{-1}ml^{-1}). Addition of urea (0.2%) and KH_2PO_4 (0.4%) into the fermentation medium increases enzyme activity (2.01 and 2.96 µmoleHQMin^{-1}ml^{-1}, respectively) while $MgSO_4 \cdot 7H_2O$ was found to inhibit GOX production by *A. niger*.

Key words: Glucose oxidase, *Aspergillus niger*, glucose oxidase.

INTRODUCTION

Glucose oxidase (GOX) (β-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 (Coxon and Schaffer, 1971), in the measurement of glucose level in blood. GOX is also used as an antioxidant (Berg et al., 1992) and development of bio-electrochemical cell (Lannc et al., 1984). GOX is mainly available from microbial sources and is normally produced by aerobic fermentation of *Aspergillus niger* and *Penicillium species* (Fiedurak, 1996; Lu et al., 1996; Plush Kell et al., 1996; Rando et al., 1997). It has high specificity for β-D-glucose (Kuly and Cenas, 1983). Glucose oxidase is produced from most of the microorganisms such as *Penicillium notatum*, *Penicillium chrysosporium*, *A. niger* and *Botrytis cinerea* (Liu et al., 1998).

The glucose oxidase from *A. niger* is an intracellular enzyme present in the mycelium of the organism (Willis, 1966). The present research project was designed for optimization and production of enzyme based glucose oxidase from *A. niger* for ultimate use in glucose estimation kit. The synthesis of glucose oxidase and catalase by *A. niger* was investigated by (Liu, 1999) using resting culture system without growth being established. $CaCO_3$ induced the synthesis of both enzyme and $CaCl_2$ inhibited it. Production was also promoted by addition of manganese, cobalt, thioglycolic acid, and gluconic acid according to (Liu et al., 2001) who studied the effect of metal ions on simultaneous production of glucose oxidase.

Fiedurek et al. (1986) found after screening different fungi that *A. niger* is the best source for the production of this enzyme. For commercial production of enzyme, the first step should be the screening of different native strains for the optimal production of enzyme. So in this study different strains of *A. niger* were examined. Furthermore, growth conditions for the optimal production of glucose oxidase are also studied. This will help the commercial production of glucose oxidase in Pakistan.

Mudeppa et al. (2003) studied the thermal inactivation of glucose oxidase from *A. niger* both in absence and presence of additives. Sukhacheva et al. (2004) reported a method for isolation and purification of extracellular GOX from *Penicillium funiculosum* 433. The enzymes studied displayed a high thermostability, resistant to metal ions and performance in wide range pH. Clarke et al. (2006) studied the location of glucose oxidase during

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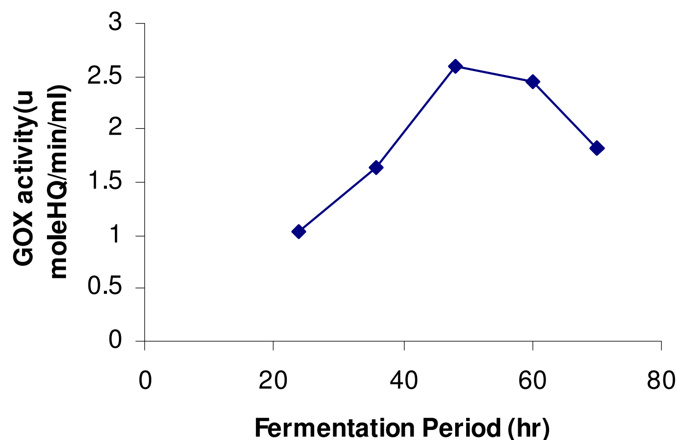

Figure 1. Effect of fermentation period on GOX production.

production by *A. 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 *A. niger*. The present research project was designed for optimization of pH, glucose concentration, fermentation period, urea, KH_2PO_4 and $MgSO_4.7H_2O$ for the maximum production of GOX from *A. niger.*

