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Strain Improvement Studies of *Aspergillus flavus* for Enhanced β-galactosidase Production

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The β-galactosidase producing fungus *Aspergillus flavus* was isolated from marine samples, in our laboratory was used for strain improvement by physical and chemical methods. A wild isolate of *Aspergillus flavus*, was subjected to mutagenic treatment, by ultraviolet light and nitrous acid. Survival curves were obtained and mutants were selected using different mutagenic doses which gave 1 to 5% rate of survival. The mutated (*A. flavus*) strains of UV1 and UV11 were selected after the UV treatment. These two strains UV1 and UV11 were again treated with nitrous acid for further improvement of â-galactosidase yield. After physical and chemical mutation, 2.16 times increase in the production of intracellular β-galactosidase and 1.58 times increase in the extracellular β-galactosidase was obtained.

Key words: β-galactosidase, *Aspergillus flavus*, Nitrous acid treatment, UV treatment.

Strain improvement platform is proven cost effective way to improve fermentation process. The exponential increase in the application of β-galactosidase in various fields demands extension in both qualitative and quantitative enhancement. Quantitative enhancements require stain improvement and medium optimization for the production of the enzyme, as the quantities produced by wild strains are usually too low. Wild strains in general are not used for industrial fermentation processes, as the metabolite concentrations produced by wild strains are usually too low for economical processes, so the mutants which are specifically adapted for fermentation process are derived (Crueger and Crueger 2000). The major motivation for industrial strain development is economic, i.e. the developed strain can reduce the cost with increased productivity and can also possess certain additional desirable characteristics (Ellaiah and Prabhakar *et al.,* 2002). The use of β-galactosidase (EC 3.2.1.23) in the hydrolysis of lactose in milk and milk products is one of the most promising applications of enzymes in the food industry. β-galactosidase occurs in variety of microorganisms including yeasts, molds, bacteria, actinomycetes. However, the properties of the β-galactosidases produced by different microorganisms vary widely, especially in their pH and temperature optima and intracellular and

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extracellular location of the enzyme. In the hydrolysis of galactose in acid whey, mold β-galactosidases are most suitable because they have acidic pH optima and are not sensitive to changes in pH (4.0 to 7.5) and temperature (40 to 65° C). Moreover, the β-galactosidase produced by many *Aspergillus* species (*Aspergillus oryzae* and *Aspergillus niger*) is extracellular, which is an additional advantage in industrial applications. The mutants of *Aspergillus* species (Nevalainen *et al.,* 1981) produced elevated levels of β-galactosidase.

In this work the production of both intracellular and extracellular β-galactosidase by *A. flavus* was improved by mutagenesis. A rapid and effective screening method for the recognition of mutant colonies with elevated enzyme production is presented. However, when screening mutants for improved enzyme production, it is often almost impossible to distinguish high yielding β-galactosidase producers based on morphological characteristics.

MATERIAL AND METHODS

Chemicals

ONPG (Ortho nitro phenyl- β-D-galactopyranoside) discs were purchased from Sigma Chemical Co, 2-nitro phenyl- β-D-galactopyranoside was purchased from Carbosynth Limited, U.K. and Ortho nitro phenol was obtained from Merck. All the other chemicals and medium constituents used in the present study were obtained from M/S Hi-media, Mumbai, Merck and s.d fine chemicals.

Micro-organism

Aspergillus flavus was isolated from marine sediments collected from Bay of Bengal near Visakhapatnam in August 2006, using dilution-plate method on PDA medium. It was identified according to its morphological characteristics and confirmed by IMTECH, Chandigarh. Working stocks were prepared on Potato Dextrose Agar slants and stored at 4° C.

