RESEARCH ARTICLE

Md. Emam Hossain [1]
Md. Firoz Alam [2]
Mazedul Anwar Razib [2]
Mohammad Taufiq Alam [1]
Md. Shameem Ahsan [1]

Optimization of the submerged steady-state fermentation process for glucose oxidase production by *Aspergillus niger* isolated from local onion

Authors' addresses:
[1] Department of Applied Chemistry and Chemical Engineering, Faculty of Engineering, University of Rajshahi, Rajshahi-6205, Bangladesh.
[2] Department of Biotechnology and Genetic Engineering, Faculty of Applied Science and Technology, Islamic University, Kushtia-7003, Bangladesh.

Correspondence:
Md. Shameem Ahsan
Department of Applied Chemistry and Chemical Engineering
Faculty of Engineering, University of Rajshahi, Rajshahi-6205, Bangladesh
Tel.: +8801711359523
Fax: +880721750064
e-mail: shameem@ru.ac.bd

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ABSTRACT
Glucose oxidase (GOX) is an enzyme with large scale applications in various industries as a biosensor for the detection of the glucose level of body fluids. The present study aims to optimize the cost-effective steady-state fermentation process for the maximum production of glucose oxidase by *Aspergillus niger* isolated from a locally contaminated onion. For low-cost enzyme production, the possibility of a submerged steady-state fermentation process was evaluated. The enzyme production was assessed by using local low-cost saccharose ($C_{12}H_{22}O_{11}$) as carbon source and sodium nitrate ($NaNO_3$) as the nitrogen source. Effect of peptone and urea on GOX production by *A. niger* were also examined. The initial pH for the optimal production of GOX was found 5.5. The addition of K^+ in fermentation medium increases enzyme activity while Mg^{2+} was found to inhibit glucose oxidase production by *A. niger*. Although Ferric ion (Fe^{3+}) showed the highest GOX productivity, it forms reddish-brown colour which hinder colour measurement in GOX activity. Incubation time and culture morphology were also examined to increase glucose oxidase production.

Key words: Glucose oxidase, *Aspergillus niger*, Steady-state fermentation, Czapek Dox Medium.

Introduction

With the increased environmental concern and depletion of crude energy, biological processes have begun to raise great attention instead of chemicals that can be used as building blocks in the industrial sector (Holladay et al., 2007). In recent years, it has increased great interest in so-called "green technologies" including new strategies to use of the enzyme in industrial sectors as well as the diagnosis process (Islam et al., 2020). A suitable strain and industrial process are the key factors for microbial production to meet the rising demand in the world. Filamentous fungi are of considerable biotechnological importance because they have been well studied as cell factories for the production of a broad range of metabolites and enzymes for decades. They have the potential abilities to utilize a variety of carbon sources and naturally accumulate high amounts of specific products under stressed conditions (Yang et al., 2014).

Glucose oxidase (β-D-glucose: oxygen oxidoreductase, EC 1.1.3.4) is an oxidizing enzyme that catalyzes the oxidation of glucose to gluconic acid in the presence of O_2 as an electron acceptor and produces hydrogen peroxide (Garay-Flores et al., 2014; Haq et al., 2015). This enzyme has a broad range of applications including food and beverage preservation by removal of oxygen and glucose, improvement of foods texture, flavour and colour (Wong et al., 2008). It has also been used in conjunction with catalase as an automatic glucose assay kit and used as a biosensor for the detection of glucose in body fluids such as urine and blood (Petruccioli et al., 1999). Recently, GOX has also been used in biofuel cells (Zhu et al., 2006).

Most of the commercially produced glucose oxidase is isolated from mycelium of *Aspergillus niger*, grown principally for the production of gluconic acid or its salts such as sodium gluconate or calcium gluconate (Gunasundari, 2014). This enzyme is also produced by some other microbes including *Saccharomyces and Penicillium* species (Hamid et al., 2003). Currently, *Aspergillus niger* is preferred for the industrial production of glucose oxidase because of the strain makes itself more economical for the ability to utilize a wide range of waste products as a nutrition source (Yoon et al., 2010, Toscano et al., 2011) and contaminated onion is a good source for *A. niger* (Samuel &

Ifeanyi, 2015). During fermentation, the most typical problems are the high cost of the substrate, low productivity and simultaneous production of other enzymes. To overcome these problems, it is crucial to investigate the economical and commercially available cheap selective medium composition and low-cost fermentation process. In respect of industrial demand, different researchers have isolated several microbial strains and evaluated their enzyme production. But there is no evidence to optimize submerged fermentation with the steady-state condition to minimize the cost of glucose oxidase production. After all, it was the first attempt in our country for isolation of microbial strain from contaminated onion and evaluation of their glucose oxidase production capability by the low-cost steady-state fermentation process. Locally available low-cost saccharose as a sole carbon source, the effect of peptone and urea was evaluated. The fermentation conditions were optimized to enhance the GOX production concerning various parameters like pH, temperature, inoculum properties, incubation time, cultivation temperature and metal ions.

