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Research Article

Improvement of *Bacillus pumilus* for Higher Xylanase Production by Mutation

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Abstract

Mutation can cause a raise in the enzyme production by the favourable changes in the genotype of a bacterial strain. The objective of the study was to obtain a better xylanase producing *Bacillus pumilus* mutant strain by exposing the wild-type strain to UV-irradiation, heat shock and EMB chemical treatment. When *Bacillus pumilus* was subjected to UV-irradiation for 3 cycles, xylanase production was increased by 1.22 times in the first two cycles and there was no increase in the third cycle. But when this mutant was further subjected to heat shock and chemical mutation by EMS, there was no significant improvement in the xylanase production. After the selection of mutant xylanase, the two type of xylanases, wild-type and mutated need to be purified and characterized for further comparative studies.

Keywords: *Bacillus pumilus*; Xylanase; EMS; Heat Shock; UV-Irradiation

Introduction

Xylanase enzymes have great potential in the industrial application. Mutation is one of the techniques used in the industry to increase the enzyme production by microbes. Mutation can occur either by natural environmental factors, UV irradiation or by chemical treatment. UV-irradiation with short wave lengths (254 nm) affects the DNA of organisms mainly by the formation of thymine-thymine dimers [1]. Such changes would cause acceptable mutation and bring about industrially important mutants with high performance and desired characters. Changes in genotype are caused by mutation and genetic recombination. The ultraviolet (UV) mutation was used to improve the microorganisms in various microbial productions for industrial purposes. In these studies, the strain *Bacillus pumilus,* isolated from opened xylan agar plate medium was tried to further increase the xylanase production by using UV-mutation, heat shock and chemical mutation. The synthesis of heat shock proteins rapidly increases in cells under a broad range of stress conditions created by heat shock [2]. Their induction requires activation of the transcription of heat shock genes by heat shock factors. These proteins play an important role in providing cells with a protective mechanism against environmental insults and in increasing the enzyme production. There might be a possibility of denaturing of proteins by heat shock [2]. Chemical mutation causes mainly transition and transversion in the nitrogen bases of DNA molecules. Alkylating agents such as ethyl methane sulphonate (EMS) act primarily on purine bases. EMS converts especially guanine to adenine and modifies the DNA. Xylanases are used to convert the xylan to xylose in the paper-pulp industry, to treat the agricultural wastes and recently to improve the bread quality [3]. The objective of the study was to obtain a better xylanase producing *Bacillus*

pumilus mutant by exposing the wild type strain to UV-irradiation, heat shock and chemical treatment.

Materials and Methods

Microorganism

Among the xylanase producing bacterial strains, available in the Biochemistry laboratory, Faculty of medicine, the strain isolated from opened xylan agar plate medium and confirmed as *Bacillus pumilus* was used [4].

Chemicals and Media

All the chemicals used were from standard sources. The activation medium contained (gL^{-1}) xylan 20.0 and nutrient broth 25.0. Fermentation medium contained (gL^{-1}) xylan, 20.0; peptone,20.0; yeast extract,2.5; $CaCl_2.2H_2O$,0.005; $MgCl_2.6H_2O$, 0.005; $FeCl_3$, 0.005; K_2HPO_4, 2.5; KH_2PO_4,1.0; NaCl, 0.1 and $(NH_4)_2SO_4$, 2.0 and 100rpm based on the experiment [5].

Production of Xylanase and Measurement of Xylanase Activity

To the fermentation medium, 20% (v/v) inoculum was added and incubated at 40°C in a rotatory shaker (100rpm). Samples were taken at 42h and centrifuged. The Supernatant was used as xylanase source. Enzyme was diluted with Tris aminomethane buffer (pH-8.5). Enzyme assay was carried out at pH 8.5 and 60°C by preincubating the enzyme extract with $10gl^{-1}$ xylan in 0.01M Tris aminomethane buffer (pH-8.5) for 5 minutes. Then 0.5ml of the enzyme was mixed with 0.5ml substrate and incubated for 5 minutes at 85°C. One unit of xylanase activity is defined as the amount of enzyme that produces one µmol of reducing sugar in one minute at pH 8.5 and 60°C with 20 gL^{-1} xylan.

UV-Irradiation Induced Mutation

Method of Leuchtenberger and Mayer [6] was followed. *Bacillus pumilus* was subjected to UV-irradiation through three cycles and the mutants giving high yields of xylanase were selected.