Rapid method for screening of mutants for β-galactosidase activity

After mutation all the isolates were screened for the β-galactosidase production by using ONPG discs. Each isolate was plated on PDA medium and incubated at 28 °C for 5 days. Colonies from each plate were picked up and transferred to a test tube of containing 0.1 ml of sterile saline solution. The sterile ONPG disc was transferred aseptically to the saline solution and incubated at 28°C for 24 hr. β-galactosidase producing colonies release Ortho-nitro phenol (ONP) resulting in the formation of yellow coloured solution. The intensity of the colour depends on the amount of β-galactosidase produced by the isolate. Out of the 33 isolates selected after U.V treatment 13 promising isolates were selected on the basis of the intensity of yellow colour produced. These isolates were further tested for β-galactosidase activity by submerged fermentation. Same procedure of screening was employed for isolating the mutants after nitrous acid treatment.

Spore suspension

The viable spore number on a PDA slant was determined by colony count technique. The spores were suspended in 10 ml of distilled water containing 0.01% Triton- X 100, using a sterile transfer needle and diluted serially. 1 ml of spore suspension was poured onto sterile Petri-plates, containing sterile potato dextrose agar (PDA) medium and spread uniformly. The inoculated Petri-plates were incubated at 28°C for 5 days. A plate that developed between 10 to 30 colonies was selected for counting. The spore density was calculated as the count multiplied by the dilution factor.

Inoculum preparation

Five ml of sterile distilled water containing 0.01% Triton-X 100 was added to the above full grown slant, and growth was dislodged and transferred into 250ml Erlenmeyer flask containing 50 ml of sterile inoculum medium. The composition of the inoculum medium is (g/L): Lactose, 10.0; peptone, 10.0; Yeast Extract, 10.0; Dextrose, 20.0; $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 1.0; K_2HPO_4 3.0; $MgSO_4.7H_2O$, 0.5. The flasks were incubated on a rotary shaker (150 rpm) at 28^0 C for 48 hr.

At the end of the incubation, the cell mass was collected by centrifuging the medium in a refrigerated centrifuge (4^0 C) at 3000 rpm for 20 minutes. The cell mass was washed twice with sterile distilled water, suspended in 10 ml of sterile distilled water and used as inoculum.

Production of β-galactosidase by submerged

fermentation

The inoculum (5% level) was transferred aseptically to 100 ml production medium contained in 500 ml EM flask. The composition of the production medium is same as inoculum medium, except dextrose. The flasks were incubated on a rotary shaker (150 rpm) at 28 °C for 5 days. The samples (5 ml) were withdrawn every 24 hr and centrifuged at 15000 rpm for 15 minutes. The clear supernatant was separated and 0.5 gm of cell mass was used for the detection of intracellular enzyme activity. Cell free supernatant (CFS) was used for the detection of extracellular enzyme activity. The enzyme assay was carried out as described below.

β-galactosidase Assay

β-galactosidase was determined by measuring the release of o-nitro phenol from ONPG (o-nitro-phenyl- β-D-galactopyranoside) at 420 nm.

For intracellular β-galactosidase activity 0.5 gm of cells were washed with distilled water twice and treated with 0.5 ml of toluene at 37 °C for 5 minutes. 2 ml of ONPG (4mg/ml) prepared in phosphate buffer (pH 6.5) was added and incubated at 40 °C for 15 minutes. The reaction was stopped by the addition of 2.5 ml 10% (w/v) Na_2CO_3. The release of O-nitro phenol from ONPG was measured at 420 nm with the help of spectrophotometer (UV-VTS Spectrometer 117, Systronics). Similarly extracellular enzyme activity was measured using 1ml aliquots of cell free supernatant (CFS).

Strain improvement studies

Strain improvement was carried out by UV- irradiation and nitrous acid treatment.

Preparation of spore suspension for physical and chemical mutation

The organism was grown on PDA slants and the spores were transferred into sterile water containing Triton X-100 (0.01%) to give uniform suspension. The spore suspension was then filtered through a thin sterile cotton wad into a sterile tube, to eliminate vegetative cells from the suspension. It was used for mutagenic treatment.

