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RANDOM MUTAGENESIS OF *ASPERGILLUS NIGER* AND PROCESS OPTIMIZATION FOR ENHANCED PRODUCTION OF GLUCOSE OXIDASE

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Abstract

The study deals with the improvement of wild strain *Aspergillus niger* IIB-31 through random mutagenesis using chemical mutagens. The main aim of the work was to enhance the glucose oxidase (GOX) yield of wild strain (24.57±0.01 U/g of cell mass) through random mutagenesis and process optimization. The wild strain of *Aspergillus niger* IIB-31 was treated with chemical mutagens such as Ethyl methane sulphonate (EMS) and nitrous acid for this purpose. Mutagen treated 98 variants indicating the positive results were picked and screened for the glucose oxidase production using submerged fermentation. EMS treated E45 mutant strain gave the highest glucose oxidase production (69.47 + 0.01 U/g of cell mass), which was approximately 3-folds greater than the wild strain IIB-31. The preliminary cultural conditions for the production of glucose oxidase using submerged fermentation from strain E45 were also optimized. The highest yield of GOD was obtained using 8% glucose as carbon and 0.3% peptone as nitrogen source at a medium pH of 7.0 after an incubation period of 72 hrs at 30ºC.

Introduction

Glucose oxidase (β-D-glucose: oxygen oxidoreductase; EC 1.1.3.4) also known as glucose aerodehydrogenase is a flavin protein that catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone using oxygen as an electron acceptor and simultaneously producing hydrogen peroxide. D-glucono-δ-lactone is later converted into gluconic acid. This enzyme only acts on β-D-glucose and not on it's α anomer. Glucose oxidase is a glycoprotein having two identical 80 kDa subunits linked through two disulphide bridges (Wohlfahrt *et al.,* 2003).

Glucose oxidase can be obtained from a number of different sources including red algae, citrus fruits, insects, bacteria and molds but industrially fungal sources are preferred mainly from the genus *Aspergillus* (Hatzinikolaou and Macris, 1995; Zia *et al.,* 2012a; Zia *et al.,* 2012b) and *Penicillium* (Sukhacheva *et al.,* 2004), of which *A. niger* is the most commonly utilized for the production of glucose oxidase (Pluschkell *et al.,* 1996). The preferred use of *Aspergillus niger* lies in its easy handling, high production of enzyme, easy recovery of the enzyme and metabolic versatility of the strain.

Due to its wide spread application in different industries glucose oxidase needs to be produced extensively. At the same time the methods of production should also be cost effective and economical. Various methods have been used to enhance the production of glucose oxidase including the classical screening method and mutagenesis (Zia *et al.,* 2010). Two different types of mutagenesis can be used for the enhancement of production i.e. the site directed mutagenesis and the random mutagenesis. While the mutagens used for the random mutagenesis can also be of two types i.e. physical mutagens e.g. ultraviolet, gamma and X-irradiation and chemical mutagens like ethyl methane sulfonate (EMS), ethidium bromide, nitrosotrimethyl guanidine (NTG), N-methyl N-nitro N-nitrosoguanidine (MNNG), nitrous acid etc. Different mutagens have different modes of action (Rowlands, 1984; Iftikhar *et al.,* 2010).

The natural function of glucose oxidase is to act mainly as an antibacterial and antifungal agent through the production of hydrogen peroxide. While industrially glucose oxidase is very important because of its plentiful applications in various fields including chemical, pharmaceutical, food, beverages, clinical chemistry, biotechnology and other industries. It is used as a biosensor in diagnostic kits for quantitative determination of glucose in biological fluids. In food industry, it is used for the removal of glucose or oxygen to improve color, flavor, texture and shelf life of various products. Apart from this it is also used as food additive, as acidity controller and in biofuel cells (Wong *et al.,* 2008).

Materials and Methods

Isolation and screening of microorganism: Collection of soil samples was carried out from the vicinities of different food industries. Soil samples were used for isolation of fungal strains using PDA medium after performing the serial dilution. On the basis of morphological analysis, colonies showing resemblance with *Aspergillus niger* were picked up and transferred to slants. All the isolates were subjected to screening which was done using submerged fermentation in 250 ml Erlenmeyer flasks. The strain yielding best and reproducible results was further confirmed using morphological techniques and biochemical testing as *Aspergillus niger.* The strain was cultured on PDA and was incubated at 30^{o}C for 72 hrs. The cultures were preserved at 4^{o}C in cold cabinet and for maintaining the vitality of the cultures; sub culturing was done after 7 days interval.