Effect of UV-Irradiation Time on the Viability of *Bacillus Pumilus*

Two loops full of *Bacillus pumilus* was inoculated into 20mL activation medium in a 250mL conical flask and it was incubated at 40°C and 100 rpm in a shaker water bath for 16h. Sample (5mL) was taken and poured in a Petridish and UV-irradiation (254 nm) was applied from 6 cm distance under dark and sterile condition by UV lamp (UV SYSTEMS LS-16X lamp, 18 W). Aliquots (1.0 mL) were collected at different time intervals (0, 10, 20, 40, 60, 80 min) and kept in dark for 30 min. UV-irradiated bacterial cells (1 mL) were diluted serially and inoculated by spreading on nutrient-agar plates in pentuplicate (from 10^3 to10^6 dilution). They were incubated for 24h at 37°C and the number of colonies was counted in each plate and the percentage of the survived cells was estimated.

$$\text{Viability of cells (\%)} = \frac{\text{Number of colonies obtained from UV-irradiated sample at a particular time}}{\text{Number of colonies obtained from non UV - irradiated sample}} \times 100$$

Improvement of Bacillus Pumilus by First Cycle of UV-Radiation and Screening the Best Xylanase Producer

The UV-radiated bacterial cells (1.0mL) were diluted serially and inoculated (1mL) by spreading on nutrient-agar plates in pentuplicate (10^5 dilution) and were incubated for 24h at 37°C. Each twelve colonies were selected randomly from 60 and 80 min UV-radiated samples (UVA1 to UVA12). Another twelve colonies selected from 80min UV-irradiated cells were labelled as from UVB1 to UVB12. The selected colonies were grown and xylanase assay was carried out by each of the twelve strains which were selected randomly from 60 and 80min UV-radiated samples and by the parent strain (non UV-radiated). Best xylanase producing strains were selected among the 60 and 80min UV-irradiated strains.

Improvement of Strains UVA2 and UVB9 by Second Cycle of UV-Radiation and Screening the Best Xylanase Producer

The selected best xylanase producers (UVA2 and UVB9) were subjected to second cycle of UV-irradiation. Each twelve colonies which had rough surface type and diameter similar to the parent were selected randomly from each parent (UVA2 and UVB9). The twelve colonies selected from parent UVA2 were labelled as from UVA2/1 to UVA2/12. Another twelve colonies selected from parent UVB9 were labelled as from UVB9/1 to UVB9/12. The selected 24 colonies were streaked on nutrient-agar slants and cultivated at 37°C for 24h for five times on slants. Xylanase production and assay were carried out by each of the twelve strains which were selected randomly among the UVA2 and UVB9 UV-irradiated strains and parents (UVA2 and UVB9).

Improvement of the Strain UVB9/8 by Third Cycle of UV-Radiation and Screening the Best Xylanase Producer

Best xylanase producing variant (UVB9/8) was selected from the parent UVB9 and it was subjected to third cycle of UV-radiation for further improvement, as said in the 2nd cycle. Eight

colonies that were similar to the parent were selected randomly for xylanase production from parent mutant UVB9/8. The eighteen colonies selected from parent UVB9/8 were labelled as from UVB9/8/1 to UVB9/8/18. The selected variants were streaked on nutrient-agar slants and cultivated at 37°C for 24h for five times to obtain stabilized mutants. Xylanase production and assay was carried out by each of the eighteen strains which were obtained by the mutation of UVB9/8 and parent (UVB9/8).

Application of Heat Shock

Improvement of the Strain UVB9/8 by Heat Shock and Screening the Best Xylanase Producer

Twenty loop full stains of UVB9/8 were inoculated into 100 mL activation medium in two 500mL conical flasks and incubated at 40°C for 16h. Heat shock was given by keeping the temperature of the shaker water bath at 80°C for 30 minutes to the first flask. Second flask was treated as the control (No heat shock). From the heat shocked culture and control culture 1mL aliquots were taken in triplicates and inoculated by spread plate method and 0.1 mL aliquots were taken in triplicates and inoculated by spreading on nutrient-agar plates and incubated at 37°C for 24h. Eight colonies which had rough surface type and diameter similar to the parent, were selected randomly from parent mutant UVB9/8 and labelled as from UVB9/8/19 to UVB9/8/26. Xylanase production and assay was carried out by each of the eight strains which were obtained by the application of heat shock of UVB9/8 and parent (UVB9/8).