Mutation and selection

UV irradiation of parent strain and selection of mutants

Strain improvement for the *A. flavus* was done by mutation and selection. The organism was subjected to UV irradiation. The dose survival curve was plotted for time of exposure against percentage of survivals. Mutation frequency was mentioned to be high when the survival rate was between 0.1 and 10% (Hopwood *et al.*, 1985).

The spore suspension of wild strains was prepared in sterile distilled water and 4 ml quantities were pipetted aseptically in to sterile flat bottomed petri dishes of 100 mm diameter. The exposure to U.V. light was carried out in a "Dispensing- Cabinet" fitted with TUP 40W Germicidal lamp that has above 90 % of its radiation at 2540-2550 A°. The exposure was carried out at a distance of 26.5 cm away from the center of the germicidal lamp. The exposure was carried out for 0, 5, 10, 15, 20, 25 and 30 minutes respectively. UV lamp was stabilized previously for 30 minutes. During the exposure the lid of the petri dish was removed. Hands were covered with gloves and the plates were gently rotated so as to get uniform exposure of the contents of the petri dish. During the treatment, all the other sources of light were cut off and the exposure was carried out in dark (using red light). The treated spore suspensions were transferred in to sterile test tubes covered with a black paper and kept in the refrigerator over night, to avoid photo-reactivation.

Each irradiated spore suspension was serially diluted with sterile distilled water, plated on to the PDA medium and incubated at room temperature for 5 days. The number of colonies in each plate was counted. It was assumed that each colony was formed from a single spore. The number of survivals from each exposure time is represented in Fig 1.1. Plates having less than 1% survival rate (25 and 30 minutes) were selected for isolation of mutants. The isolate was selected on the basis of macroscopic differential characteristics. The selected isolates were subjected to fermentation and further tested for β-galactosidase production capacities as described as earlier. The results were shown in the Fig 1.2. The best β-galactosidase producing U.V. mutants are UV1 and UV11, which were selected for nitrous acid treatment.

Nitrous acid treatment of UV mutants

The UV mutants UV1 and UV11 were sub-cultured on PDA slants, and incubated at 28^0 C for 5 days. The spore suspensions were prepared as described earlier. The spores were re-suspended

in 0.1 ml of sodium nitrate (5mg/ml), which generates mutagenic agent (nitrous acid) in 0.9 ml acetate buffer. The suspension was incubated at room temperature for different time intervals ranging from 15-240 minutes. After incubation the suspension was centrifuged at 10,000 rpm, and pellet was washed twice with phosphate buffer (pH 6.5) and finally re-suspended in phosphate buffer. The samples were adequately diluted and plated on PDA medium. The plates were incubated at room temperature for 5 days and colony counts were made. The dose-survival curve was constructed as described earlier (Data not shown). The colonies were selected on the basis of morphology, size, and shape. Selected colonies were sub-cultured on to the PDA slants and evaluated for their β-galactosidase producing capabilities. UV11-N8 showed the highest yield of β-Galactosidase.

RESULTS AND DISCUSSION

A total thirty five mutant strain obtained after UV and nitrous acid treatment were tested for beta-galactosidase activity by submerged fermentation. The enzyme activities were calculated as units per gram dry cell weight. After UV treatment, plates having less than 1% survival rate (30 minutes), as shown in Fig 1.0, were selected for the isolation of mutants of *A. flavus*. A total of 13 mutants were selected and these were labeled as UV1 to UV13**.** These mutants were tested for their both intracellular and extracellular β-galactosidase producing capabilities by submerged fermentation and the results of intracellular β-galactosidase are presented in Fig 1.1 and extracellular were presented in Table 1.1. The results indicate that among the UV

