USE OF CHEAP MEDIA TO ENHANCE GLUCOSE OXIDASE PRODUCTION DURING BATCH CULTIVATION OF *ASPERGILLUS NIGER*

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ABSTRACT

The study was carried out to check the parameters affecting glucose oxidase production, optimizing the growth culture conditions and to establish a cost effective medium for industrial production of this enzyme. In this study, *Aspergillus niger* PTTC 5012 strain was grown on different simple and complex sources of carbon and nitrogen in shaking flasks. Different culture conditions were also applied to find optimal values of pH, temperature, $CaCO_3$ concentration and culture time for growth and enzyme production. Beet molasses and corn steep liquor showed to be very high promising carbon and nitrogen sources for glucose oxidase production. Using 50 g l^{-1} molasses and 10 g l^{-1} corn steep liquor and at optimal conditions of pH 5.5, temperature 30 ^oC, $CaCO_3$ concentration of 50 g l^{-1}, culture time of 72 h and with previously optimized conditions of oxygenation, the activity of glucose oxidase reached to around 550 U ml^{-1}. The results of the present study could be scaled-up for a commercial production of glucose oxidase at a satisfactory productivity level using molasses and corn steep liquor as the basal medium.

INTRODUCTION

Aspergillus niger is well known to produce a lot of organic acids, enzymes, plant growth regulators, mycotoxins and antibiotics. The industrial importance of *A. niger* is due to its capacity for synthesis of more than 35 native products. During the past few years numerous studies have been presented on this most important fungus for production and secretion of proteins (Jeenes *et al.* 1991). The application of *A. niger* as a host organism for production and secretion of homologous and heterologous proteins demonstrates many advantages. *A. niger* is a prodigious exporter species of homologous proteins and is able to produce certain enzymes in quantities of kilograms per cubic meter under the right conditions (Finkelstein 1987). *A. niger* has a long history of usage within the fermentation industry and is generally regarded as safe (GRAS). The fermentation industries are very familiar with the conditions required to maximize production of homologous proteins in *Aspergillus*. Its species are effective secretors of proteins, often in a native and correctly folded form. They do not tend to accumulate large quantities of the protein intracellularly, in form of inclusion bodies, as some bacteria and yeast do. Glucose oxidase (β-D-glucose: O_2 1-oxidoreductase, E.C. 1.1.3.4) is one of the most important enzyme produced by this fungi in both submerged (Singh 2006; Witteveen *et al.* 1992) and solid state fermentation (Ramachandran *et al.* 2007). Glucose oxidase catalyses the oxidation of

β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide and finally to gluconic acid using molecular oxygen as electron acceptor (Leskovac *et al*. 2005). It is widely used in the removal of traces of oxygen or glucose from different foods such as dried egg, beer, wine and fruit juices, as a source of hydrogen peroxide in food preservation and in gluconic acid production (Kapata *et al*. 1998). Glucose oxidase was also found to be antagonistic against different food-borne pathogens such as *Salmonella infantis, Staphylococcus aureus, Clostridium perfringens, Bacillus cereus, Campylobacter jejuni* and *Listeria monocytogens* (Kapata *et al*. 1998). It has been also used as an ingredient of toothpaste (Petruccioli *et al.* 1999). It is also a key enzyme, which is being exploited commercially in biosensors for monitoring the glucose level in blood, as well as in fermentation broth for on-line estimation of residual glucose (Petruccioli *et al*. 1999; Sierra *et al.* 1997).

In the present study, the effect of culture conditions and media composition on glucose oxidase production by *Aspergillus niger* was investigated. We have already reported the influences of aeration and agitation as two important parameters in this fermentation (Jafari *et al*. 2007). However, for designing industrial production processes the selection of medium compositions is crucial. Different sources of carbon and nitrogen in different conditions will be examined in the laboratory in order to achieve optimal production conditions. Economical and commercially available media would be considered to reduce production costs. Corn steep liquor and molasses are the most usual substrates, which are used to formulate the low cost media during an industrial fermentation. Molasses is a carbon source whereas CSL is usually considered as a nitrogen source. However, both of them are rich sources of nutrients, vitamins and minerals.

The major goal of this work was to enhance the production of glucose oxidase by optimization of different parameters and using these low cost substrates.

