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Original Research Article

Effect of UV and nitrous acid treatment on production of Xylanase enzyme by *Acinetobacter sp*

Lipika Mishra, Madhavi Vernekar, Mugdha Harmalkar*

Department of Biotechnology and Bioinformatics, Padmashree Dr. D.Y Patil University, Sector 15, Plot no 50, CBD Belapur, Navi Mumbai 400 614, India
Corresponding author

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A B S T R A C T

A novel xylanase producer isolated from soil near garbage site viz *Acinetobacter sp* was exposed to UV rays and nitrous acid and its effect on production of xylanase enzyme was studied. Screening of mutants showed that in all UV mutants a considerable increase in xylanase activity of 150-200% was seen and these mutants were relatively stable in enzyme production upto number of generations studied. Treatment with nitrous acid yielded only few mutants with enhanced xylanase activity (NA6, NA7, NA9, NA10 and NA12) but none of these NA mutants were stable for xylanase production as a gradual decrease was seen in xylanase activity. To enhance the stability of NA mutants for xylanase production, NA6 mutant was subjected to additional mutation by exposure to UV rays. The double mutants (DM) obtained did show a significant increase in xylanase activity as compared to NA6 mutant however none of these mutants were stable for the xylanase production and xylanse activity in these mutants was found to decrease with increase in the number of generations. Thus, UV treatment alone was found to be effective mutagen for enhancement of xylanase production in *Acinetobacter sp* as compared to treatment with nitrous acid or nitrous acid + UV treatment.

Introduction

Xylan is one of the most abundant polysaccaharide of plant origin after cellulose, accounting for 20-40% of plant dry matter. Xylan comprises of β-1,4-linked xylose residues in the backbone, to which short side chains of o-acetyl, α-L-arabinofuranosyl, D-α-glucuronic and phenolic acid residues are attached (Coughlan and Hazelwood 1993).

Xylanases are hemicellulases which degrade xylan. The xylan hydrolysis end product have considerable industrial applications in biofuel, artificial sweetener, animal feed production, baking, textile, clarification of fruit juices and coffee extraction etc. Besides, xylanases showed interesting application as eco-friendly bleaching agent in paper pulp

industries (Viikari *et al*., 1994, Li *et al*., 2010).

Microorganisms are preferred sources of xylanases because of their easy availability, large quantities, their specificity and low cost of production (John et al.2001). Many microorganisms including bacteria, fungi and actinomycetes are known to produce different type of xylanases and the nature of enzymes varies among different organisms (Garg et al. 1998, Gessesse and Mamo et al. 1998, Inagaki et al. 1998, Ishihara et al. 1997, Lopez et al. 1998, Puchart et al. 1999).

Large-scale cultivation of fungi is often difficult because of slow generation time, coproduction of highly viscous polymers, and poor oxygen transfer (Margaritis and Zajic 1978). Xylan degrading bacteria includes strains of *Aeromonas*, *Bacillus*, *Bacteroides*, *Cellulomonas*, *Microbacterium*, *Paenibacillus*, *Ruminoccocus* and *Streptomyces* (Rapp and Wagner 1986). Most of the wild strains which have potential use in industrial fermentation processes are subjected to industrial strain improvement programme to make the fermentation process economical. Although genetic engineering has made a significant contribution to strain improvement, random mutagenesis is still a cost-effective procedure for reliable short-term strain development and is frequently the method of choice. In the present study, we have studied the effect of UV rays and Nitrous acid on production of xylanase enzyme by *Acinetobacter* sp.

Materials and Methods

Xylan (birchwood) was obtained from SRL. All the other chemicals were of analytical grade and were procured from reputed commercials. Culture media ingredients of bacteriological grade were procured from reputed commercials.

Microorganism and growth media

Acinetobacter sp was grown on Xylan complete media (XCM) (Xylan 1%, Peptone 0.5%, $CaCl_2$ 0.2 g/l, K_2HPO_4 0.2 g/l, $MgSO_4$ 0.2 g/l, $MnCl_2$ 0.02 g/l, Agar 2%) at 37°C for 24h. (Singh D. et al 2012). Subculturing of cells was done at an interval of 15 days.