MATERIAL AND METHODS

Microorganism

The pure culture of A*. niger* (isolated from potato) was obtained from Fungal Culture Bank, University of the Punjab Lahore, Pakistan. This was grown on malt extract agar slants. The malt extract agar slants were prepared by the mixing of 30 g malt extract and 15 g agar and 5 g peptone in one litre of distilled water. The media was heated to boiling, with constant stirring for 20 min, and was poured (5 – 7 ml) into the clean test tubes. The tubes were plugged with sterilized cotton and autoclaved at 15 lbs PSI at 120°C for 20 min. After autoclaving, the tubes were placed in satiating position for 24 h propagation of strains on fresh medium was continued after every two weeks. These pure and identified propagated colonies were kept in the refrigerator at 4°C for storage and further procedure (Khawar, 1990).

Fermentation

This enzyme was produced by submerged fermentation of *A. niger* in 250 ml shake flask. Conical flask with 50 ml fermentation medium containing different concentrations of micro- nutrients were inoculated with 5 ml of spore suspension (107 - 108 spore in 1 ml) and 10 ml of different concentration of glucose. The flasks were incubated at pH 5.3 (after optimizing it) and 30°C on a shaker (120 rpm) for optimum fermentation period that is 48 h (after optimizing it). The fermented biomass in each case was filtered and then blended to extract intracellular enzyme. Finally the filtrates were centrifuged. The supernatant was ultra filtered through filter paper and the filtrate was assayed for GOX.

Enzyme extraction

After 48 h incubation in shaking incubator at 30°C. The mycelia were collected by filtration and washed with 0.1% saline water. The collected mycelia were weighed. The washed mycelia were crushed in water in homogeniser at 1500 rpm for 30 min and centrifuged at 10,000 rpm for 20 min. Mycelium debris was separated and kept for dry mass estimation. The filtrate was precipitated with ethyl alcohol. The filtrate was mixed with ethyl alcohol in 1:4 ratio and kept at 4°C over night. The precipitate was collected by centrifugation at 10,000 rpm for 15 min and pellets of each sample were dissolved in 2 ml of distilled water and refrigerated till further use (Khawar, 1990).

Optimization of fermentation medium conditions

The growth medium of glucose was fermented with *A. niger* for different fermentation periods with varying the concentration of glucose, pH, urea (nitrogen source), KH_2PO_4 and $MgSO_4.7H_2O$ in shake flask. The experiments were carried out in such a way that the parameter optimized in one experiment was maintained in the subsequent investigation.

Enzyme assay

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 (Cicucu and Patrescu, 1984).

RESULTS AND DISCUSSION

Fermentation period

The duplicate growth media containing 4% glucose as substrate were autoclaved, inoculated and incubated for 24, 36, 48, 60, and 72 h at pH 5.3 and 30°C for the optimization of fermentation period. It was observed that production of glucose oxidase increased with an increase in fermentation period from 12 - 48 h, reached maximum at 48 h and decreased thereafter (Figure 1). These

Table 1. Production of GOX with verifying glucose concentration.

Glucose concentration (%)	Enzyme activity (µmoles HQMin^{-1}ml^{-1})
4.0	0.31±0.023
5.0	0.82±0.001
6.0	0.89±0.008
7.0	1.03±0.002
8.0	1.04±0.002
9.0	1.20±0.008
10.0	1.59 ±0.011
11.0	1.34±0.006
12.0	0.75±0.011
13.0	0.475±0.005
LSD:	**0.01765**

LSD: Least significant difference

Table 2. Effect of pH on GOX production by *A. niger*.

PH	Enzyme activity (µmoles HQMin^{-1}ml^{-1})
3.4	0.32±0.0208
4.0	0.76±0.011
4.5	0.93±0.0086
5.0	1.26±0.00568
5.5	1.56±0.0100
6.0	1.03±0.03
6.5	0.38±0.007549
7.0	0.181±0.007
7.5	0±0.00
8.0	0±0.00
LSD:	**0.023**

LSD: Least significant difference.

results are in accord with (Willis, 1966) work where he obtained highest GOX yield after 48 h of fermentation, while Hamid et al. (2003) obtained highest GOX yield after 36 h of fermentation.