MATERIALS AND METHODS

Microorganism: Aspergillus *niger* PTTC 5012 strain from IROST collection in Iran was used, the slant prepared on PDA for vegetation of spores and maintained at 4°C.

Growth media and culture conditions: The strain was initially grown in a 500 ml preculture flask containing 100 ml of the medium with following composition (in grams per liter): $(NH_4)_2HPO_4$, 0.4; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.2; Peptone, 10 and Sucrose, 70. The pH was adjusted to 5.5, with 1 N HCl, prior to sterilization. After inoculation, the preculture was incubated for 24 h on a rotary shaker operating at 225 rpm and 30°C. The culture flasks contained the mentioned preculture medium plus 4% (w/v) of $CaCO_3$, which were inoculated at the same conditions with 10 % (v/v) of this preculture for different fermentation time periods with varying the type of carbon and nitrogen sources and levels of substrates. Glucose, fructose, galactose, sucrose, molasses and starch as carbon sources and yeast extract, peptone, corn steep liquor (CSL), $NaNO_3$, $(NH_4)_2SO_4$ and NH_4NO_3 as nitrogen sources were investigated. Beet molasses and CSL were obtained from Karaj Ghand factory (Karaj, Iran) and Glucosan company (Ghazvin, Iran) respectively. The levels of pH, temperature and $CaCO_3$ concentration were also considered to optimize the production conditions. All media were sterilized at 121 ^oC for 20 min. The experiments were carried out in such a way that the parameter optimized in one

experiment was maintained in the subsequent investigation.

Fermentation procedure: Fermentation was also carried out in a 5-l stirred glass vessel bioreactor (Chemap AG, Switzerland) with a working volume of 3 l. The bioreactor was equipped with two rushton turbine blades impellers with 80 mm diameter. The following probes were installed on the top plate: Mettler Toledo sterilizable Inpro 6000, pH-electrode, pt-100-temperature sensor and a conductive foam sensor. The fermentation parameters were controlled by a digital measurement and control system. The fermentation medium was made based on the optimum results obtained from shaken flasks. The fermentation conditions were as follows: the agitation and aeration had been set up at 300 rpm and 1.5 vvm respectively; temperature, 30 ^{o}C; liquid silicone was used as antifoam agent. The fermentation was inoculated with 10% (v/v) from the 24-h inoculum culture. The pH of culture was kept at value of 5.5 and maintained with pH adjusting from controller.

Biomass measurement: Samples were withdrawn in different intervals of time. Biomass was measured by conventional method of filtration as cell dry weight. Before filtration, the $CaCO_3$ was converted to soluble form of $CaCl_2$ by 4 N HCl and reduce pH to 2.5 as recommended by Hatzinikolaou *et al.* (1995). The fungal biomass was separated from the culture fluid by filtration and washed with distilled water several times and then dried in 80°C. Concentration of biomass was determined by weighing dried mycelia.

Crude extract of intracellular and extracellular enzyme preparation: Mycelia from the culture liquid were collected, washed two times with distillated water on a sieve (whatman paper No. 42), and suspended in 0.1 M citrate-phosphate buffer, pH 4. The mycelia were incubated into a freezer in -20 °C for 24 hours. Before disruption, 1 mmol l^{-1} of PMSF and 3 mmol l^{-1} EDTA reagents were added to samples in order to inactivate intracellular protease enzyme and chelating the metal ions respectively. Then mycelia were disrupted with glass beads using mechanical force. After disruption the aliquots of culture fluid were clarified by centrifugation at 6000 rpm for 15 min at room temperature and enzyme activities were measured in the clear supernatant. The obtained data for intracellular activity showed that the concentration of intracellular glucose oxidase was less than half of its extracellular enzyme and could be ignored after 20 h of culture. So that, the enzyme assays was limited to activity of the extracellular source of glucose oxidase. For extracellular enzyme determination, aliquots of culture fluid were clarified by centrifugation at 6000 rpm for 15 min at room temperature and the clear yellow supernatant was used as the source of glucose oxidase.

