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Enhanced Production of Glucose Oxidase Using *Penicillium notatum* and Rice Polish

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Summary

Glucose oxidase (GOD) is an important enzyme that finds a wide range of applications in food and pharmaceutical industry. In this investigation the feasibility of using rice polish as a substrate for the production of GOD by *Penicillium notatum* in submerged fermentation (SmF) has been evaluated. The intention was to enhance total GOD activity by the selection of economical substrate, microorganism and consecutive optimization of various cultural conditions. Maximum GOD activity of (112±5) U/mL was achieved under optimum growth conditions: rice polish 5 g, incubation period 72 h, buffering agent 3 % (by mass per volume), incubation temperature (30±1) °C and pH=6.0. Addition of carbon and nitrogen sources further enhanced the enzyme yield, indicating an economically attractive process for GOD production.

Key words: glucose oxidase, *Penicillium notatum*, submerged fermentation, rice polish, culture conditions

Introduction

Glucose oxidase (GOD, β-D-glucose:oxygen-1-oxidoreductase, EC 1.13.4) is an enzyme which catalyses the oxidation of β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide using molecular oxygen as the electron acceptor. D-glucono-δ-lactone, a weak competitive inhibitor of GOD, is hydrolyzed non-enzymatically to gluconic acid and the reduced $FADH_2$-enzyme is reoxidized by molecular oxygen (*1*). GOD is commercially used in pharmaceutical industry as a biosensor in diagnostic kits (*2–4*) and in the food industry for the removal of glucose or oxygen to improve colour, flavour, texture and shelf life of various products (*5–7*). Recently, GOD has also been used in biofuel cells (*8*).

The most common microbial sources for GOD production are selected strains of *Aspergillus niger* and *Penicillium amagasakiense*, although a high level of production has also been reported from *Penicillium variabile* (*9,10*). The enzyme from *Aspergillus niger* is considered to be an intracellular enzyme, while the enzyme from *Penicillium* sp. has been generally regarded as an extracellular enzyme.

Typical problems that are usually encountered during their production are high cost of the substrate, low productivity and simultaneous production of other enzymes such as catalase (*1*). To overcome these problems, it is suggested that economical and commercially available media be investigated to reduce the production costs. Some of the commercial substrates exploited for production of GOD are molasses (*11*) and corn steep liquor (*12*). The selection of a particular strain, however, remains a tedious task, especially when commercially significant enzyme yields are to be obtained. Rice polish is the flour taken from the basic brown rice during the process of making white rice. It contains parts of the rice germ and bran, and provides a high content of vitamins and iron. A reduction of the medium cost suggests that rice polish is a cost effective medium for fermentation.

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In this paper, an effort was made to optimize the culture conditions for enhanced production of glucose oxidase from *Penicillium notatum* using low cost rice polish as carbon source.

Materials and Methods

All the chemicals used were of analytical grade and mainly purchased from Sigma Chemical Company, USA. Rice polish was procured from a local rice mill.

Organism and maintenance

Penicillium notatum was obtained from National Fungal Culture Collection of Pakistan (NFCCP), Department of Plant Pathology, University of Agriculture, Faisalabad. The culture was maintained on potato dextrose agar (PDA) slants and subcultured once a month with storage at 4 °C.

Inoculum development

The inoculum was developed by transferring spores from 5- to 6-day-old slant culture into 500-mL Erlenmeyer flask containing 150 mL of sterile culture medium. Composition of the culture medium was (in g/L): glucose 20, trisodium citrate 2.5, KH_2PO_4 5, NH_4NO_3 2, $(NH_4)_2SO_4$ 4, $MgSO_4 \cdot 7H_2O$ 0.2, peptone 2, microelement solution 10 mL and vitamin solution 5 mL. The solution of trace elements had the following composition (in g/L): $CuSO_4$ 0.08, H_2MoO_4 0.05, $MnSO_4 \cdot 4H_2O$ 0.07, $ZnSO_4 \cdot 7H_2O$ 0.043 and $Fe_2(SO_4)_3$ 0.05, whereas the vitamin solution contained (in g/L): biotin 0.2, folic acid 0.2, thiamine-HCl 0.5, riboflavin 0.5, pyridoxin-HCl 1, cyanocobalamine 0.1, nicotinic acid 0.5, Ca-pantothenate 0.5, *p*-aminobenzoic acid 0.5 and thioctic acid 0.5. The pH of the medium was adjusted to 5.0 using 1 M HCl/1 M NaOH. The flasks were incubated on a rotary shaker at 150 rpm at (30±1) °C for 48 h to get a homogenous spore suspension (*13*).

