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Up-Streaming Process for Glucose Oxidase by Thermophilic *Penicillium* sp. in Shake Flask

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Abstract

The present study is concerned with the production of glucose oxidase (GOD) from thermophilic *Penicillium* sp. in a 250 mL shake flask. Fourteen different strains of thermophilic *Penicillium* sp. were isolated from the soil and were screened for glucose oxidase production. IIBP-13 strain gave maximum extra-cellular glucose oxidase production as compared to other isolates. The effect of submerged fermentation in shaking and static conditions, different carbon sources and incubation period on the production of extra-cellular glucose oxidase was studied. A maximum yield (0.325 U/mL) of extra-cellular glucose oxidase was obtained after 72 h of incubation at 45 °C using submerged fermentation under shaking utilizing sucrose as a carbon source.

Keywords: Glucose oxidase, thermophilic, *Penicillium*, submerged fermentation

Introduction

Glucose oxidase (E.C. 1.1.3.4) is a very important non-hydrolytic enzyme that belongs to the oxidoreductase family. Glucose oxidase is also called as glucose aerodehydrogenase [1,2]. Glucose oxidase acts as a catalyst and oxidizes β-D-glucose to gluconolactone and reduces the molecular oxygen to hydrogen peroxide. Then the gluconolactone is hydrolyzed spontaneously and gluconic acid is produced [3]. Several enzymes are known which oxidize glucose among which glucose oxidase is the best known enzyme [4].

Glucose oxidase has applications in the pharmaceutical and food industries. In the pharmaceutical industry, it is used as a biosensor and it determines enzymatically the presence of glucose in liquor fermentation, body fluids and beverages [5-7]. In the food industry, it is used to remove glucose and oxygen to enhance the color, taste and consistency of different products [8,9]. It also improves the shelf life of food industry products and prevents the food from rotting e.g. it can be used to preserve stored foods and food products. Glucose oxidase has also emerged for use in biofuel cells [10].

Various organisms have the capability to produce glucose oxidase and among these fungi have an eminent status. Mostly different species of *Penicillium* and *Aspergillus* are used for this purpose. *Penicillium adametzii* is an effective fungus for the production of extracellular glucose oxidase [11]. Other than *Aspergillus niger* some species from the genera *Penicillium, Gliocadium, Scopulariopsis*, and *Gonatobotyrs* also produce gluconic acid along with glucose oxidase. Many bacterial species also produce glucose oxidase and among these *Gluconobacter oxydans*, *Zymomonas mobilis*, *Acetobactor methanolicus*, *Pullularia*, *Micrococcus*, *Enterobacter* and *Scopulariopsis* are the most dominant. Although, glucose oxidase can be produced from many species of bacteria and fungi, fungi are considered to be of more interest than bacteria for the production of this enzyme on an industrial scale [12]. The ability of filamentous fungi to secrete large amounts of extracellular

protein has made them well suited for industrial enzyme production [13].

Various techniques can be employed for the production of enzyme glucose oxidase which includes solid state and submerged fermentation. Submerged fermentation is found to be more effective in the production of glucose oxidase and other industrial enzymes because it is easier to control the environmental factors in this technique compared to solid state fermentation [14]. The present study was conducted with the aim of isolating a potent strain of thermophilic *Penicillium* from the local habitat and optimizing the production of glucose oxidase using the isolated strain.

Materials and methods

Isolation of thermophilic *Penicillium* sp.

Different thermophilic *Penicillium* species were isolated from soil samples taken from different places by using the serial dilution method described by Omar and Lee [15]. The soil or compost samples were collected from different places in Pakistan and were kept in sterilized polythene bags. Serial dilutions were made by adding 1.0 g of soil sample in 100 mL of sterilized distilled water and dilutions were made up to 10^7. Then 1 mL from the 10^6 dilution was added to the petri plate containing 4.0 % potato dextrose agar medium (pH: 5.5) and the plates were incubated at 45 °C for 48 to 72 h for the growth of fungi. The fungal colonies were further purified from bacterial contaminants by adding antibiotic amoxicillin (10 - 30 mg/L) in the culture medium. Different isolates of fungi were identified by examining them under a compound microscope (Model: XSA-107BN, China) after the work of Konemann and Van [16]. Isolated colonies were transferred to potato dextrose agar slants for culture maintenance. The cultures were stored in a refrigerator at 4 °C until further studies were carried out.