Application of Chemically Induced Mutation (Ems)

Effect of Ethyl Methane Sulphonate (EMS) Treatment on the Survival of the Strain UVB9/8

The method described by Ingolia and Wood [7] was used. UVB/9/8 was selected from the parent UVB/9 and it was subjected to chemical mutation for further improvement. Two loops full of UVB/9/8 was inoculated to 20mL activation medium from the slant culture and incubated at 40°C and 100 rpm for 16h. To the cells, 60µl of EMS was added and incubated at 40°C and 100 rpm for 105 min. Samples (1mL) were taken at different time interval (0, 15, 30, 45, 60, 75, 90 and 105min) and 5mL 5% of Sodium thiosulphate was added to stop the mutagenesis. Dilution plating was carried out by plating 50µl of 10^2, 10^3, 10^4, 10^5 and 10^6 dilutions of the EMS treated cells on nutrient-agar plates, and they were incubated at 37°C for 24h. Number of colonies to each plate was counted. Percentage of survived cells was estimated.

$$\text{Survival rate} = \frac{\text{Number of colonies obtained from EMS treated sample at a particular time X100}}{\text{Number of colonies obtained from fresh sample}}$$

Survival rate was used to denote the lethal effect of EMS to the cells, in terms of percentage of viable cells after the treatment.

Improvement of UVB9/8 by EMS Treatment and Screening the Best Xylanase Producer

Two loops full of strain UVB9/8 was inoculated to 20 mL activation medium from slant culture and incubated at 40°C and 100 rpm for 16h. To the cells, 60µL of EMS solution was added and incubated at 40°C and 100 rpm for 105 min. The experiment was proceded as mentioned above. Numbers of 9 colonies were selected randomly from 105min EMS treated culture sample and they were labelled as from UVB9/8/27 to UVB9/8/35. The nine variants obtained by the EMS treatment of UVB9/8 and parent (UVB9/8) were assayed for xylanase.

Results and Discussion

Improvement of Bacillus pumilus by first cycle of UV-Radiation

The cells in the inoculum of *Bacillus pumilus*, was UV-irradiated for 60 and 80 min Due to UV-irradiation for 60 and 80 min 99.2 and 99.96% of the cells were killed (Figure 1). Thus it was expected that the survived cells might have been mutated. Due to the mutation xylanase productivity of mutated variants would also have improved, diminished or not affected. Hence twelve colonies were selected randomly from each 60 and 80 min UV-irradiated sample. Slant cultures were prepared from the colonies and the strains were cultivated for five times to stabilize the mutants.

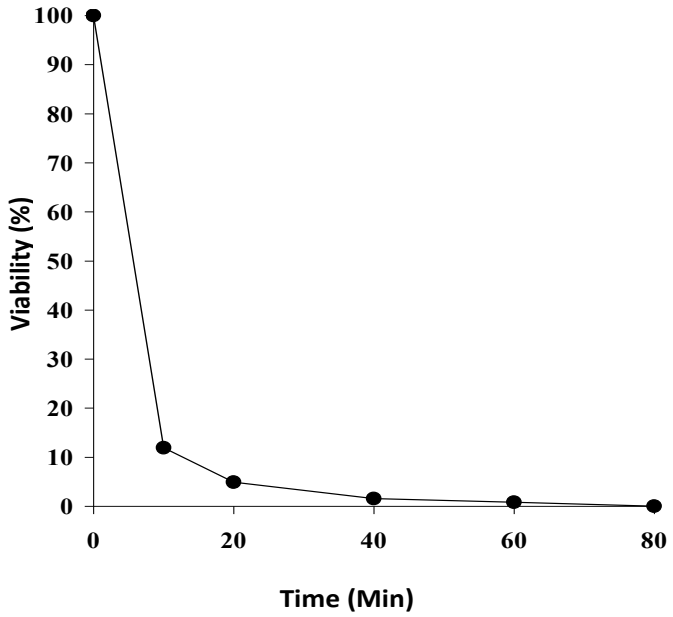

Figure 1. Effect of UV-irradiated time on the survival of *Bacillus pumilus*.