Treatment of Acinetobacter sp with U.V rays

10 ml of 16 h old culture (O.D. 0.4 at 600 nm) was centrifuged at 2500 rpm for 10 min. The cells were washed and re-suspended in saline. 1ml of cell suspension was dispensed in flat bottom sterile petridishes and exposed to UV rays (40W lamp pre-stabilized for 30 min) for varying time intervals (20, 30, 40, 50, 60 and 120 sec) at a distance of 30 cm (all other light sources were cut off during UV treatment). UV exposed cells were kept in dark for an hour to avoid photoactivation, appropriately diluted in saline and plated on the XCM. The plates were incubated at 37°C for 24 h.

The survival colonies were counted and the dose survival curve was plotted for time of UV exposure against percent survivals. The mutation frequency was mentioned to be high when survival rate was between 0.1% and 10% (Hopwood et al. 1985). Colonies were selected from the plates showing less than 1% survival rate and were screened for xylanase enzyme using enzymatic assay.

Treatment of Acinetobacter sp with Nitrous acid (NA)

Cells were centrifuged and the cell pellet obtained was washed twice with phosphate buffer (0.2 M pH 7). 10^8cells/ml were re-suspended in a mixture of 0.9 ml of acetate buffer (0.1 M, pH 6.8) and 0.1 ml of sodium nitrite (5mg/ml), which generates mutagenic agent (nitrous acid). The cells were incubated in this mixture at 37°C for varying time intervals from 30-120 min. At each time interval, cells were washed with phosphate buffer (pH 7.0) to remove the traces of mutagen, and finally re-suspended in 1ml of phosphate buffer. Cells were adequately diluted and plated on XCM agar plates. After incubation at 37°C for 24 h, the survival colonies were counted and the dose survival curve was plotted for time of NA exposure against percentage of survivals. The plates showing 1% survivors were further screened for xylanase activity.

Treatment of Nitrous acid (NA) mutant with UV rays

The NA6 mutant was used for UV treatment. 10^8 cells/ml was exposed to UV rays for 1 min at a distance of 30 cm. The cells after UV exposure were treated as mentioned above. The suvival colonies obtained were designated as double mutants (DM) which were further screened for xylanase enzyme.

Xylanase activity assay

Xylanase activity was assayed using 1% solution of birchwood xylan as a substrate and the amount of reducing sugar released was determined by the dinitrosalicyclic acid (DNSA) method (Miller 1959), using xylose as standard. 0.3 ml of the culture supernatant was mixed with 1.6 ml of 1% w/v xylan dissolved in phosphate buffer (0.2 M pH 7) and incubated at 50° C in a water bath for 30 min. Inactivated enzyme samples (100° C/ 10 min) were used as blank. After 30 min incubation, 1 ml of the reaction mixture was mixed with 1 ml of DNSA solution, boiled for 15 minutes and the absorbance was measured at 540 nm against enzyme blank. One unit of xylanase activity was defined as the amount of enzyme required to liberate 1µM xylose per minute under given experimental conditions. Substrate alone and inactivated enzyme were used as controls.

Stability studies of mutants

The production of xylanase by UV mutants, nitrous acid mutants and double mutants was studied for a period of 5 months (7-12th generations). Mutants were maintained on XCM and sub-cultured after every 15 days for each batch of enzyme assay.

Statistical analysis

The xylanase activity was assayed at each generation and was expressed as mean± S.E. The increase in xylanase activity was calculated as percentage of control.

Results and Discussion

Effect of UV rays and nitrous acid on cell viability and xylanase production

A novel isolate of xylanase producing organism, *Acinetobacter sp* was exposed to UV rays and NA for varying time intervals and the percent survival was calculated. Almost 99% lethality was seen when exposed to UV rays for 120 sec and for 120 min when exposed to nitrous acid (Fig 1and Fig 2).The survival population

after UV exposure varies with the type of strain under study. Mohamed et al. 2011 reported 0.39% viability of *Streptomyces sp* when exposed to UV rays for 180 sec. Vegetative cells of *Bacillus thuringiensis* were shown to be sensitive to UV and nitrous acid as shown by high frequency of lethality (94-99%) (Ghribi et al. 2004).