Carbon source level (glucose concentration)

The maximum enzyme activity (1.59 µ mole HQMin-1ml-1) was observed with 10% glucose in continuous shaking medium as shown in Table 1. These results are in agreement with the results of Mischak (1985) , Markwell (1989) and Rogalski et al.(1988) while Hatzinikolaou and Macris (1995) found enhanced enzyme activity at 3% molasses.

pH effect

In this experiment duplicate media of glucose 10% were adjusted at different pH values;3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The results showed maximum activity of GOX (1.564 µ moles HQMin-1ml-1) at pH 5.5 in Table 2. This result is in agreement with the result of Rogalski et al. (1988). They determined that the optimum pH for glucose oxidase production was in the range pH 5 to 5.8. Fiedurek and Gromada (2000) reported an increased in intracellular GOX and Catalase production at pH.5.

Effect of urea

The concentration of urea (nitrogen source) has a considerable influence on GOX production. The maximum enzyme activity of glucose oxidase was observed with 0.2 % urea (Table 3) in continuous shaking fermentation medium. Further increase in urea concentration resulted in a decrease in enzyme activity.

Table 3. Effect of concentration of Urea on GOX production by *A. niger.*

Urea concentration (%)	Enzyme activity (µ moles $HQMin^{-1}ml^{-1}$)
0.1	1.65 ±0.00702
0.2	2.01 ±0.00264
0.3	1.59 ± 0.0073
0.4	1.51 ±0.0203
0.5	1.47±0.00624
LSD:	**0.01928**

LSD: Least significant difference.

Table 4. Effect of different concentration of KH_2PO_4.

KH_2PO_4 conc. (%)	Enzyme activity (µ mole $HQMin^{-1}ml^{-1}$)
0.2	2.58±0.00264
0.4	2.96±0.0203
0.6	2.59± 0.0073
0.8	2.440.00624
LSD:	**0.0198**

LSD: Least significant difference.

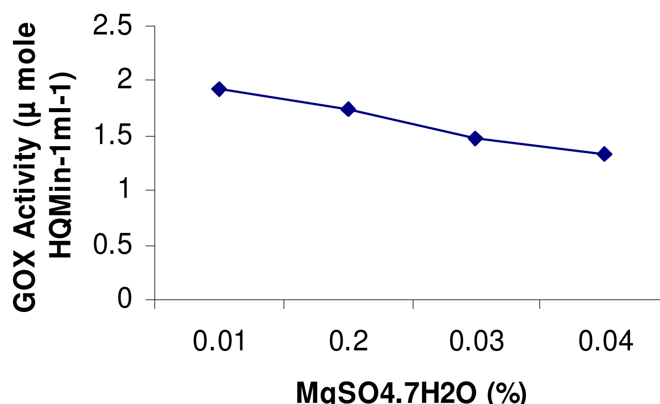

Figure 2. Effect of different concentration of $MgSO_4.7H_2O$ on GOX production by *A. niger*.

This result is in agreement with the work of (Ray and Banik (1999). They added urea up to 0.14% concentration for maximum yield of the enzyme.

Effect of KH_2PO_4

The effect of different concentrations of KH_2 PO_4 was studied on GOX production in medium containing optimum concentrations of glucose, urea, $MgSO_4.7$ H_2O at pH.5.5. The results showed maximum activity of glucose oxidase (2.96 µ mole $HQMin^{-1}ml^{-1}$) at 0.4% KH_2PO_4 in the medium under preoptimized culture conditions and then decreased thereafter as shown in (Table 4). The results of Sidney and Northon (1955) were similar with what we obtained.

Effect of $MgSO_4.7H_2O$

Different concentrations of $MgSO_4.7H_2O$ were examined for GOX production in preoptimized culture medium with 10% glucose, 0.4% KH_2PO_4, 0.2% urea at pH 5.5 and 30ºC. It was observed that with the increase of $MgSO_4.7H_2O$ concentration GOX activity decreases gradually (Figure 2). So very minute amount of $MgSO_4$ $.7H_2O$ is recommended. Yang et al. (1996) and Hamid et al.(2003) also showed that addition of Mg^{+2} in the medium strongly inhibited the production of glucose oxidase.

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