Morphologically parent and 24 variants looked alike. Both had dentated margin, yellow in colour and round shaped colonies. But the diameter and surface appearance of the colonies differed. In the selected 24 mutants, diameters of 13 colonies were same as that of the parent strain (4.6±0.2mm) and 7 colonies were larger than parent strain (4.9 to 5.7 mm) and rest of the 4 colonies were less (3.7 to 4.3 mm) than that of the parent strain. The morphological type of colonies were observed on enzyme production of *Bacillus* was circular, opaque with a rhizoid border, slightly raised and has a granular shiny surface [8]. Similar results for *Bacillus* sp have been reported [9] on different enzyme production.

Screening the Best Xylanase Producer among the Selected Strains Obtained by the first Cycle of UV-Radiation

Among 24 mutants, five mutants produced higher xylanase activity at 48h than the parent strain (26.44UmL^{-1}). The five variants which gave higher xylanase activity than the parent strain gave the colonies with rough surface and diameter similar to the parent. Among them, the strains UVA2 and UVB9 were selected for further improvement. The strains UVB5 and UVB11 gave higher xylanase activity (27.18 UmL^{-1} and 28.59 UmL^{-1} respectively) than that of parent strain and UVB7gave nearly same amount of xylanase activity (26.73 UmL^{-1}, Figure 2) as that of the parent strain and all the other 19 mutants gave the xylanase production less than the parent strain. Therefore, xylanase production was improved only in five selected mutants. Two mutants UVA2 which gave the highest xylanase production (among which obtained after 60min UV-irradiation) and UVB9 which gave the highest xylanase production (among which obtained after 80min UV-irradiation) were selected for the second cycle. The selected mutants (UVA2 and UVB9) and parent strain showed similar xylanase production patterns. The xylanase activity produced by the strains UVA2 and UVB9 were 1.05 and 1.14 times higher than that produced by the parent strain (Figure 3).

Improvement of the Strains UVA2 and UVB9 by the Second Cycle of UV-radiation and Screening the Best Xylanase Producer

The strains UVA2, UVB9 from the first cycle were used as parent strains and subjected to the second cycle of UV-irradiation. The number of colonies with nearly same morphological characters was obtained in nutrient-agar plates. Among them, twelve colonies were selected randomly from each parent (UVA2 and UVB9) as they had rough surface type and diameter similar to the parent. Among the 12 strains which were obtained by the second cycle of UV-radiation and the parent strain UVA2, the xylanase production by all the mutants was less than the parent strain (28.92 UmL^{-1}, Figure 4), at 48h. The results indicated that the xylanase production was not improved and was diminished in all the 12 strains, obtained from UVA2.

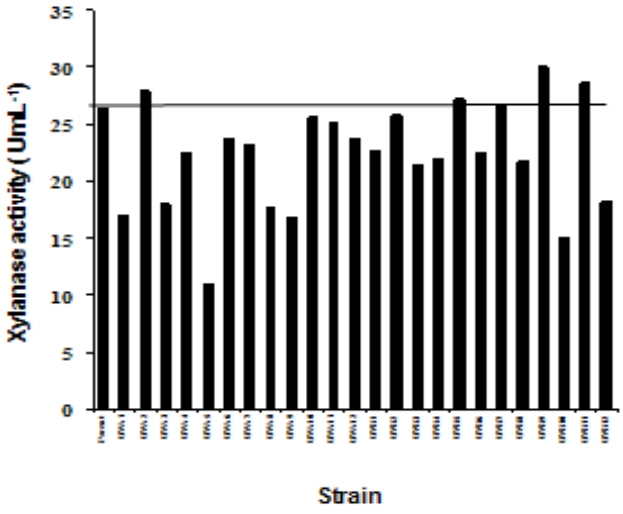

Figure 2. Xylanase activities produced by the strains of *Bacillus pumilus* obtained by the first cycle of UV-radiation and parent strain (control) at 40oC and 100 rpm.