Strain improvement

Nitrous acid mutation: Fully grown *A. niger* slants were used to prepare the spore suspension. Spore suspension having 1.2 x 10^7 spores was subjected to

centrifugation and the pellets obtained were washed twice with 0.1 M phosphate buffer (pH 7.0). After that pellets were treated with 0.1 M sodium acetate buffer and subsequently $NaNO_2$ was added to adjust the final concentration to 0.8, 0.9, 1.0 and 1.1 M. The reaction mixture was vigorously mixed for 10 min and reaction was stopped by adding 0.1 M phosphate buffer. Pellets obtained after centrifugation of reaction mixtures were washed and re-dissolved in phosphate buffer. 100 µl of it was poured onto PDA plates and incubated at 30°C for 48 hrs (Mala *et al.,* 2001).

EMS mutation: The spore suspension of *A. niger*, as prepared above was serially diluted to have final concentration of 1.2 x 10^7 spores/ml. 250µl of the diluted spore suspension was transferred to 3 different eppendorf tubes and 50µl of EMS was added in every tube to make the final concentration of 200mM. Treatment with EMS was done at time interval of 5, 10 and 15 min. Reaction was stopped by adding sodium thiosulphate to the treatment tubes. Reaction mixture was centrifuged and the obtained pellet was washed twice using phosphate buffer. 100µl of re-dissolved pellet was poured on PDA plates (Lotfy *et al.,* 2007).

Inoculum preparation: Three days old *A. niger* slant was taken and 10 ml of sterilized distilled water was added to the slant. The conidia were scratched off from PDA matrix using a sterilized inoculating loop and then the spore suspension was vortexed for homogenization. This spore suspension was used as inoculum for fermentation.

Submerged fermentation: Glucose oxidase production was done using the the medium having 8% Glucose, 0.3% peptone, 0.0388% $(NH_4)_2HPO_4$, 0.0188% KH_2PO_4, 0.0156% $MgSO_4.7H_2O$ and 3.5% $CaCO_3$ (Fiedurek & Sczodrak, 1995). The medium was autoclaved, cooled and then aseptically inoculated with 1ml of spore suspension as prepared earlier. The inoculated flasks were placed in a shaking incubator at 250 rpm and 30°C for 72 hrs.

Extraction of enzyme: The fermentation broth was filtered using cheese cloth and the mycelium was crushed and homogenized in 0.1 M chilled citrate phosphate buffer having pH 7.0. The crude extract was centrifuged at 6000 rpm for 10 min. The extract obtained was further used for the estimation of glucose oxidase along with total protein content.

Glucose oxidase assay: O-dianisidine method was used for estimation of glucose oxidase (Bergmeyer *et al.,* 1974). 2.5 mL O-dianisidine (0.1mg/ml), 0.3 mL glucose (10%) and 0.1 mL peroxidase solution (1 mg/mL) were added to the cuvette and allowed to equilibrate at 25°C. It was used as a blank, and then 0.1 ml of sample extract was added to the cuvette, the reaction mixture was thoroughly mixed and absorbance of both the blank and sample was taken at 436nm. The absorbance per min was determined using 7 cycles of 00.10 seconds each, using spectrophotometer.

One unit of glucose oxidase is defined as the amount of enzyme catalyzing one micromole of glucose per min under assay conditions.

$$\text{GOX activity} = \frac{\Delta OD/min \times TV \times \text{dil. factor}}{8.3 \times SV \times \text{cell mass (g)}}$$

where:
$\Delta OD/min = OD_2 - OD_1 / T_2 - T_1$
TV = Total Volume (3.0 ml)
8.3 = millimolar extraction coefficient for O-dianisidine (oxidised)
SV = Sample Volume
Total protein content of the extract was estimated using Bradford assay (Bradford, 1976).

Results and Discussion

One hundred and sixty fungal isolates were obtained from the soil samples. These isolates were subsequently screened for the production of glucose oxidase using submerged fermentation (Fig. 1). Out of 160 fungal isolates, 27 showed production of glucose oxidase and among these 27 isolates, the strain IIB-31 gave the highest yield of glucose oxidase i.e., 24.57±0.1 U/g of cell mass. It was identified as *A. niger* using morphological and molecular techniques.

The wild strain of IIB-31 was then treated with various mutagenic agents including nitrous acid and EMS (ethyl methane sulphonate). 98 variants from parent strain were obtained and screened for glucose oxidase activity (Figs. 2 and 3). The E-45 strain gave the highest yield of glucose oxidase and was consequently selected for further work.