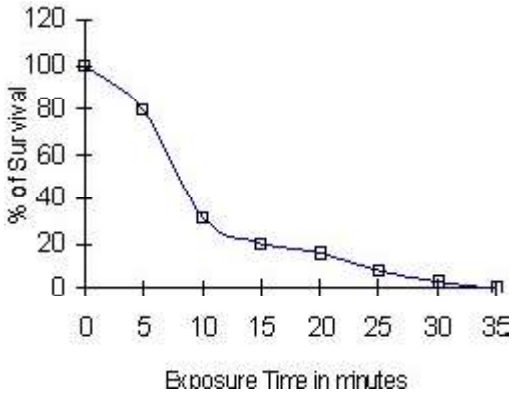


Fig. 1. Surival curve of fungal isolate *A. flavus* after UV irradiation

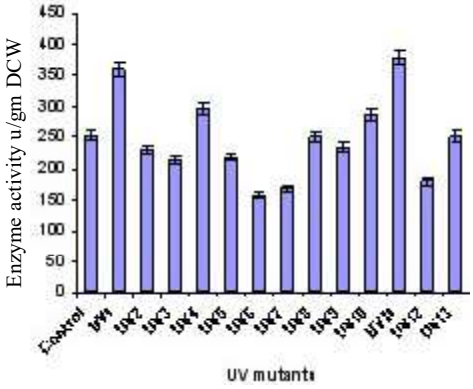


Fig. 2. UV mutants and thei beta galactosidase activity

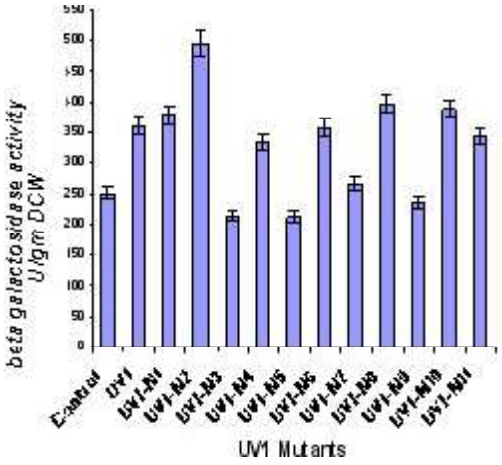


Fig. 3. Nitrous and mutants ov UV 1

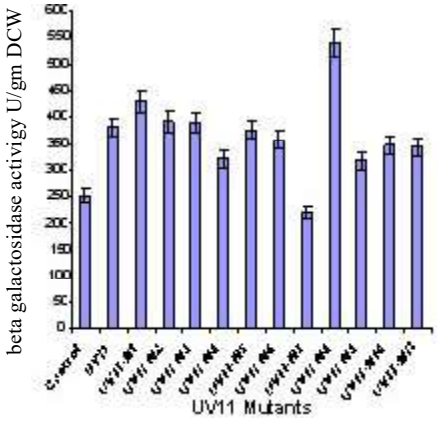
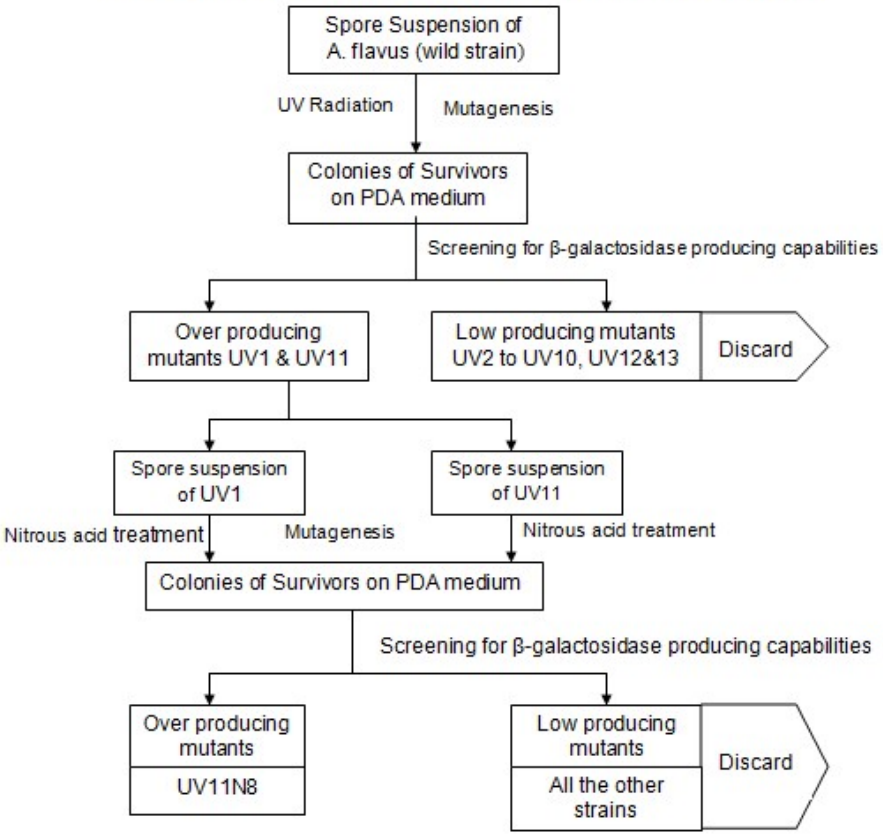