Inoculum preparation

10 mL of sterilized distilled water was transferred to each slant having profused conidial growth on its surface. The conidia were scratched with a sterilized inoculating needle and the tubes were shaken gently to break the clumps of conidia. The conidial suspension was used as an inoculum. The size of the inoculum was known by measuring the conidial density e.g. conidial number per unit volume with the help of Haemocytometer, Neubauer improved, precicdor HBG, Germany (Tiefe depth profondeur 0.100 mm and the area of 0.0025 mm^2).

Fermentation

Submerged fermentation was carried out in the present studies 25 mL of fermentation medium containing a carbon source (8.0 %), K_2HPO_4 (0.125 %), KH_2PO_4 (0.125 %), $MgSO_4.7H_2O$ (0.05 %), $NaNO_3$, (0.5 %), yeast extract (0.1 %), KCl (0.05 %), $FeSO_4$ (0.001 %) and $CaCO_3$ (0.3 %), pH 5.5 was transferred to each 250 mL conical flask and were cotton plugged. The flasks were sterilized in an autoclave at 121 °C, 15 lb/in^2 for 15 min. The flasks were cooled to room temperature and were inoculated with 1.0 mL conidial suspension. The flasks were incubated at 45 °C for 48 to 72 h in a shaking incubator (200 rpm). After the incubation, the fermented broth was centrifuged at 6,000 rpm for 10 min and the supernatant was used for analytical purposes.

Enzyme assay

The enzyme assay was performed by mixing 2.5 mL of *o*-dianisidine solution, 0.1 mL of peroxidase, 0.3 mL of glucose solution and 0.1 mL of enzyme solution in a cuvette. It was then incubated at 45 °C for 10 min in the incubator and the optical density at 436 nm measured using a spectrophotometer. One unit of glucose oxidase activity is defined as the "amount of enzyme that will convert one mole of D-glucose to product in 1 min under the given conditions of the assay".

Optimization of fermentation parameters

Different carbon sources like sucrose, fructose, lactose, glucose and cane molasses were added to the fermentation medium to test their effect on the production of extracellular glucose oxidase by thermophilic *Penicillium* species IIBP-13. Time course study for the production of extracellular glucose oxidase was carried up to 96 h. The effect of static and stirred fermentation was also studied on the production of extracellular glucose oxidase. All the experiments were carried out in triplicate.

Statistical analysis

A comparison of treatment effects was made using the methods of Snedecor and Cochrane [17].

The importance was presented as a Duncan's multiple range test and it was presented in the form of values of the probability (*P*).

Results and discussion

Thermophilic fungi are potential sources of heat-stable enzymes and valuable chemicals. The suitability of an enzyme for practical functions is dependent on its thermal stability [18]. So, various thermophilic *Penicillium* species were isolated from the soil samples taken from different places in Lahore, Pakistan and were screened for the biosynthesis of glucose oxidase. When the enzyme assay was carried out, different strains of thermophilic *Penicillium* species showed some differences in the values of the glucose oxidase production. Analysis of these values showed that IIBP-13 gave the best production of extracellular glucose oxidase (0.202 ± 0.018 U/mL) in submerged fermentation, while all other isolates gave less production of glucose oxidase (**Table 1**). Different scientists worked on different species of fungi for glucose oxidase production. Sukhacheva *et al.* [19] investigated the production and purification of extracellular glucose oxidase from *Penicillium funiculosum* 433. Study of various fungi was carried out for the natural genetic variability that may provide possibilities for selecting more potent producers from the population [20]. *Penicillium* has four different types or variants which have different characteristics and vary in their ability to produce glucose oxidase. In the same way different strains differ from each other in their capacity to produce glucose oxidase [21].

Table 1 Screening of thermophilic *Penicillium* sp. for glucose oxidase production.

***Penicillium* strains**	**Glucose oxidase activity (U/mL)***
IIBP-1	0.149 ± 0.009^{e}
IIBP-2	0.06 ± 0.005^{h}
IIBP-3	0.125 ± 0.01^{f}
IIBP-4	0.166 ± 0.019^{c}
IIBP-5	0.148 ± 0.009^{e}
IIBP-6	0.021 ± 0.0012^{j}
IIBP-7	0.09 ± 0.0015^{g}
IIBP-8	0.122 ± 0.009^{f}
IIBP-9	0.147 ± 0.012^{e}
IIBP-10	0.156 ± 0.009^{d}
IIBP-11	0.063 ± 0.0025^{h}
IIBP-12	0.187 ± 0.012^{b}
IIBP-13	0.202 ± 0.018^{a}
IIBP-14	0.036 ± 0.005^{i}

* ± indicates the standard deviation among the three parallel replicates. Values followed by letters differ significantly at $p \leq 0.05$. Incubation temperature = 45 °C, Fermentation time = 48 h.