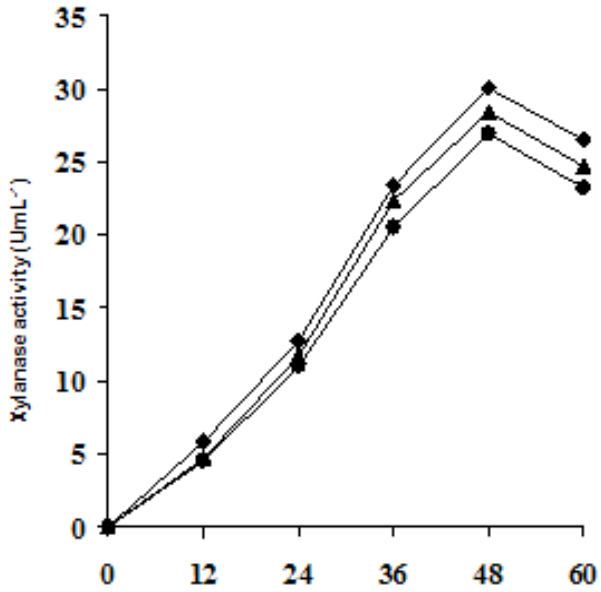

Figure 3. Xylanase activities produced by selected strains (▲) UVA2 and (●) UVB9 of Bacillus pumilus obtained by the first cycle of UV-irradiation and (♦) parent strain (control) at 40oC and 100 rpm.

Among the another 12 strains which were obtained by the second cycle of UV-radiation and the parent strain UVB9, the xylanase production was higher in two strains than the parent strain (29.18 UmL^{-1}), which were UVB9/7 (29.31 UmL^{-1}) and UVB9/8 (31.09 UmL^{-1}) at 48h (Figure 5). Other 10 mutants produced xylanase less than the parent strain. The results in-

dicated that the xylanase production by the two strains such as UVB9/7 and UVB9/8 was improved by 1.004 and 1.06% respectively and diminished in 10 strains. Hence, among the mutants obtained by the 2nd cycle, the strain UVB9/8 improved and gave 31.09 UmL^{-1} xylanase activity and it was selected for the third cycle of UV-radiation. The parent strain (UVB9) and the selected mutant (UVB9/8) showed similar xylanase production patterns (Figure 6). Thus the strain UVB9/8, which showed the xylanase production 1.06 times higher than that of parent strain, was subjected to UV-irradiation for the third cycle.

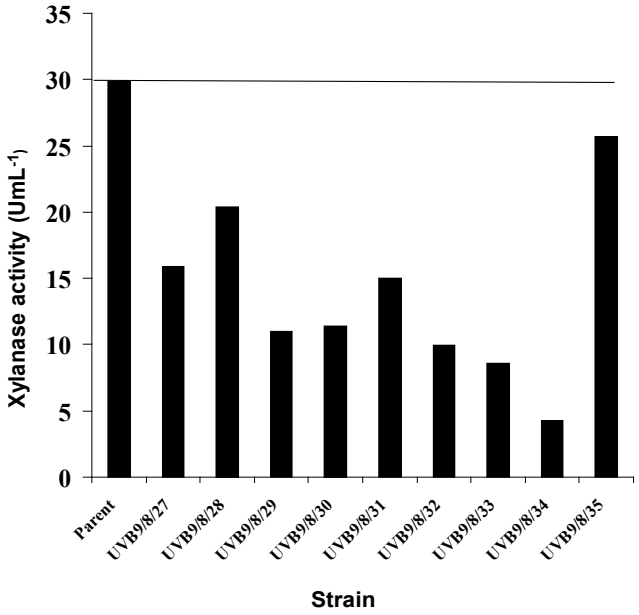

Figure 4. Xylanase activities produced by the varients of *Bacillus pumilus* obtained by the second cycle of UV-radiation and parent strain (UVA2; control) at 40°C and 100 rpm.

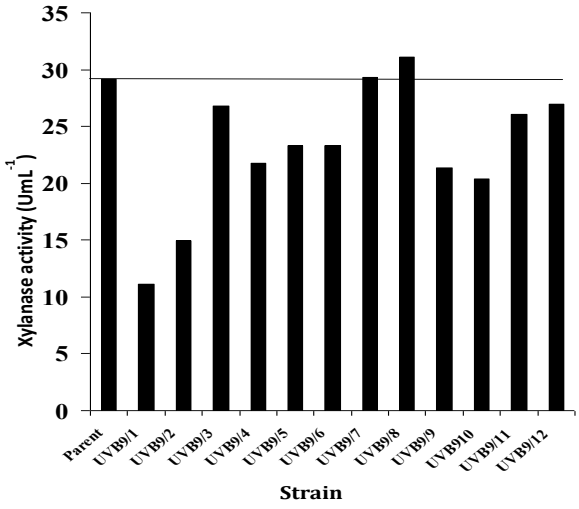

Figure 5. Xylanase activities produced by variants of Bacillus pumilus obtained by the second cycle of UV-irradiation and parent strain (UVB9; control) at 40oC and 100 rpm.