The conditions for the production of glucose oxidase from IIB-31 strain and E-45 strain were optimized. The effect of different carbon and nitrogen sources, incubation time, temperature, pH, size of inoculum used and the concentration of the inducer i.e. calcium carbonate in the medium, was studied.

Effect of different incubation periods on the production of glucose oxidase was studied using time periods of 24, 36, 72 and 96 hrs (Fig. 4). The maximum activity for both the wild IIB-31 strain (20.58±0.01 U/g of cell mass) and mutant E-45 strain (64.95±0.01 U/g of cell mas) was seen after 72 hrs of incubation. The decrease in enzyme production started after 72 hrs which was a result of the depletion of nutrients and decrease in pH of the fermentation media. Similar types of results were reported by Hatzinikolaou & Macris (1995) and Petruccioli *et al.,* (1999).

Different carbon sources were used for the investigation including glucose, maltose, sucrose, fructose and lactose (Fig. 5). Glucose gave the maximum yield of glucose oxidase in both the mutant E-45 strain (67.95±0.01 U/g of cell mas) and wild IIB-31 strain (23.58 ± 0.01 U/g of cell mass). This might be due to the reason that glucose can be easily metabolized by the organism used as compared to the other carbon sources. The results are similar to those reported by Rogalski *et al.,* (1988).

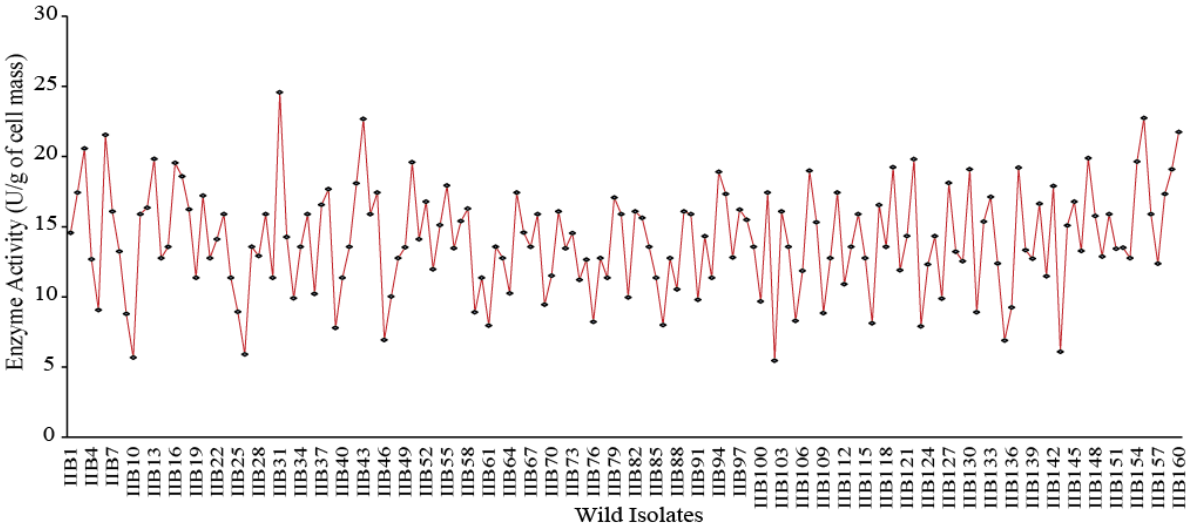

Fig. 1. Screening of the *A. niger* isolates for glucose oxidase production.