The effect of various carbon sources like sucrose, fructose, lactose, glucose, and cane molasses was studied on the production of extracellular glucose oxidase by thermophilic *Penicillium* species IIBP-13 (**Figure 1**). Sucrose was found to be the best carbon source for the production of extracellular glucose oxidase by IIBP-13 using submerged fermentation (0.315 ± 0.007 U/mL) while all other carbon sources gave a lower production of glucose oxidase. Rando *et al.* [22] also found sucrose as the best carbon source for the production of glucose oxidase *by Penicillium pinophilum*. Hatziuikolaou and Macris [4] showed that various carbohydrates like sucrose, glucose, fructose, lactose, maltose and xylose can be used as a carbon source for glucose oxidase production but among these only glucose, fructose and sucrose were found to be useful.

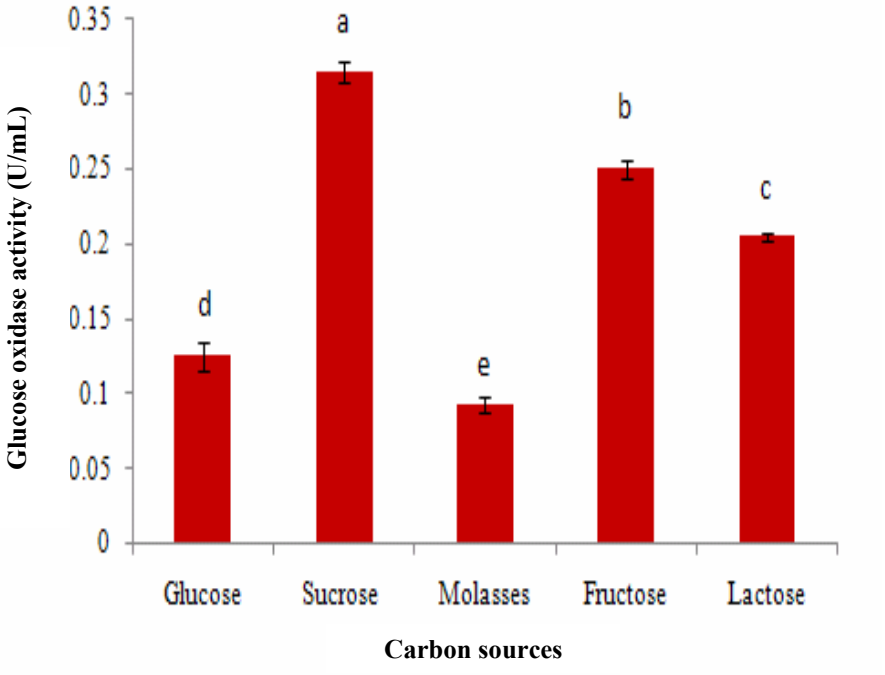

Figure 1 Effect of carbon source on glucose oxidase production by IIBP-13 (± indicates the standard deviation among the three parallel replicates. Values followed by letters differ significantly at $p \leq 0.05$, Incubation temperature = 45 °C, Fermentation time = 72 h.

The effect of different incubation time (24 - 96 h) was studied on the biosynthesis of extracellular glucose oxidase by thermophilic *Penicillium* species IIBP-13 using submerged fermentation (**Figure 2**). The results obtained after the statistical analysis showed that the maximum production of glucose oxidase (0.325 ± 0.009 U/mL) was obtained after 72 h of fermentation. Further increase in incubation time resulted in decreased glucose oxidase production. The decrease in the glucose oxidase production may be due to the depletion of substrate or macro- and micro nutrients in the fermentation medium with the lapse in time, which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes or accumulation of metabolic waste, toxic products or inhibitors (such as aldehydes and organic acids) in the culture medium, the hydrolytic action of proteases or oxygen depletion due to limitation of living space due to increase in number of organisms [23-25]. Jafari *et al.* [26] obtained maximum production of glucose oxidase by *A. niger* after 72 h of fermentation and the rate of activity remained constant after this time period.