Fig. 4. Nitrodus acid mutatnts of UV 11

Table 1. Extracellular β-galactosidase activity of all the mutants

Isolate no	Extracellular activity U/L	Isolate no	Extracellular activity U/L
Wild strain	39.8	UV1-N5	36.5
UV1	42.1	UV1-N6	42.1
UV2	37.5	UV1-N7	38.0
UV3	32.9	UV1-N8	45.3
UV4	40.6	UV1-N9	33.2
UV5	35.5	UV1-N10	41.1
UV6	22.2	UV1-N11	40.8
UV7	31.2	UV11-N1	57.1
UV8	42.2	UV11-N2	52.9
UV9	44.3	UV11-N3	55.3
UV10	34.2	UV11-N4	56.2
UV11	45.4	UV11-N5	60.1
UV12	22.1	UV11-N6	43.9
UV13	39.2	UV11-N7	39.5
UV1-N1	41.9	UV11-N8	62.0
UV1-N2	59.0	UV11-N9	54.3
UV1-N3	32.3	UV11-N10	54.9
UV1-N4	39.4	UV11-N11	43.9



Flow diagram for wild strain of *A. flacus* undergoing mutation process

mutants, UV1 and UV11 were found to be the highest β-galactosidase producers with activities of 360 and 380 U/gm respectively compared to the parent wild strain (254 U/gm). â-galactosidase production in UV1 improved by 41.73% and in UV11 by 49.61% over the parent wild strain. Hence these two mutant strains (UV1 and UV11) were selected for nitrous acid treatment as described by Puvanakrishnan R et al (2001). The Plates having less than 1% survival were selected for the isolation of mutants. A total of 22 mutants were selected after nitrous acid treatment, eleven from UV1 and eleven from UV11 and were labeled as UV1-N1 to UV1-N11 and UV11-N1 to UV11-N11. All these strains were evaluated for their β-galactosidase production capabilities by submerged fermentation. The results are presented in the Fig 1.2 & 1.3. The results indicated that the mutant UV11-N8 (from the parent UV11, 380 U/gm) yielded 540 U/gm. while the mutant UV1-N2 (from parent UV1, 360 U/gm) yielded 495 U/gm. Thus the nitrous acid treatment resulted in mutant UV11-N8 (yielding 540 U/gm) which 42.11% higher over the parent UV11 (380 U/gm). It is a significant increase in the enzyme yield. The other mutant UV1-N2 (495 U/gm) showed 37.50% improvement over the parent UV1 (360 U/gm). From the results, it is evident that UV and nitrous acid were effective mutagenic agents for strain improvement of *Aspergillus flavus.*

The mutant UV11-N8 (540 U/gm) with the highest β-galactosidase activity was selected for subsequent studies.

CONCLUSION

The over all strain improvement program increased the β-galactosidase activity 2.13 times with respective to the parent wild strain of *A. flavus*. The mutated strain UV11-N8 yields 540 U/gm of intracellular β-galactosidase activity and 58.5 U/L of extracellular â-galactosidase activity.

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