Survivor colonies were randomly selected and screened for xylanase production by enzymatic assay. All the UV mutants showed considerable increase in xylanase activity of 150-200% as compared to the wild type strain (Fig 3). Amongst the various UV mutants, UV2 mutant showed a significant increase in xylanase activity with an average percent increase of 178% whereas UV1, UV4, UV5 and UV7 also showed considerable increase in xylanase activity with an average percent increase of 150 -160% as compared to wild type strain.

The stability of these UV mutants for xylanase production was studied using enzymatic assay. All the UV mutants showed stable xylanase production for number of generations studied suggesting the stability of these mutants for xylanase production (Fig 3). The UV2 mutant showed a maximum of 230% increase in xylanase activity as compared to wild strain. Thus UV irradiation was found to be effective mutagen in enhancing the xylanase production in *Acinetobacter sp*. UV muatagenesis studies was also done by many other groups for strain improvement. UV muatagenesis studies of *Penicillium purpurogenum* was also shown to enhance xylanase activity by 181% as compared with the wild type strain (Steiner 1998). The UV mutants obtained in our study showed 2-3 fold increase in xylanase activity as compared to wild strain whereas in another study a 1.5 fold increase in xylanase activity was seen in *Thermomyces lanuginosus* upon UV mutation (Kumar et al. 2009). UV mutagenesis had also been used for enhancing yield of other microbial enzymes e.g., cellulase production by *Penicillium pinophillum*, dextranase production by constitutive mutant of *Lipomyces starkeyi* and amylase production by *Aspergillus foetidus* (Talkhan 2000). However UV treatment failed to enhance the yield of α-amylase in *B. amyloliquifaciens* as reported Haq et al. 2010.

NA mutants showed varied enhancement of xylanase activity. Some NA mutants showed poor to marginal increase in enzyme activity (data not shown) while NA6, NA7, NA9, NA10 and NA12 mutants showed a considerable increase in xylanase activity with an average percent increase of 130 -180% as compared to wild strain (Fig 4). The stability of these mutants for xylanase production was assayed till 12^{th} generation. NA mutants which showed poor to marginal increase in xylanase activity, a further drop in enzyme activity was seen (data not shown). Although NA6, NA7, NA9, NA10 and NA12 showed considerable increase in xylanase activity, none of these mutants were stable for xylanase production and showed a gradual decrease in xylanase activity as the number of generations increased (Fig 4). In another study for enhancement of lipase production in *Aspergillus niger* by ultraviolet (UV) and nitrous acid mutagenesis, enhanced lipase production was observed in nitrous acid mutants exhibiting 18.4% increased activity compared with the wild strain (John et al. 2001**).**

Figure.1 Effect of UV rays on *Acinetobacter sp*. Cells were exposed to UV source (40 W lamp at a distance of 30 cm) and the percent survival population plotted at different time intervals showed 99% lethality at exposure time 60 to 120 sec.

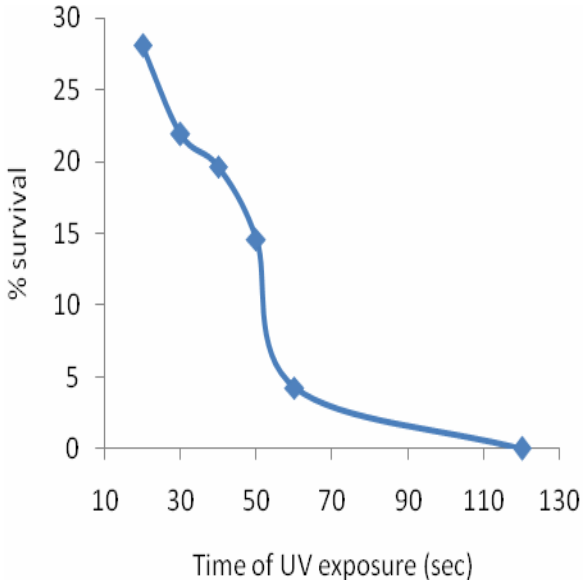


Figure.2 Effect of nitrous acid *on Acinetobacter sp.* Cells were treated with a mixture of acetate buffer and sodium nitrite for varying time intervals. The percent survival population was plotted against time shows 99% lethality at exposure time of 60 to 120 min.