Materials and Methods

Isolation of the fungal strain

The fungal strains were isolated from different contaminated onions for the production of glucose oxidase. The collected fungi were inoculated to Potato Dextrose Agar (PDA) plate and mature culture was sub-cultured onto a fresh plate until pure strain was obtained. The pure culture was maintained on PDA slants and stored at 4°C for further use.

Nutrient medium optimization

To enhance the yield of glucose oxidase with locally available cheap raw materials, different four types of media were designed based on nitrogen source to compare with Czapek Dox Medium (CDM). Synthetic nutrient medium with peptone ($g.l^{-1}$): Glucose: 50.0, KCl: 0.20, KH_2PO_4: 0.15, $MgSO_4$: 0.12, $NH_4H_2PO_4$: 0.60, Peptone: 2.0, pH: 5.0±0.2; Synthetic nutrient medium without peptone ($g.l^{-1}$): Glucose: 50.0, KCl: 0.20, KH_2PO_4: 0.15, $MgSO_4$: 0.12, $NH_4H_2PO_4$: 0.60, pH: 5.0±0.2; Fermentation medium ($g.l^{-1}$): Glucose: 50.0, KH_2PO_4: 0.14, $MgSO_4$: 0.06, Urea: 0.5, pH: 5.0±0.2; Fermentation medium without urea($g.l^{-1}$): Glucose: 50.0, KH_2PO_4: 0.14, $MgSO_4$: 0.06, pH: 5.0±0.2. Czapek Dox Medium ($g.l^{-1}$): Saccharose: 30.0, $NaNO_3$: 2.0, K_2HPO_4: 1.0, $MgSO_4$: 0.5, KCl: 0.5, $FeSO_4$: 0.01.

Carbon and Nitrogen source optimization

To determine the effect of different carbon sources, Lactose ($C_{12}H_{22}O_{11}$), Maltose ($C_{12}H_{22}O_{11}$), Saccharose ($C_{12}H_{22}O_{11}$), Fructose ($C_6H_{12}O_6$), was examined instead of glucose ($C_6H_{12}O_6$). Different carbon sources were examined with CDM at a concentration of 30 $g.l^{-1}$. For the selection of nitrogen source Sodium Nitrate ($NaNO_3$), Urea (CH_4N_2O), Di-Ammonium Hydrogen Phosphate [$(NH_4)_2HPO_4$], peptone and Lead Nitrate [$Pb(NO_3)_2$] were tested at 2.0 $g.l^{-1}$ in the same medium.

Optimization of other parameters

For optimum fermentation conditions, different other parameters such as inoculum culture, pH, incubation time & temperature, the effect of metal ions were also tested in submerged fermentation. The experiments were carried out in such a way that the parameter optimized in one experiment was maintained in the subsequent investigation.

Enzyme assay

After specific incubation, the culture broth was filtered and used for enzymatic activity. The activity of glucose oxidase was determined by the O-dianisidine method (Bergmeyer et al., 1974). The basic principle is the detection of hydrogen peroxide in the presence of peroxidase, indirectly indicated by a colour change which is analyzed by spectrophotometer (UV-1650PC, Shimadzu, Japan) at A_{500nm}. One unit of glucose oxidase is defined as the amount of enzyme catalysing one micromole of glucose per minute.

Results and Discussion

Isolation and identification of Aspergillus niger from onion bulbs

For glucose oxidase production, a number of *Aspergillus* spp. were isolated from 11 different onion samples. The black mold of onion caused by fungus *Aspergillus niger* was collected from local areas. Colonies from onion bulbs were grown rapidly on PDA medium and reached 21-23 mm in diameter after 5 days. The colonies were initially white and quickly became black with the conidial production. Hyphae found to be septate, double-layered and hyaline (Figure 1). A similar type of finding was reported by Haq et al., (2014) for the identification of *Aspergillus niger*. The major morphological distinction of *A. niger* from other species of *Aspergillus* is the presence of carbon black or dark brown spores on biseriate phalides, which are arranged in a globose head radiating from a vesicle conidiophore.

