

## Short Communication

# Enhanced production of glucose oxidase from UV-mutant of *Aspergillus niger*

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UV rays were used as mutagen in wild type strain of *Aspergillus niger* for enhanced production of glucose oxidase. After mutagenization and selection, mutant *A. niger* strains, resistant to 2-deoxy-D-glucose were obtained. The mutants showed 1.57 and 1.98 fold increase in activities of extra and intra cellular glucose oxidase respectively in comparison with the parental strain. Out of 6 mutants, mutant U-6 was selected as the best producer of glucose oxidase after 36 h of fermentation using black strap molasses as substrate.

**Key words:** *Aspergillus niger*, glucose oxidase, mutagenesis, UV.

## INTRODUCTION

Glucose oxidase [EC 1.1.3.4] catalyzes the oxidation of β-D-glucose, via D-glucono-δ-lactone to D-gluconic acid (GA) and hydrogen peroxide using molecular oxygen as an electron acceptor (Worthington, 1988). Glucose oxidase has found several commercial applications mainly in food industries and in clinical analysis (Bucke, 1983; Röhr et al., 1983), and due to increasing number of potential uses as an ideal diagnostic tool (Field et al., 1986).

Several attempts have been made to improve glucose oxidase production through *Aspergillus niger* by strain selection using mutagenesis classical screening techniques (Fiedurek et al., 1986; Markwell et al., 1989; Witteveen et al., 1990). Traditionally, strain development requires painstaking lengthy and tedious procedures to identify superior isolates among a mutagen-treated population. Rational selection procedures are considerably more efficient than random screening for selecting improved producers, and usually have a biochemical basis. Special environmental condition, toxic to the majority of cell types but less toxic or non-toxic to a desired minority of cells have been often employed to enrich a cell population to obtain desired mutants (Elander, 1980; Elkeles et al., 1994). The great advantage of this screening method is its simplicity; it does not require any

profound understanding of molecular biology and physiology of the micro-organisms being manipulated.

In the present study an effort was made to improve the enzyme glucose oxidase production from mutagenization of *A. niger*, resistant to a range of metabolic inhibitors, as a means of obtaining isolates with high yields of glucose oxidase. The best mutants were compared with the parental strain for the production of glucose oxidase using fermentation biotechnology processes.

## MATERIALS AND METHODS

### Organism

*A. niger* wild type strain, forward from Department of Microbiology, was used and maintained on potato dextrose agar (PDA) slants, stored at 4°C in a refrigerator. All the media, unless otherwise stated, were sterilized at 1.05 kg/cm<sup>2</sup> pressure at 121°C for 15 min.

### Inoculum preparation

Vogel's medium of 50 ml (0.5% trisodium citrate, 0.5% KH<sub>2</sub>PO<sub>4</sub>, 0.2% NH<sub>4</sub>NO<sub>3</sub>, 0.4% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.02% MgSO<sub>4</sub>, 0.1% peptone, 0.2% yeast extract) pH 5.5 was used as inoculum into a 300 ml conical flask. Chronic acid washed marble chips (3 - 5 in number) were added in each flask (to break the mycelial colonies) and were autoclaved. 2 ml of sterilized 50% (w/v) stock solution of glucose were aseptically added to the autoclaved Vogel's medium as a carbon source. The flasks were inoculated with a loop full *A. niger* spores under aseptic conditions. The inoculum was kept at 30°C on

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a rotary shaker (180 rpm) for 36 h (Haq et al., 2001). The cell were harvested, centrifuged at  $8331\times g$  for 15 min, washed twice with saline and resuspended in saline solution. The optical density was measured calorimetrically (at 610 nm) and maintained at  $0.5 \times 10$  dilution. The above fungal population was used for mutational work or to inoculate the glucose oxidase fermentation medium.

### Improvement techniques

The spore suspension of 36 h culture of *A. niger* was transferred to sterile Petri dishes and was exposed to UV irradiations ( $10^7$  spores  $ml^{-1}$ ) using a 20 w UV lamp (Gromada and Fiedurek, 1997). The dose of exposure to suspension was  $1.2 \times 10^6$  J/m<sup>2</sup>/s for different time intervals (15 - 180 min). The survival curve was prepared and time of exposure giving 3 log kill was selected for mutation of the organism (Haq et al., 2001). At this stage the drop in viability was about 90%. After mutagenization the surviving spores were grown on PDA medium containing ox gall (1 - 2%) as colony restrictor along with 2-deoxy D-glucose ( $1 \text{ mg ml}^{-3}$ ) as metabolic inhibitor. So, the appeared mutant colonies were picked and used for the glucose oxidase production.

### Screening procedures

Mutants *A. niger* producing hyper glucose oxidase was identified on agar plate containing  $0.1 \text{ gL}^{-1}$  O-dianisidin and  $6000 \mu\text{ml}^{-1}$  of horse radish peroxidase giving rise a brown color. Six strains showed the greatest diameter of the enzymatic zone in comparison with parent strain.

### Glucose oxidase production

The entire selected mutants were used for glucose oxidase production through liquid state fermentation using molasses (2%) as substrate in fermentation medium. The pH of the medium was adjusted to 5.5. The flasks were incubated, in triplicate, on an orbital shaker (180 rpm) at 30°C for 36 h. The data given here are the average of the measurements.