Optimization of conditions under submerged fermentation

All the growth experiments for GOD production were performed under submerged fermentation (SmF). SmF was carried out considering different process parameters like substrate amount (0.5–7 g/50 mL culture medium), initial pH (4–7), fermentation period (12–120 h), effect of temperature (25–40 °C), effect of buffering agent ($CaCO_3$) and various carbon (maltose, fructose, glucose, sucrose and starch) and nitrogen (urea, peptone, cotton meal and yeast extract) additives that affect the production of GOD. Conditions were optimized by adopting the search technique of varying parameters one at a time (*11*). All the experiments were conducted in 250-mL Erlenmeyer flasks containing substrate and 50 mL of basal medium. Composition of the basal medium was (in g/L): trisodium citrate 2.5, KH_2PO_4 5, $MgSO_4 \cdot 7H_2O$ 0.2. Flasks were plugged with cotton and sterilized by autoclaving for 15 min at 121 °C. SmF conditions included agitation at 150 rpm, inoculum size 3 mL (10^6–10^7 spores/mL) under various experimental conditions. Optimum conditions achieved in each step were fixed for subsequent studies. All experiments were conducted in triplicate and the results were reported as mean ± SD.

Enzyme isolation

Crude extract of extracellular GOD was prepared by filtering the culture fluid through a Whatman No. 4 filter paper. After the removal of cell debris by centrifugation (39 200×*g* for 10 min at 4 °C), clear supernatant was used as enzyme source to determine its activity.

Enzyme assay

GOD activity was determined with the help of a coupled *o*-dianisidine-peroxidase reaction (*14*). Appropriately diluted enzyme (100 μL) was added to dianisidine buffer mixture (pH=6.0) containing glucose and peroxidase after proper mixing. The increase in absorbance at 460 nm was monitored with the help of Hitachi UV-VIS spectrophotometer, thermostated at 25 °C. One unit (U) of GOD activity was defined as the amount of enzyme required to oxidize 1 μmol of glucose/(mL·min) under the above assay conditions.

Results and Discussion

The production of GOD by *Penicillium notatum* was studied in submerged SmF. Shake flask experiments were conducted to optimize the culture conditions for its enhanced production. Results regarding the effect of the amount of rice polish are shown in Fig. 1. High enzyme activity of (60±3) U/mL was achieved with 5 g of rice polish as compared to lower or higher amount at 30 °C for 48 h. Significant difference ($p<0.05$) in GOD activity was observed with various amounts of rice polish. The amount of substrate per unit area of working volume of the flask may influence the porosity and aeration of the substrate, which ultimately influences enzyme biosynthesis by the microorganism (*15*,*16*). Moreover, a high concentration of rice polish may have inhibitory effect on the growth of organism. A GOD activity of 2.17 U/mL has been reported with *Aspergillus niger* using sucrose (*11*). Similarly, GOD activities as high as 3.42 U/mL were produced at 2 % substrate level (rice polish) by *Aspergillus niger* (*17*). After optimizing the amount of substrate (5 g), pH of the medium was optimized to get max-

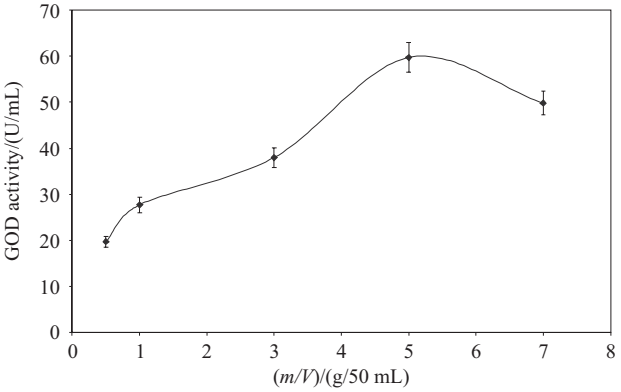

Fig. 1. Effect of the amount of substrate on GOD production by *P. notatum* under optimum conditions: pH=5.0, temperature (30±1) °C, inoculum size 3 mL, incubation time 48 h