Effect of inoculum on culture morphology and enzyme production

The origin of inoculum showed a great influence on cell growth and subsequent glucose oxidase production (Munk et al., 1962). If stored (4°C) culture was used as inoculum, the aggregation between spore was less and formed in a non-filamentous network. But in the case of fresh inoculum, the affinity between spores to aggregation increased and the pellet form growth with a micro-filamentous network was observed. The activity of the enzyme produced in the culture medium was more than two times higher in filamentous

Figure 1: *Onion sample and isolated Aspergillus niger.*

grown culture compared to the small aggregated non-filamentous one (Figure 2). The lower yield in small aggregation was due to the composition of biomass inside the non-filamentous network that can be divided into an active and inactive fraction (Domingues et al., 2000).

Effect of nutrient medium

For optimizing the production medium with local cheap raw materials, four types of media were examined. We conclude that synthetic nutrient medium without peptone showed better yield (6.2 U.ml-1) compare with other media. In the case of peptone in the medium, the initial cell growth (biomass) is high but the ultimate enzyme yield is low although peptone promotes increasing microbial biomass. But all the four-medium showed a lower yield of glucose oxidase compare with CDM (Figure 3). On CDM, the early growth phase indicates the rapid production of glucose oxidase than other media (Khurshid et al., 2011). After 48h, the enzymatic activity remains almost constant or lower due to the fact that the pH of the medium decreases by the transformation of glucose to gluconic acid.

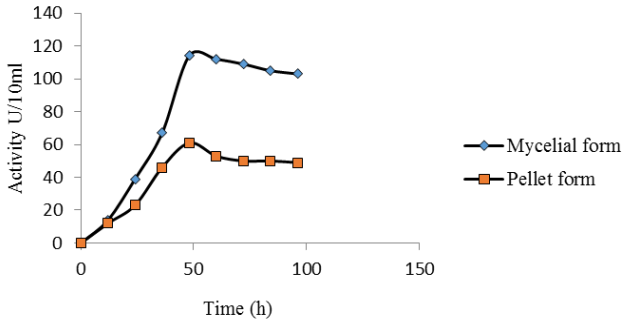

Figure 2. *Changes of enzymatic activity in the mycelium and small aggregate form).*

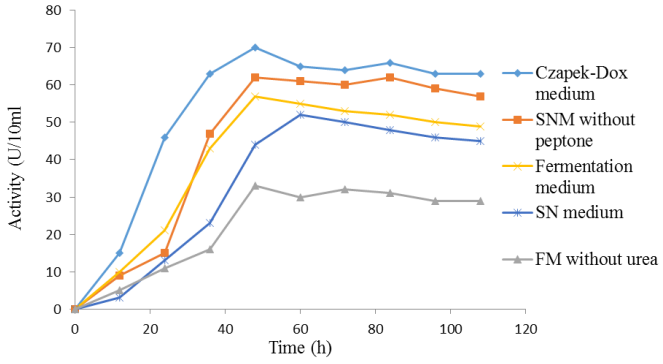

Figure 3. *Glucose oxidase production using different culture mediums.*

Effect of different carbon sources

Carbon source not only acts as a major constituent for the building of cellular material but is also used in the synthesis of polysaccharide and as an energy source (Stanbury et al., 1997). The maximal enzymatic activity 7.8 $U.ml^{-1}$ was obtained after 48h by using saccharose as carbon source which is pretty cheaper than glucose, lactose, maltose and fructose (Figure 4). The maximum result obtained with saccharose could probably be explained by the fact that GOX is glycoproteins that usually show this sugar as the main carbohydrate component (Hayashi & Nakamura, 1976). In our experiment, pH was easily observed between 4.7 to 5.7 by using saccharose whereas fructose and maltose containing medium was dropped down to 3.40 ~ 3.85.

Effect of different nitrogen sources

The choice of nitrogen source is an important factor that influencing the subsequent growth productivity and cost of enzyme production. The results showed that sodium nitrate favoured and promoted glucose oxidase production (7.10U/ml) compared with other nitrogen sources (Figure 5). Sandip et al., (2009) showed in their experiment that sodium nitrate was the best nitrogen source for glucose oxidase production by *Aspergillus niger*. In most culture processes, the concentration of the nitrogen in the medium is increased to secure high productivity.