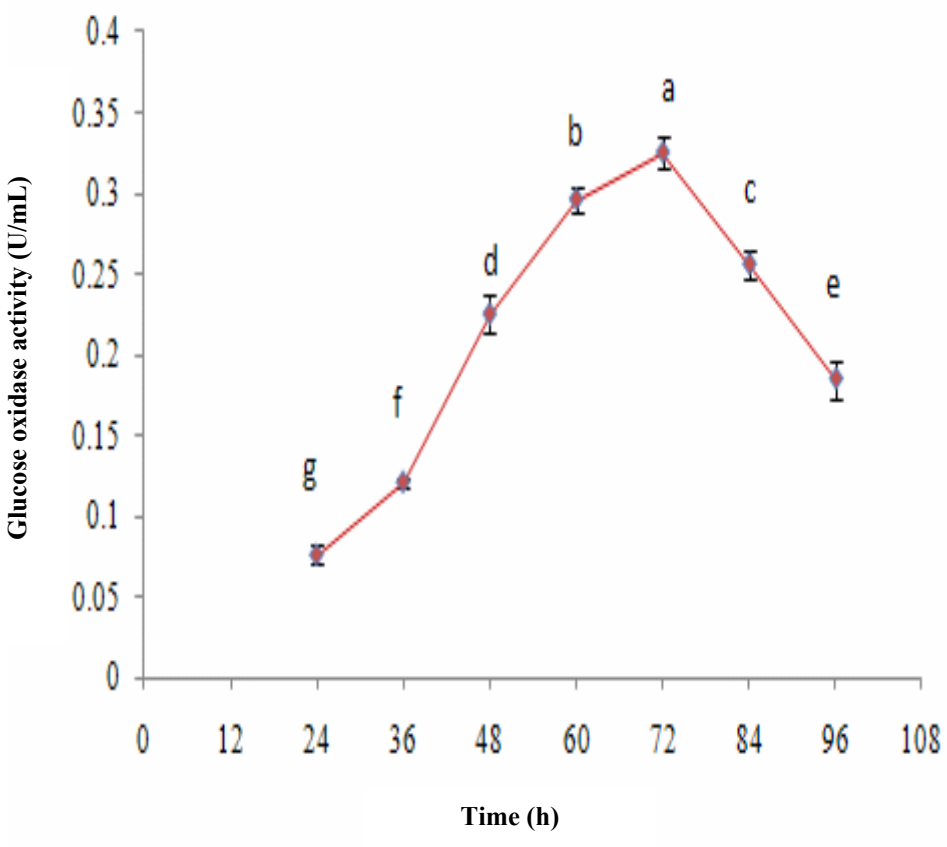

Figure 2 Effect of different fermentation time on glucose oxidase production by IIBP-13. (± indicates the standard deviation among the three parallel replicates. Values followed by letters differ significantly at $p \leq 0.05$. Incubation temperature = 45 °C, Carbon source = sucrose.

The effect of static and stirred fermentation was studied on the extracellular glucose oxidase production by thermophilic *Penicillium* species IIBP-13. Submerged fermentation was performed in both static and shaking incubator to study the effects of both of these types of incubations on extracellular glucose oxidase production. It was found that stirred fermentation was better for the production of glucose oxidase as compared to static fermentation (**Table 2**). Stirred fermentation at 200 rpm and 45 °C gave more production of glucose oxidase (0.320 ± 0.001 U/mL) as compared to static fermentation (0.195 ± 0.018 U/mL) after 72 h of incubation. Hamid *et al*. [27] inoculated the flasks with the pure culture of *Aspergillus* species and incubated the flasks in a rotary shaker at 150 rpm for 36 h for the biosynthesis of glucose oxidase. Sandip *et al.* [28] used the submerged fermentation technique for the production of glucose oxidase by *A. niger* in the shaker at 180 rpm and at 30 ± 2 °C for 96 h.

Table 2 Effect of culturing technique on glucose oxidase production by IIBP-13.

Culturing Technique	Glucose oxidase activity (U/mL)*
Static	0.195 ± 0.018^{b}
Stirred	0.320 ± 0.001^{a}

± indicates the standard deviation among the three parallel replicates. Values followed by letters differ significantly at $p \leq 0.05$. Incubation temperature = 45 °C, Carbon source = sucrose, Fermentation time = 72 h.

Conclusion

The effect of carbon sources, time of incubation and type of submerged fermentation on the biosynthesis of extracellular glucose oxidase by thermophilic *Penicillium* species IIBP-13 was investigated in this study. The study showed that submerged fermentation in a shaker at 200 rpm for 72 h at 45 °C was best for the production of extracellular glucose oxidase (0.325 ± 0.009 U/mL) by thermophilic *Penicillium* sp IIBP 13 and this study will help in the development of inexpensive and cell free enzymatic processes for the production of the gluconic acid.

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