imal enzyme yield. Maximum GOD activity of (66±3) U/mL was observed at pH=6.0, 3 mL of inoculum size and an incubation period of 48 h. Statistical analysis showed the significant difference ($p<0.05$) among the activities of GOD at varying pH values (4–7). A wide range of pH from 5.5 (*11*) to 7.0 (*12*) affecting the production of GOD by various microorganisms has been reported in the literature. A pH value of 6.5 has been reported for GOD production by *Penicillium variabile* (*18*). Optimum pH is very important for growth of organisms and their metabolic activities. As the metabolic activities of the organisms are very sensitive to changes in pH, GOD production by *Penicillium notatum* was found to be affected by higher or lower pH levels compared to the optimum value.

The effect of incubation time on GOD production is shown in Fig. 2. Results revealed that an incubation period of 72 h was necessary for maximum GOD activity of (94±6) U/mL. After 72 h, GOD activity started decreasing with a value of (72±4) U/mL after 120 h. Statistical analysis indicated a significant difference ($p<0.05$) in GOD activities during various time course studies. It is a well-known fact that the cultivation time depends on the nature of the microorganism, nature of the fermenting material, concentration of the nutrients and physiological conditions. Generally, the growth period in synthetic media by filamentous fungi varies from 48–72 h. A strain of

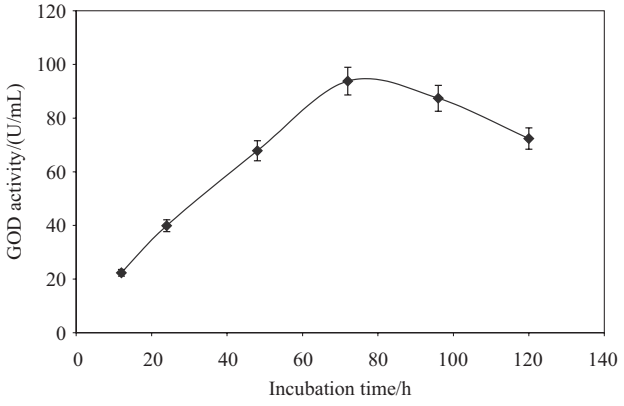

Fig. 2. Effect of incubation time on GOD production by *P. notatum* under optimum conditions: pH=6.0, temperature (30±1) °C, inoculum size 3 mL, substrate 5 g

Aspergillus niger (G-IV-10) produced maximum GOD activity (both intracellular and extracellular) after 48 h of incubation (*19*). Petruccioli *et al*. (*10*) reported an optimum growth period of 72 h for maximum (65.1 % more than the control) GOD activity. The effect of growth temperature on GOD production by *Penicillium notatum* is depicted in Fig. 3. Maximum GOD activity of (95±5) U/mL was achieved in the medium incubated at (30±1) °C. Higher growth temperatures resulted in lower enzyme synthesis by mesophilic fungi. Incubation temperature is a critical process variable that varies from organism to organism, and slight changes in growth temperature may effect GOD production. At higher temperature, due to the production of large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation (*15*). Growth temperatures of 27.5 and 30 °C have been reported for total glucose oxidase production by *Aspergillus niger* (*6*,*11*).

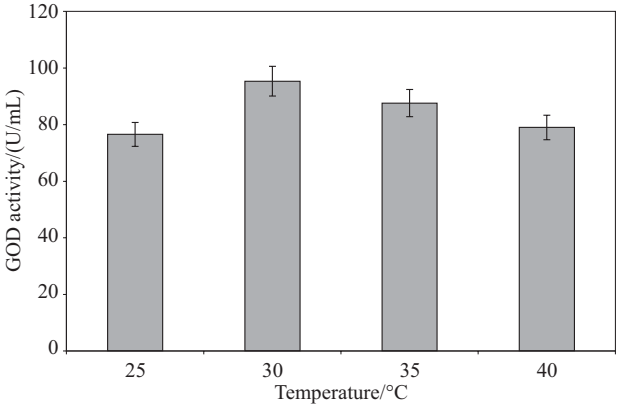

Fig. 3. Effect of initial incubation temperature on GOD production by *P. notatum* under optimum conditions: pH=6.0, inoculum size 3 mL, substrate 5 g, incubation time 72 h