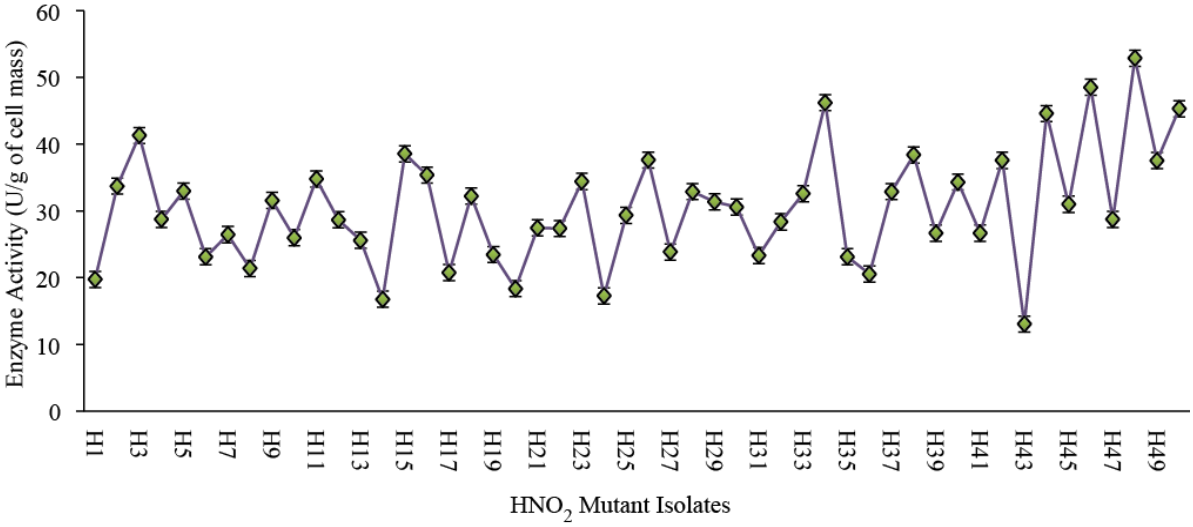

Fig. 2. Glucose oxidase production by nitrous acid mutants.

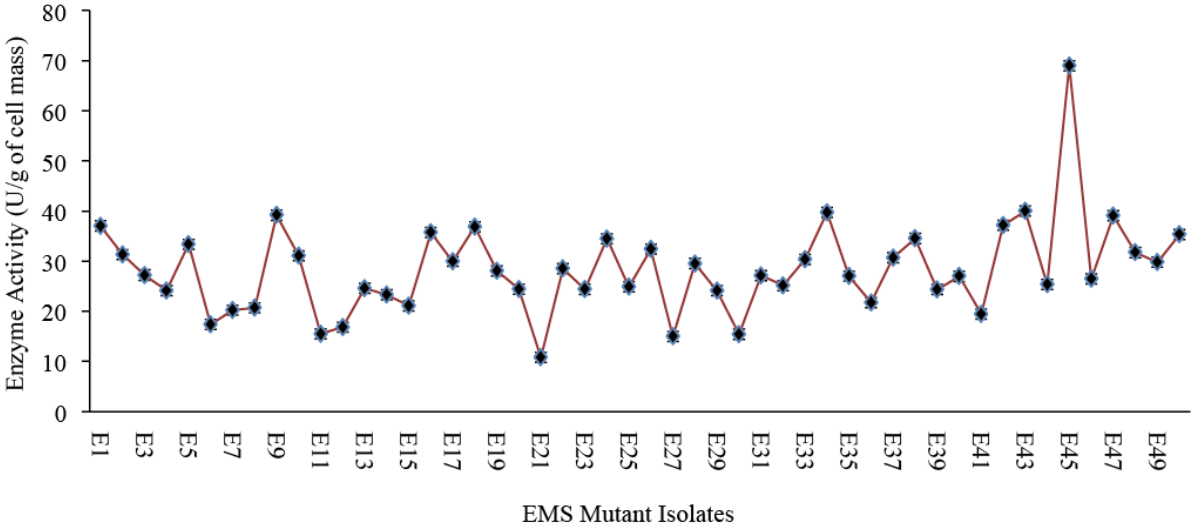

Fig. 3. Glucose oxidase production from EMS mutants.

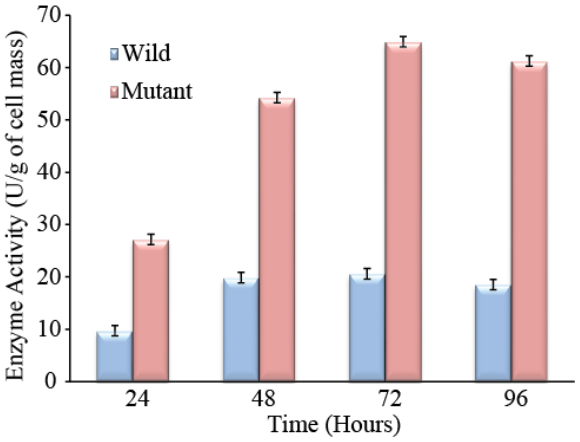

Fig. 4. Effect of Incubation time on the production of glucose oxidase.
The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars.