### Enzyme assay

Mycelia from the culture liquid were collected and suspended in potassium phosphate buffer (10 mM, pH 5.0). To determine the intracellular enzyme activity the mycelia were disrupted in a commercial blender (Perkin Elmer 053257), operating at maximum speed for 3 min and resulting suspension was subjected to sonication at 5°C speed  $10,000\times g$  for 15 min (Heraeus Omnifuge, Germany). To determine extracellular enzyme activity aliquots of culture were clarified and enzyme activity was measured in the clear suspension. The linear rate of glucose consumption was determined at 460 nm wavelength. One unit of glucose oxidase activity was defined as the amount of enzyme required to oxidize 1  $\mu$  mole of glucose per minute.

## RESULTS AND DISCUSSION

Screening of the best mutant was carried out on a number of *A. niger* strain in order to isolate those with the highest total glucose oxidase activity. Six strains showed increased glucose oxidase activity. Out of six strains mutant U-6 was characterized by the best growth on the medium containing 0.1% 2-deoxy-D-glucose, showed the greatest diameter of the enzymatic zone compared with

**Table 1.** Total glucose oxidase activities.

Type	Extracellular activity (U ml <sup>-1</sup> )	Intracellular activity (U ml <sup>-1</sup> )
Mutant U1	10.51	47.05
Mutant U2	12.02	35.02
Mutant U3	13.43	49.01
Mutant U4	7.68	18.67
Mutant U5	17.55	41.12
Mutant U6	20.51	51.81
Mutant U7	15.43	33.00
Mutant U8	9.45	22.49
Parent	11.25	28.86

Values represent means of three cultures at 30°C for 36 h of fermentation.

the parent strain. The results showed that the mutant strain U-6 obtained the highest glucose oxidase activity (Table 1).

A number of specific selection schemes have been adapted to improve the production of enzyme. Resistance to the toxic glucose analogue (2-deoxy-D-glucose) has been used as a criterion to select mutants showing increased rates of glucose oxidase (Fiedurek et al., 1987). High levels of chymosin derivative have been isolated from the culture of *A. niger* on the basis of resistance to sodium orthovanadate, a toxic analogue of inorganic phosphate, and poison phosphorylated enzymes such as ATPase. Selection of mutants resistant to antibiotics is sometimes effective for strain improvement.

A number of carbon sources were tested in order to determine their effect on growth and total glucose oxidase production by selected strain. The results are summarized in Table 2 and showed the best activities were obtained with glucose, sucrose and molasses. Infact, the microorganism was able to grow in all carbon source tested, but glucose oxidase was produced at significant levels only in three types of carbon sources mentioned in the Table 2. This result indicates that glucose is a principle inducer of the glucose oxidase gene.

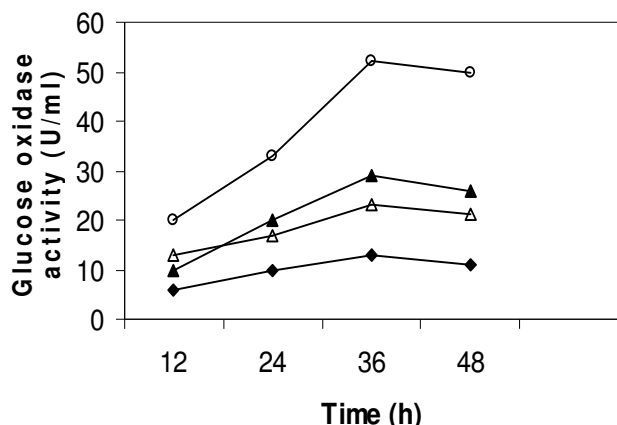
The dynamics of enzyme activity of the most active mutant *A. niger* U-6 and its parental strain is presented in Figure 1. The intracellular and extracellular concentration of glucose oxidase continued to rise with time in both cultures to reach the maximum activity. Compared with the parent strain, intra and extracellular glucose oxidase activity increased about 1.57 and 1.98 fold, respectively.

Literature reports the possibility of obtaining high effectiveness of glucose oxidase synthesis in *A. niger* and *P. notatum* mutants, for instance, by induction with various mutagens (Fiedurek et al., 1990; Markwell et al., 1989; Petruccioli et al., 1995). In this respect, our results can be compared with data compiled by Gromada and Fiedurek (1997) who improved glucose oxidase production over 125% after mutagenesis of *A. niger*. Similar results (over twofold increase of glucose oxidase

**Table 2.** Effect of certain carbon sources on growth of *Aspergillus niger* and total glucose oxidase production<sup>a</sup>.

Carbon source	Biomass (mg ml <sup>-1</sup> )	Total enzyme activity (U ml <sup>-1</sup> )
Fructose	7.5	11.15
Galactose	6.3	0.00
Glucose	6.9	21.74
Lactose	8.2	1.00
Molasses	5.9	21.83
Sucrose	6.1	12.17
Starch	7.3	10.05

<sup>a</sup>The cultivation time was 2 days. Molasses was best as a carbon source. The CaCO<sub>3</sub> concentration was 5%.



**Figure 1.** Dynamics of extracellular glucose oxidase production by the most active *A. niger* (parent; ■). Intracellular glucose oxidase activity of *A. niger* (parent; ▲). Extracellular glucose oxidase activity of *A. niger* (mutant; △). Intracellular glucose oxidase activity of *A. niger* U-6 (mutant; ○).

activity) were reached by Markwell et al. (1989) and Witteveen et al. (1993) after mutagenization of *A. niger*.

The studies presented above seem to confirm a high effectiveness of deletion in some kinds of biochemical mutants with regard to increased glucose oxidase activity in the *A. niger* fungus. This mutational work has given a potent organism for production of glucose oxidase from molasses. Further work is needed to improve the substrate consumption rate by isolating mutants which are resistant to higher concentration of 2-deoxy-D-glucose and are aspartate-requiring. Such mutants have been shown to exhibit high substrate consumption and product formation rates.

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