Effect of fermentation period

The time course of glucose oxidase production in the range of 24-100 h was investigated and maximum production

was observed after 48h (7.0 U.ml^{-1}). These results are in accord with Willis, (1966) and Khurshid et al., (2011) where the highest GOX yield after 48h, while Hamid et al., (2003) obtained the highest yield after 36h of fermentation. It was observed that the production of glucose oxidase increased with an increase in the fermentation period from 18 - 48h and reached the maximum at 48h and decreased thereafter (Figure 6). Further incubation did not show any increment in the level of enzyme production due to depletion of nutrients and an increase in toxic unwanted wastes in the medium, which leads to decreased growth of microbes and enzyme production.

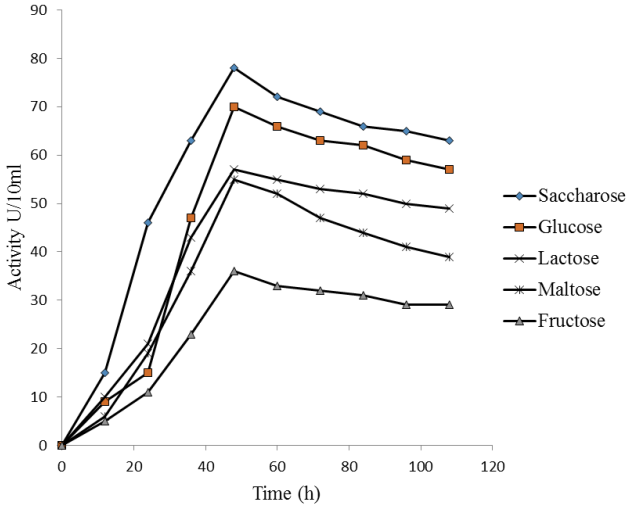

Figure 4. *Effect of different carbon sources on glucose oxidase production.*

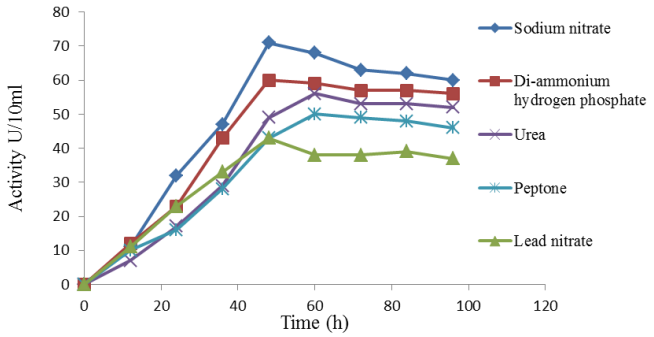

Figure 5. *Effect of different nitrogen sources on glucose oxidase production.*

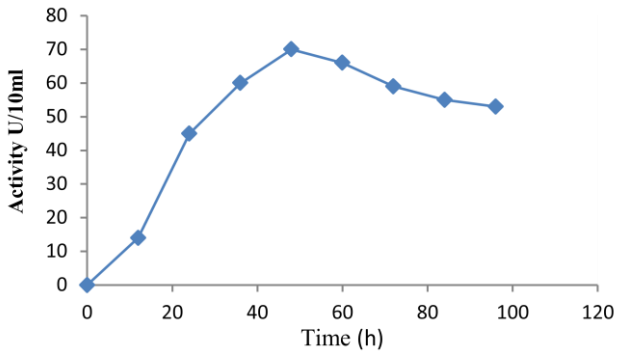

Figure 6. *Effect of incubation time on glucose oxidase production in CDM.*

Effect of pH

Initial pH has a strong effect on the uptake of mineral nutrients for microbial growth from medium and subsequent enzyme production. The optimum pH for glucose oxidase production found was 5.2 (Figure 7). According to Khurshid et al., (2011) the optimum pH for glucose oxidase production