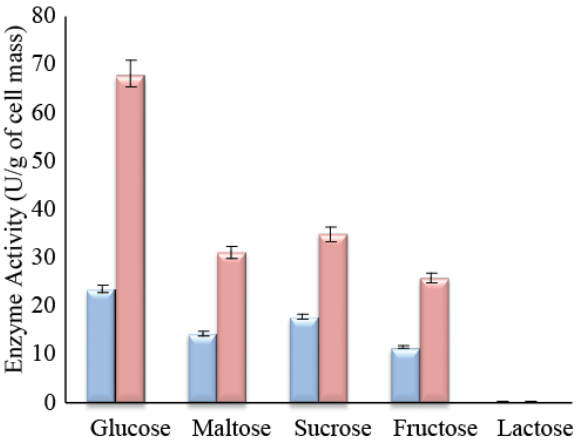

Fig. 5. Effect of different carbon sources on the production of glucose oxidase.
The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars.

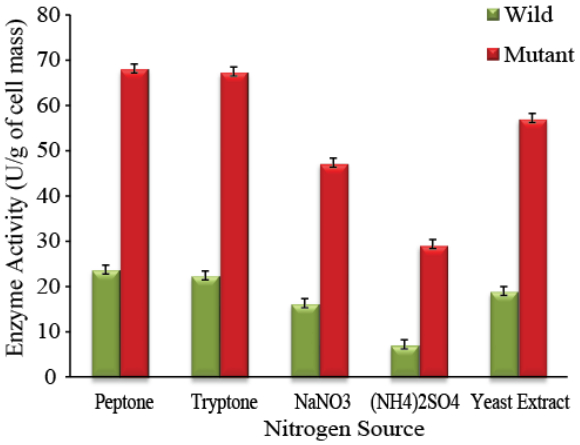

Fig. 6. Effect of different nitrogen sources on the production of glucose oxidase
The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars.

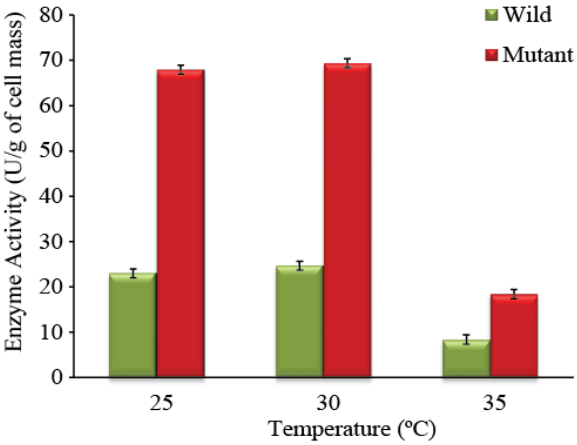

Fig. 7. Effect of incubation temperature on the production of glucose oxidase
The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars.

The ideal nitrogen substrate for the production of glucose oxidase was determined by using peptone, tryptone, yeast extract, ammonium sulfate $(NH_4)_2SO_4$ and sodium nitrate $NaNO_3$ (Fig. 6). Peptone gave the maximum activity 23.75 ± 0.02 and 68.19 ± 0.01 U/g of cell mass for the IIB-31 wild strain and the E-45 mutant strain respectively, since it is rich in amino acids such as glutamic acid, aspartic acid, glycine and alanine which are structural components of glucose oxidase. The results are in accordance with the results of (Gromada & Fiedurek, 1996) and (Bankar *et al.,* 2007).

The yield of glucose oxidase at different incubation temperatures i.e., 25°C, 30°C and 35°C was observed (Fig 7). Maximum production of enzyme for both the wild IIB-31 strain (24.68 ± 0.01 U/g of cell mass) and mutant E-45 strain (69.37 ± 0.02 U/g of cell mass) were seen at a temperature of 30°C. This indicates that the microorganism used is mesophilic in nature. (Zubair *et al*., 2002) also reported similar results.

The influence of pH on the production of glucose oxidase was observed by varying the pH of the medium i.e., 4.0, 5.0, 6.0, 7.0, 8.0 (Fig. 8). Medium pH of 7.0 was recorded as the optimum pH for the production of the enzyme using both the IIB-31 strain and E-45 mutant strains, with the activity of 24.81 ± 0.02 U/g of cell mass and 69.47 ± 0.01 U/g of cell mass, respectively. The pH reported for maximum glucose oxidase activity by (Rogalski *et al.,* 1988) was ranging from 5 to 5.8, which is in contrast to the results obtained in present study. This may be due to some physiological and habitat changes.