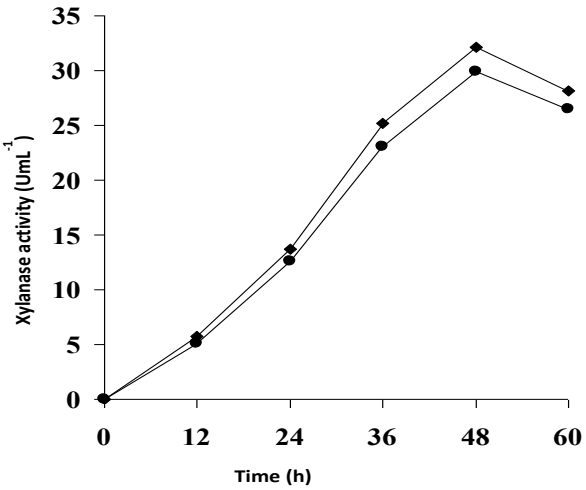

Figure 6. xylanase activities produced by the selected strain (◆) UVB9/8 of *Bacillus pumilus* obtained by the second cycle of UV-radiation and parent strains (●)UVB9 (control) at 40°C and 100 rpm.

Screening the Best Xylanase Producer among the Selected Strains Obtained by the Third Cycle of UV-Irradiation

The randomly selected eighteen strains obtained from the third cycle of UV-irradiation and parent strain (UVB9/8; control) were used for xylanase production in fermentation medium at 40°C and 100 rpm. Xylanase activity produced was determined and the efficient xylanase producers were selected. Among the UV-irradiated strains which were obtained by the third cycle, no strains gave higher xylanase production at 48h than the parent strain (UVB9/8; 29.07 UmL^{-1}, Figure 7). Only strain UVB9/8/9 gave nearly same amount of xylanase activity as that of the parent strain but the xylanase production was less (28.84 UmL^{-1}) than the parent strain and it also was not selected.

Thus the results indicated that the xylanase production was not improved by any strains obtained from the third cycle of UV-irradiation (Figure 7). In many cases, mutations are harmful, but occasionally may lead to a better adapted organism to its environment with improved biocatalytic performance. The potential of a microorganism to mutate is an important property conferred by DNA, since it creates new variations in the gene pool. The challenge is to isolate those strains which are true mutants that carry beneficial mutations [10]. UV rays are important inducers of strain mutations. The pyrimidines are especially sensitive to modifications by UV rays. This may result in the production of thymine dimers that distort the DNA helix and block future replications [11]. Sometimes UV treatment might have caused inhibition of xylanase production and disturbed the basic metabolic activities of the bacterial strain.

This may be the reason for the lower production of xylanase in most number of mutants.

Endo-xylanase by the mutant of *Humicola lanuginose* TH1 was 1.6-fold more than that produced by the parental organism in solid-state fermentation of rice bran at 45°C [12]. A newly isolated *Pseudomonas* strain was improved to produce lipase by mutation [13]. *Pseudomonas* sp. ATCC 31461 was improved by UV-mutation for polysaccharide production [14]. The UV-mutation and chemical mutation have improved the citric acid production by *Aspergillus niger* P_2 [15].

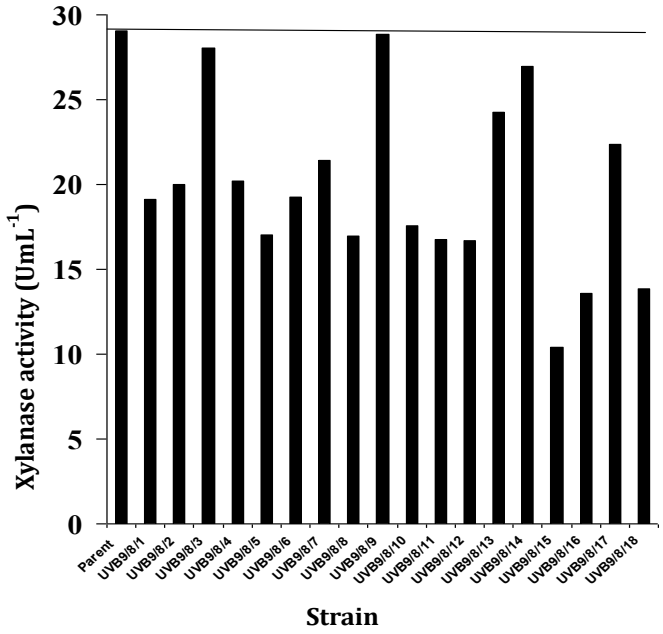

Figure 7. Xylanase activities produced by the variants of *Bacillus pumilus* obtained by the third cycle of UV-radiation and parent strain (UVB9/8; control) at 40°C and 100 rpm.