Analytical methods: All chemicals used were of analytical grade, and obtained through Merck Chemical Co. The reducing sugars concentration was measured by the DNS (3,5-dinitro salicylic acid) method using glucose as the standard (Miller 1959). The same method was used for measuring enzyme activity, measuring residual reducing sugars. The reducing sugars were treated with DNS, which is reduced to 3-amino-5-nitro-salicylic acid. The later was quantified by measuring absorbance at 540 nm using a spectrophotometer (Taktorn model 163 Varian). The reducing sugars were measured as follows: 0.2 ml reducing sugar solution, 1.8 ml distilled water and 2 ml DNS reagent were boiled for 5 min followed by cooling to room temperature and diluting to 24 ml. A standard

calibration curve was prepared using known concentration of glucose (0.5-5 g l^{-1}). From the standard curve the concentration of reducing sugar was determined (Kona *et al.* 2001).

The enzyme assay mixture consisted of 0.2 ml reducing sugar solution, 0.2 ml crude enzyme preparation, 1 ml citrate phosphate buffer (pH 5) and 0.6 ml distilled water. The citrate phosphate buffer contained 0.02 g l^{-1} sodium nitrate to inhibit catalase activity without affecting glucose oxidase activity. The reaction mixture was incubated at 30°C for 30 min. The reaction was stopped by keeping the tube in boiling water. The residual sugar then was measured by the mentioned method. Activity Unit was defined as the amount of enzyme that converts 1 µmol glucose (β-D-glucose) to gluconic acid and H_2O_2 in one minute under the above-described conditions. Protein concentration of the samples was also determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

RESULTS

Table-1 shows the cell concentration and glucose oxidase activity obtained by using different carbon sources in the media based on the mentioned preculture medium. The initial concentration of the all carbon sources was 70 g l^{-1}. For molasses, the initial concentration was adjusted to this value based on its total solids concentration. Fermentation was allowed to continue for 72 h. As seen the highest enzyme activity obtained by sucrose followed by molasses and glucose. The enzyme activities of other substrates were significantly lower than molasses. Then, conducting experiments at various initial concentrations to optimise the concentration of the molasses and sucrose showing higher activity. Figure 1 presents the resulted data, in which it is clear that the optimum levels of both substrates are not the same and molasses at the concentration of 50 g l^{-1} resulted in a higher activity than sucrose. By taking into account the prices of these substrates, molasses was considered as the definite source of carbon in this fermentation.

Table-1. Effect of different types of carbon source on the *A. niger* cell concentration and glucose oxidase production.

Carbon source	Dry cell weight/g l^{-1}	Enzyme activity/ unit ml^{-1}
Starch	10.5	220
Molasses	9.1	502
Fructose	5.6	310
Galactose	3.8	200
Sucrose	8.9	509
Glucose	8.5	494

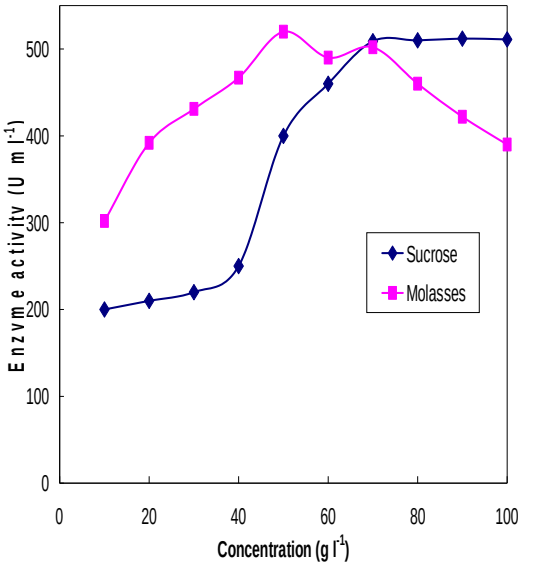


Fig 1 Effect of different concentrations of sucrose and molasses on glucose oxidase production in shaken flasks with initial CSL concentration 10 g l^{-1}, initial pH of 5.5 at 30°C and 225 rpm.

To achieve a suitable source of nitrogen, the same procedure was followed and the optimum concentration of molasses was used instead of sucrose in the preculture-based media. Table 2 shows the cell concentration and glucose oxidase activity obtained by using different nitrogen sources. The initial concentration of the all nitrogen sources was 10 g l^{-1}. For CSL the initial concentration was adjusted to this value based on its total solids concentration. As seen the highest enzyme activity was obtained by CSL followed by peptone and yeast extract. The lower enzyme activity of other sources reveals that the complex organic sources of nitrogen are more efficient than simple mineral nitrogen sources. CSL also resulted in higher cell concentration than other nitrogen sources. The concentration of CSL was then optimised by conducting experiments at different initial concentrations as presented in figure 2. The initial selected concentration of 10 g l^{-1} showed the maximum activity.