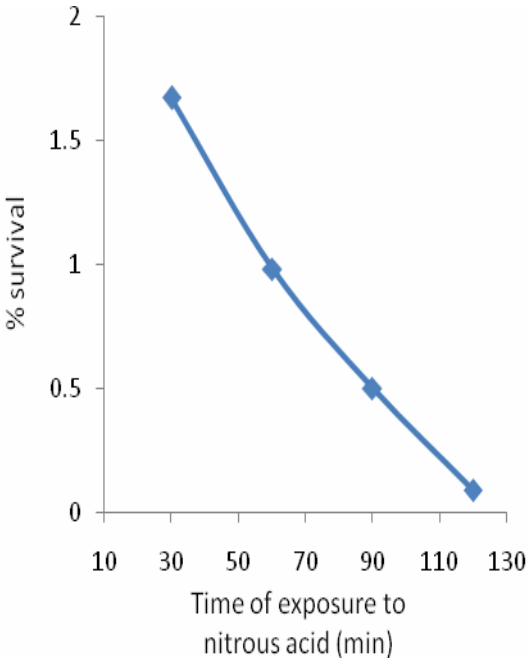


Figure.3 Xylanase activity in UV mutants. The xylanase activity (U/ml/min) in UV mutants was assayed by DNSA method. The percent increase in xylanase activity of all UV mutant at each generation was calculated against wild type strain.

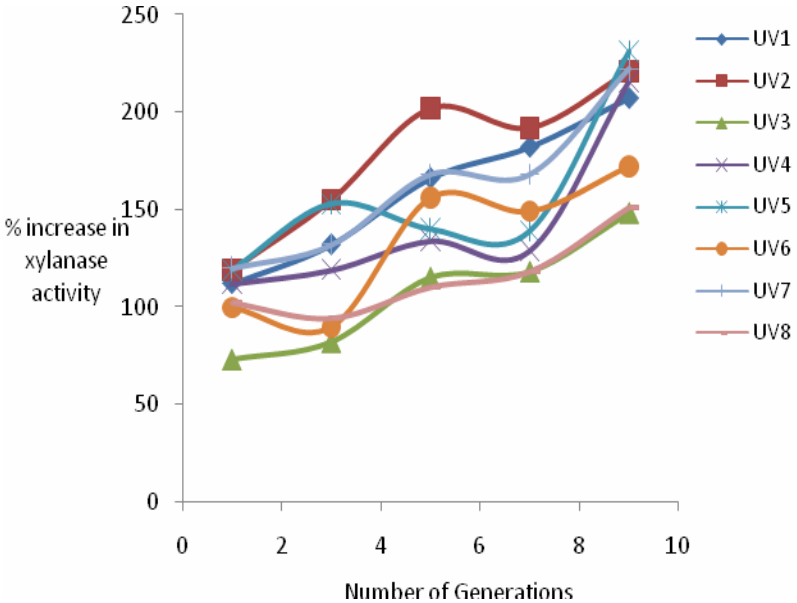


Figure.4 Xylanase activity in NA mutants. The xylanase activity (U/ml/min) in all the NA mutants was assayed by DNSA method. The percent increase in xylanase activity of all NA mutant at each generation was calculated against wild type strain.