The impact of size of the inoculum on the production of glucose oxidase was also investigated by varying the amount of inoculum used i.e. 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml (Fig. 9). The maximum yield of glucose oxidase for both the wild IIB-31 strain (24.29 ± 0.01 U/g of cell mass) and mutant E-45 strain (69.16 ± 0.02 U/g of cell mass) was obtained using 1 ml (3.85%) spore suspension to inoculate 25 ml of medium. This result is similar to that

of (Irfan *et al.,* 2011). The amount of spores present in 1 mL of inoculum are optimum while 0.5 mL is too less to utilize the media and 1.5 mL and 2.0 mL are excessive for the optimum production of glucose oxidase.

The induction capacity of $CaCO_3$ was investigated by varying the concentration of $CaCO_3$ in the media i.e., 2.5%, 3.5%, 4.5%, 5.5% and 6.5% (Fig. 10). The maximum activity for the E-45 mutant strain (69.43 ± 0.01 U/g of cell mass) was obtained at concentration of 2.5%, while the IIB-31 wild strain gave the maximum activity of 24.44 ± 0.01 U/g of cell mass at 3.5% of $CaCO_3$. The results are contradictory to the optimum $CaCO_3$ concentration that has been observed by Bankar *et al.,* (2007), according to which 3.5% $CaCO_3$ gave the maximum glucose oxidase activity. As $CaCO_3$ is added to the medium to prevent the acidification during fermentation, addition of 2.5% $CaCO_3$ was probably enough to avoid the pH drop which is not suitable for the optimum production of glucose oxidase. Further increase in concentration of $CaCO_3$ may lead to inhibition of glucose oxidase production as also reported by Hatzinikolaou & Macris (1995).

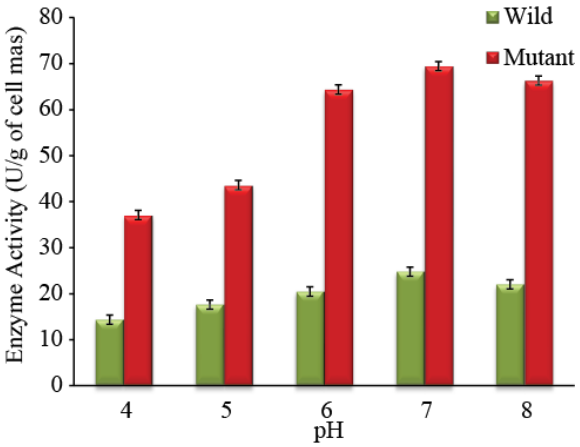

Fig. 8. Effect of medium pH on the production of glucose oxidase
The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars.

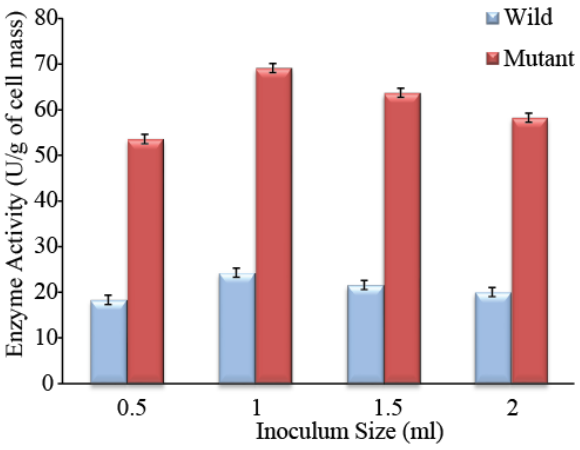

Fig. 9. Effect of inoculum size on the production of glucose oxidase
The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars.

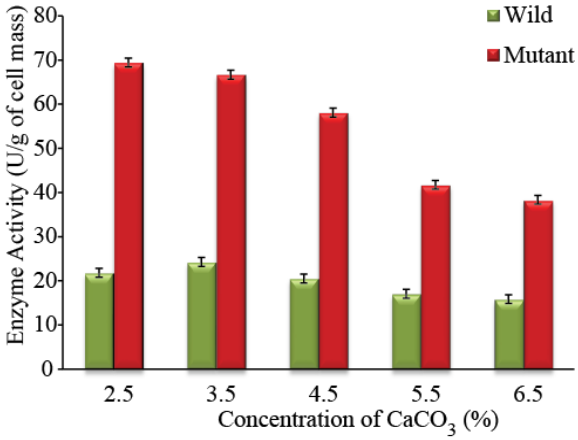

Fig. 10. Effect of $CaCO_3$ concentration on the production of glucose oxidase
The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars.

Conclusion

From the present study, it was concluded that highly potent indigenous strains of *Aspergillus niger* for glucose oxidase production are present and their enzyme producing potential can be enhanced through random mutagenesis and process optimization.

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