Table 2: Effect of different types of nitrogen source on the *A. niger* cell concentration and glucose oxidase production.

Nitrogen source	Dry cell weight/g l^{-1}	Enzyme activity/units ml^{-1}
$NaNO_3$	8.2	480
$(NH_4)_2SO_4$	3.5	402
CSL	9.1	528
Yeast extract	7.25	505
Peptone	7.8	520
NH_4NO_3	5.2	501

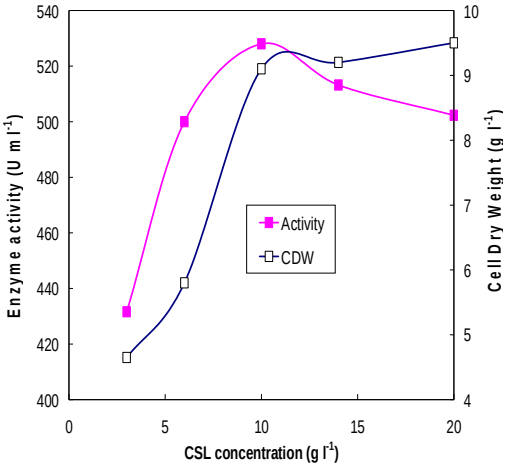


Fig-2: Cell growth of *A. niger* based on cell dry weight and glucose oxidase production in shaken flasks with different CSL conc. using optimal molasses concentration.

Another factor affecting the synthesis of glucose oxidase is pH. The shaken flasks in duplicate were prepared in different initial pH from 4 to 7. As seen in the figure 3 the maximum enzyme activity was obtained the pH 5.5.

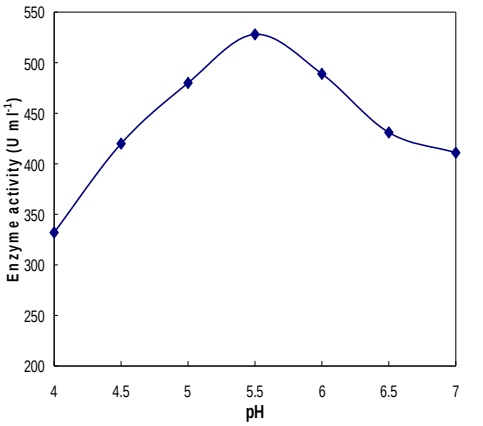


Fig-3: Effect of pH on glucose oxidase production in shaken flasks at optimal substrate concentrations of 10 g l^{-1} CSL and 50 g l^{-1} molasses.

Temperature is also an effective factor in fermentation of *A. niger*. The optimum value of this factor was also evaluated in shaken flasks incubated at different temperatures. The results were shown in figure 4 with the highest activity at 30 ^{o}C.

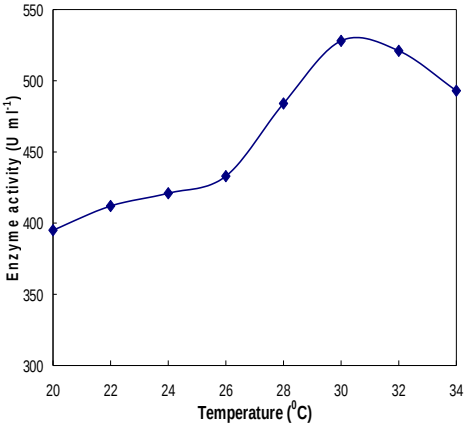
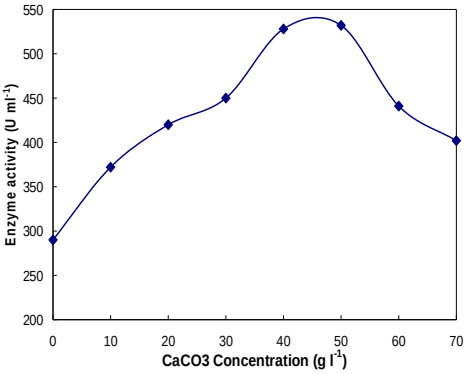