Improvement of the Strain UVB9/8 by Heat Shock and Screening the Best Xylanase Producer

Among the heat shocked strains which were obtained by the application of heat shock, no strains gave higher xylanase production at 48h than the parent strain (UVB9/8; 29.10 UmL^{-1}). Only strain UVB9/8/20 gave higher amount of xylanase activity than the other mutants, but the xylanase production was less (22.84 UmL^{-1}, Figure 8) when compared with that of the parent strain and it was not selected. Thus the results indicated that the xylanase production was not improved and all the eight strains used for heat shock, were discarded. Temperature increases, a wide variety of stress agents increases the synthesis of heat shock proteins in the bacterial cells [2]. The results suggest that the inhibitory effect of heat shock treatment may be mainly due to the inhibition of protein synthesis. Heat shock treatment of bacterial cells might have resulted in the inhibition of enzyme biosynthesis. High temperature might have denatured some proteins which are essential for the enzyme synthesis.

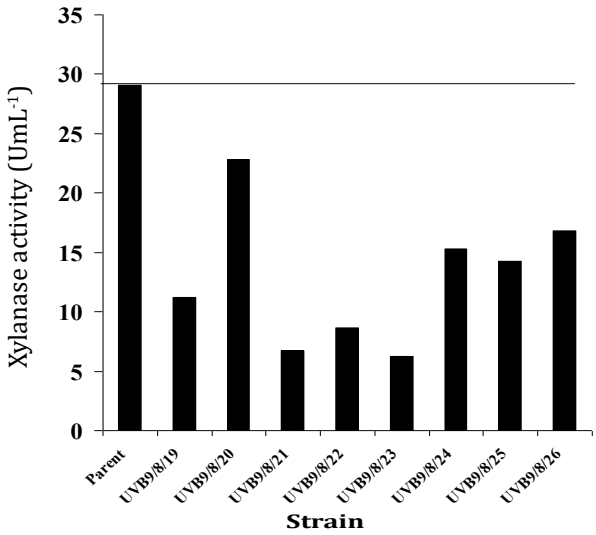

Figure 8. Xylanase activities produced by the variants of *Bacillus pumilus* obtained by the application of heat shock and parent strain (UVB9/8; control) at 40°C and 100 rpm.

Effect of Ethyl Methane Sulphonate (EMS) Treatment Time on the Survival of the Strain UVB9/8

The viability of the cells decreased suddenly to 33.8% at 15min and thereafter decreased up to 0.013% until 105 min (Figure 9). The results showed that the duration of EMS treatment was 15, 30, 45, 60, 75, 90 and 105 min, it had killed 66.2, 91.77, 95.22, 97.78, 99.04, 99.933 and 99.987% of cells in the medium. Thus the lethal effect was increased with EMS treatment time.

Hydroxylamine mutagenesis for 60 minutes supported the better mutation for the production of depolymerase enzymes by the bacterial strain *Bacillus* sp [16]. The cells which have survived after the EMS treatment might be due to the mutation occurred. However, the lethal effect was 99.987% at 105 min EMS treatment. As the 0.013% of cells found at 105 min it was decided to treat the mutant UV9/8 for 105 min.a

Improvement of the Strain UVB9/8 by Ethyl Methane Sulphonate (EMS) Treatment and Screening the Best Xylanase Producer

When the cells in the inoculum of UVB9/8 were subjected to EMS treatment for 105 min 0.013% of the cells survived. The cells which have survived might have been mutated. Hence, nine colonies were selected randomly from the sample treated with EMS for 105 min. Slant cultures were prepared from the colonies and the strains were cultivated five times to stabilize

the strains. The number of colonies with nearly same morphological characters was obtained in nutrient-agar plates. Among them, nine colonies which had rough surface type and diameter similar to the parent (4.6±0.2 mm) were selected randomly. Slant cultures were prepared from the selected nine colonies and the strains were cultivated five times to stabilize the mutants. The colony size and colour of B*acillus* sp were changed when it was subjected to chemical mutation using hydroxylamine [16]. Among the EMS treated strains, no strains gave higher xylanase production at 48h than the parent strain (UVB9/8; 29.87 UmL^{-1}). Only strain UVB9/8/35 gave higher amount of xylanase activity (25.724 UmL^{-1}, Figure 10) than the other mutants, but the xylanase production was less when compared with that of the parent strain and it was also not selected. Thus the results indicated that the xylanase production was not improved and diminished in all the nine strains obtained from the chemical mutation.