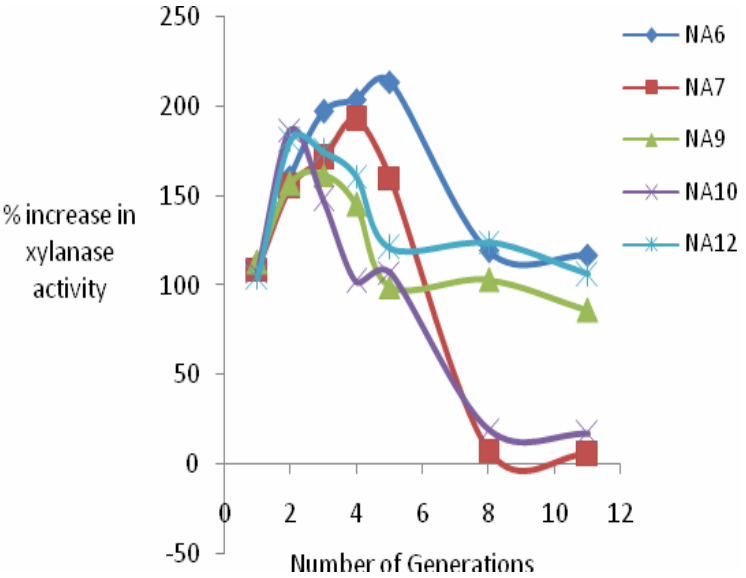
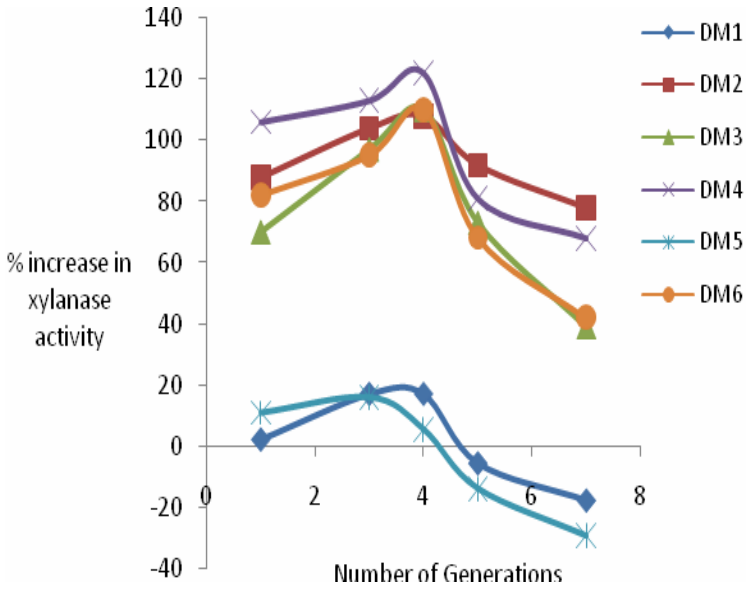


Figure.5 Xylanase activity in DM mutants. The average xylanase activity (U/ml/min) of all the DM mutants was assayed by DNSA method. The percent increase in xylanase activity of each DM mutant was calculated against NA6 mutant.



To enhance the stability of NA mutants for xylanase production, NA6 mutant was exposed to UV rays. Double mutant (DM) colonies were randomly picked up and assayed for xylanase activity. Two mutants viz DM1 and DM5 showed a negligible increase in xylanase activity whereas mutants DM2, DM3, DM4 and DM6 showed a significant increase in xylanase activity of about 80 to 120%, however xylanase activity was found to decrease after few generations suggesting the unstability of these mutants for xylanase production (Fig 5).

In one of the study, *Aspergillus flavus* when subjected to UV light and nitrous acid for further improvement of β-galactosidase yield, a 2.16 fold increase in the production of intracellular β-galactosidase and 1.58 fold increase in the extracellular β-galactosidase was obtained (Pavani et al. 2009). In our study, the combined treatment of nitrous acid and UV rays did increase the xylanase production in these mutants however the stability of these mutants for xylanase production was not improved.

Thus, the present study shows that random mutagenesis methods can yield mutants with enhanced xylanase activity and UV rays alone is a very effective mutagen for enhancement of xylanase production in *Acinetobacter sp* as compared to treatment with nitrous acid or nitrous acid + UV treatment.

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