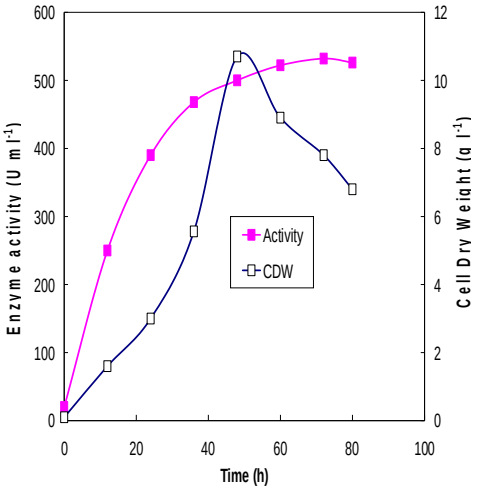
Fig-4: Effect of temperature on glucose oxidase production by *A. niger*. The enzyme production was evaluated by measuring its activity.

The effect of $CaCO_3$ was also investigated. $CaCO_3$ has been reported to be essential for the induction of glucose oxidase synthesis (Liu *et al.* 1998). The optimum concentration of $CaCO_3$ for the biosynthesis was in the range of 40-50 g l^{-1} (Fig. 5).



Fig–5: Effect of calcium carbonate concentration on glucose oxidase production in shaken flasks at optimal temperature, pH and substrates concentration.

As mentioned the cultivation times in all experiments were 72 h. However, idea about the effect of growth time on cell concentration and enzyme production, the complementary experiments were done under the above-mentioned optimal conditions. Figure-6 shows the cell concentration increased with the time till 48h of cultivation with the maximum value of 10.7 g l^{-1}. It diminished during the rest of the cultivation. The enzyme activity increased through the time with a more or less smooth profile at the end of culture.



Fig–6: Time courses of glucose oxidase activity and *A. niger* biomass concentration in term of cell dry weight. The fermentation was carried out in shaken flasks under optimal conditions.

Taking into account all of these results, an experiment was carried out in a 5-l fermentor at optimal values of 300 rpm agitation speed and 1.5 vvm aeration rate as previously reported (Jafari *et al.* 2007). In

this experiment the maximum cell concentration and enzyme activity reached to 11.9 g l^{-1} and 548 U ml^{-1} respectively.

DISCUSSION

Many researchers have tried to improve the production of biomass and glucose oxidase by applying low cost various cheap culture sources like molasses and CSL (Kona *et al.* 2001; Hatzinikolaou *et al.* 1995; Rothberg *et al.* 1999). Nakamatsu *et al.* (1975) studied the effect of different complex carbon sources as well as different nitrogen sources on glucose oxidase production. Among eight natural carbon sources, they found that beet molasses was the best to support growth and glucose oxidase production. Such complex substrates can improve the glucose oxidase production due to availability of substrate for longer time. Molasses also contain many minerals such a vitamins, trace elements and sugars mainly sucrose and rafinose and other elements which make it as an appropriate substrate for growth and production of this enzyme. The usefulness of molasses in the production of glucose oxidase has also been confirmed in the present study. Our results showed the same positive effect of molasses as reported by Hatzinikolaou *et al.* (1995). However few authors reported the negative effect of molasses on glucose oxidase production (Kona *et al.* 2001). This may be related to the differences in the kind and concentration of impurities found in molasses from diverse origins. Heavy metals such as iron, zinc, copper and manganese in molasses can cause a critical problem during fermentation and also inhibited the enzyme and these must be should be removed by suitable treatment method before adding to culture media. Bowes and Mattey (1979) reported that manganese at concentration of 10 mg l^{-1} has drastically decreased the citric acid accumulation. Investigations by Clark *et al.* (1966) and Kisser *et al.* (1980) confirmed the key regulatory nature of manganese ion. Optimization studies of glucose oxidase production and increasing enzyme activity using CSL have been reported by some authors (Kona *et al.* 2001; Park *et al.* 2000). In this study the CSL was found to be a very good nitrogen source. It supports very well *A. niger* growth as well as glucose oxidase production. It may be concluded that the complex organic substrates such as CSL is a more suitable substrate for *A. niger* growth and glucose oxidase production than the simple inorganic substance like $NaNO_3$.