Addition of buffering agent ($CaCO_3$) to the growth medium substantially induced GOD activity. A high titre of GOD ((112±5) U/mL) was observed with 3 % (by mass per volume) of buffering agent. The difference between 3 and 4 % (by mass per volume) was not statistically significant. The addition of $CaCO_3$ to prevent a pH drop had been shown to be extremely important due to gluconic acid formation during fermentation (*9*). Moreover, it has been suggested that it might give some kind of mechanical support to fungal mycelium for proper growth (*11*). After optimizing growth conditions, the time course of growth and enzyme activity of the fungus grown under optimized conditions was also investigated. An enzyme activity of (115±6) U/mL was observed after 72 h (Fig. 4).

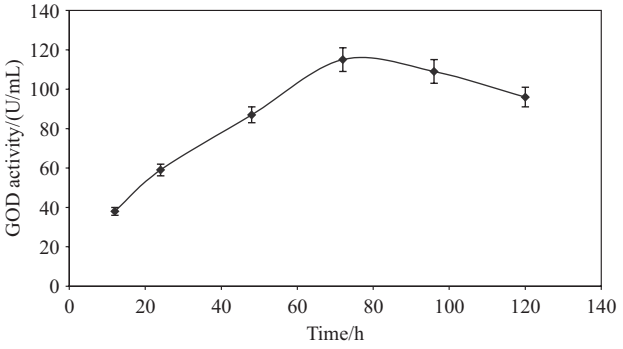

Fig. 4. Time course study of GOD production by *P. notatum* under optimum conditions: pH=6.0, inoculum size 3 mL, substrate 5 g, temperature (30±1) °C, calcium carbonate 3 % (by mass per volume)

In order to determine whether rice polish was deficient in nutrients, the substrate was supplemented with various carbon and nitrogen additives to a level of 1 % (by mass per volume). The results show that the best GOD activities were observed with sucrose ((134±6) U/mL) as compared to control (Fig. 5). Similarly, all the sources

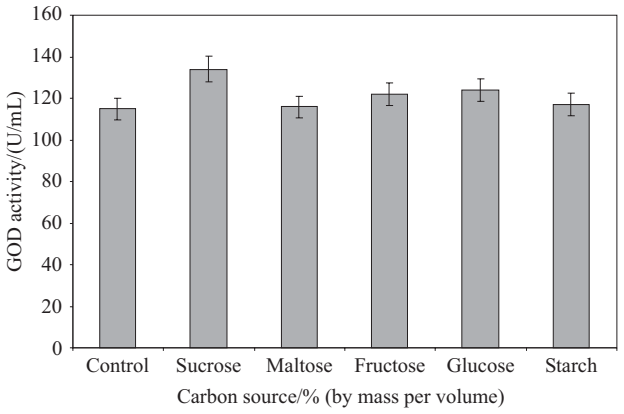

Fig. 5. Effect of different carbon additives on GOD production by *P. notatum* under optimum conditions: pH=6.0, temperature (30±1) °C, inoculum size 3 mL, substrate 5 g, incubation time 72 h, calcium carbonate 3 % (by mass per volume)

of nitrogen increased GOD production; however, peptone exhibited better glucose oxidase yield ((131±7) U/mL) followed by yeast extract ((129±7) U/mL). The microorganisms require an adequate supply of carbon as energy source and nitrogen for various metabolic activities. The effect of various carbon and nitrogen sources has been reported in several works. Hatzinikolaou and Macris (*11*) reported maximum activity of GOD by *Aspergillus niger* using glucose and peptone. *Penicillium variabile* exhibited better activity of GOD with glucose (*18*). Results of this investigation indicate that rice polish could be an attractive and promising substrate for enhanced production of glucose oxidase by *Penicillium notatum.*

Conclusions

Glucose oxidase was isolated from mycelium extracts of *Penicillium notatum.* The results provided valuable information for the production of GOD by *Penicillium notatum* using relatively inexpensive substrate rice polish. Maximum GOD activity of (112±5) U/mL was achieved under optimum growth conditions, which were: rice polish 5 g, incubation period 72 h, incubation temperature (30±1) °C and pH=6 for the maximum yield of the enzyme. Addition of carbon and nitrogen sources further enhanced the enzyme yield, indicating an economically attractive process for GOD production. These characteristics will further aid to explore the knowledge of the commercial production of glucose oxidase and its applications.

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