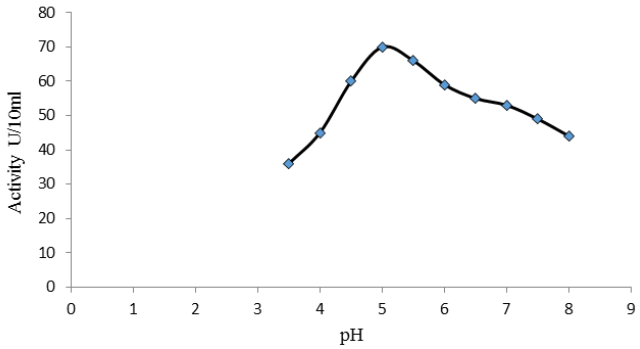

Figure 7. *Effect of pH on glucose oxidase production by A. stage.*

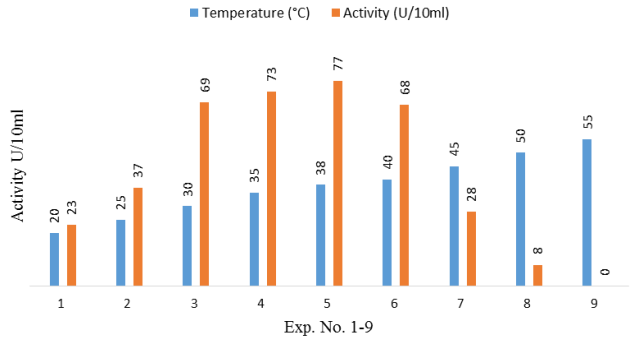

Figure 8. *Effect of cultivation temperature on glucose oxidase production.*

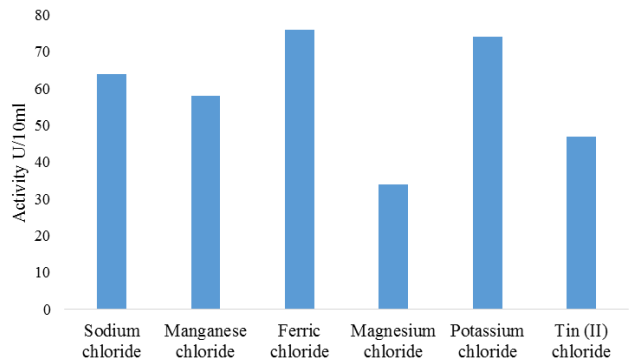

Figure 9. *Effect of metal ions on culture growth and enzyme production.*

is 5.5. Rogalski et al., (1988) determined the optimum pH for glucose oxidase was in the range of 5.0 to 5.8. At higher pH, the production of alkaline protease increases and production of glucose oxidase decreases; while at lower pH, the possibility for production of acidic protease and citric acid may be increased (Rajesh et al., 2014).

Effect of cultivation temperature

For the optimization of cultivation temperature, the fermentation medium was incubated at different temperatures (20, 25, 30, 35, 38, 40, 45, 50 and 55°C). After appropriate incubation, the optimum temperature for GOX production was observed at 38°C (Figure 8). Other researchers also reported around the same result for maximum GOX enzyme production (Traeger et al., 1991 and Caridis et al., 1991). At high temperature, due to the production of a larger amount of metabolic heat, thereby inhibiting microbial growth and ultimately enzyme production (Sabir et al., 2007).

Effect of metal ions on culture growth and glucose oxidase enzyme

The metal ion plays a significant effect on the growth of an organism and the level of glucose oxidase production (Uddin et al., 2014). In our experimentation, the extracellular glucose oxidase production was highest in the presence of Ferric Chloride ($FeCl_3$) than Potassium Chloride (KCl), Sodium Chloride (NaCl), Manganese Chloride ($MnCl_2$), Tin (II) Chloride ($SnCl_2$) and Magnesium Chloride ($MgCl_2$) (not shown), but the colour of Ferric Chloride hinders the colour of Glucose oxidase. For this reason, potassium chloride was chosen for glucose oxidase production. Figure 9 showed that glucose oxidase production was significantly increased in the presence of Fe^{3+} and K^{+} but decreased in the presence of Mg^{2+} ions. Lu et al., (1996) and Hamid et al., (2003) reported that the addition of Mg^{2+} in the medium inhibits the production of glucose oxidase by *Aspergillus niger*.

Conclusion

In this study, glucose oxidase production was evaluated by *A. niger* isolated from a contaminated onion. To abate the cost of the fermentation process, the study reported here the possibility of the steady-state fermentation process for GOX production instead of continuing shaking. Locally available cheap saccharose was selected as the best carbon source for the production of glucose oxidase by *A. niger*. Effect of organic nitrogen source peptone and inorganic nitrogen source urea was also observed on GOX production by selected strain. Further studies are being carried out to enhance the glucose oxidase production by controlling feeding, aeration, agitation, etc. indicating an economically attractive process. These characteristics will further aid to explore the knowledge of the commercial production of glucose oxidase and its application.

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