Another important factor affecting the synthesis of glucose oxidase is $CaCO_3$ concentration. It has been suggested that the induction and synthesis of glucose oxidase and catalase by $CaCO_3$ could be due to these three effects: high calcium ion concentration, high pH (as a consequence of $CaCO_3$ addition) and insolubility of salt acting as solid support for mycelial growth (Kundu and Das 1985; Rogalski *et al.* 1988). The optimal concentration of $CaCO_3$ found to be in the range of 40-50 g l^{-1}, a little higher than the optimal concentration reported by Liu *et al.* (1998). Our results also agree with Hatzinikolaou *et al.* (1995) who reported 50 g l^{-1} as optimal concentration of $CaCO_3$ in molasses media for production of glucose oxidase by *Aspergillus niger*. They identified $CaCO_3$ as a particularly strong inducer of glucose oxidase activity. The addition of $CaCO_3$, to prevent a pH drop had been shown to be extremely important. Petruccioli *et al.* (1995) tried to use different chemicals such as $CaCO_3$, NaOH, and NaOH plus $CaCl_2$ to counteract the pH drop in fermentation broth and found $CaCO_3$ the best inducer for glucose oxidase production. They suggested a possible role in enzyme

stabilization, which was played by calcium ions.

Since fungal growth itself results in a change in the pH of the culture, control of it is also necessary. Protease secretion was regulated by the carbon and nitrogen sources. It was found that all the extracellular proteases are only expressed when the preferred carbon and nitrogen sources are not available to the cell (Van den hombergh *et al.* 1997). The extracellular fungal proteases are also strictly pH regulated. Acid proteases are only expressed at acidic pH (Denison 2000). *Aspergillus niger* can predominantly produce aspartic protease, however, by regulating the pH near to 5.5 the production of this acid protease could be inhibited. Mischak *et al.* (1985) have shown that at low pH values *A. niger* produces citric acid while after the adjustment of pH to 5.5 a *de novo* synthesis of glucose oxidase occurs and most of the produced enzyme is secreted. Our result shows the same optimum pH of 5.5 for glucose oxidase production (Fig. 4). At higher pH the production of alkaline protease increases and at lower pH, as mentioned above, the possibility for production of acidic protease and citric acid may be increased. Our finding is in accordance with Liu *et al.* (1999) and Hatzinikolaou *et al.* (1995).

Temperature is also determinative for the growth rate and morphology of fungi. Most fungi are mesophilic growing at temperature between 10 and 40^{o}C. The genus *Aspergillus* grows at temperature between 15 and 30^{o}C. For *A. niger*, the optimum temperature range is 17-42 ^{o}C. The influence of cultivation temperature on growth kinetics of *A. oryzae* has been investigated by Carlsen *et al.* (1995) in a series of batch experiment. According to the authors, at higher temperature, the oxygen supply of the cells was inadequate. Therefore, the pellets were transformed to filamentous mycelium at 30^{o}C, but at 35^{o}C, clumps were formed. Temperature also affected the production of xylanase, with the maximum productivity obtained at 35^{o}C. In the investigated temperature range of 27–40 ^{o}C by Carlsen *et al.* (1995), at pH above 4.0 and with inoculums consisting of spores, pellets were formed and the pellet size distribution was found to be temperature independent. In our study the optimum temperature was 30 ^{o}C. According to our results neither high nor low temperature was suitable for *A. niger* growth and glucose oxidase production. This was in agreement with Hatzinikolaou *et al.* (1995).

The time courses of growth and enzyme production showed that the maximum biomass reach at 48 h while maximum activity of glucose oxidase was found at 72 h of fermentation. Petruccioli *et al.* (1995) found the same time of fermentation for maximum activity of glucose oxidase and reported a constant activity thereafter. It is interesting to note that the glucose oxidase continued to be produced until the last few hours of the run. Several attempts were made to make the production of enzyme continue for longer period of time, but the success was not significant (Rothberg *et al.* 1999).

It is concluded that the specified strain of *A. niger* showed to be a high promising strain for production of glucose oxidase using low cost and abundant substrates such as molasses and corn steep liquor. pH, temperature, $CaCO_3$ concentration and duration of fermentation process contribute to enhanced glucose oxidase productivity.

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