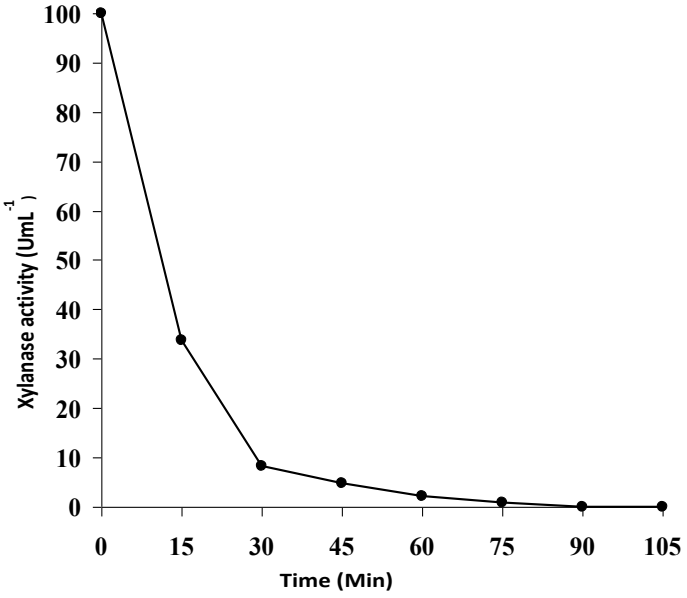

Figure 9. Effect of EMS treatment time on the survival of *Bacillus pumilus*.

EMS triggers the regulation of protein synthesis in some bacteria [17]. A chemical mutation caused by Ethyl methanesulphonate (EMS) affecting the synthesis of cellulase in *Bacillus sp* BpCRI6 was studied and the mutants have the ability of secreting significant amounts of cellulose. Cellulase production was four times higher than that of the wild type under optimum growth conditions (pH 6.5, 25°C and Ca^{2+} 1mM) [18]. However, mutants obtained by EMS treatment exhibited lower production of xylanase than the strain UVB9/8 under tested conditions. This result indicates that the reduction of xylanase production in the strain is not due to an increase in cell growth but is only due to the chemical mutagenesis affecting the synthesis of xylanase within the strain. Sometimes EMS might have caused inhibition of enzyme biosynthesis and disturbed the basic metabolic activities of the bacterial strain. This may be the reason for the lower production of xylanase by the bacterial strains.

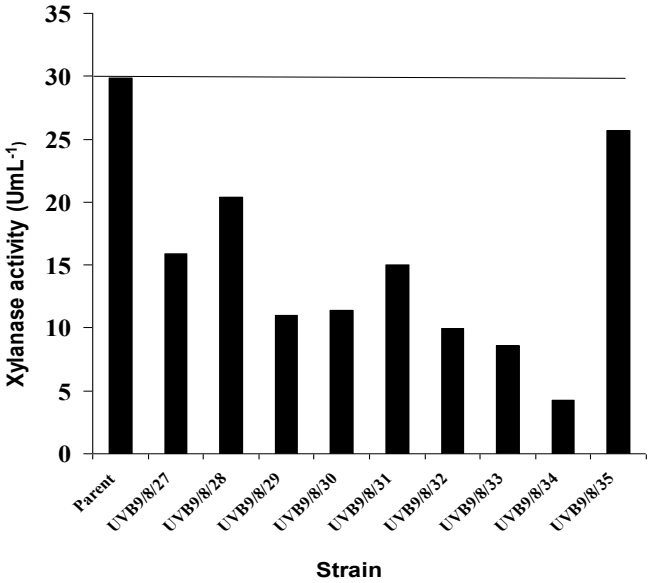

Figure 10. Xylanase activities produced by the variants of Bacillus pumilus obtained by the chemical mutation and parent strain (UVB9/8; control) at 40oC and 100 rpm.

Conclusion

Xylanase production was increased by 1.22 times in *Bacillus pumilus* in the first two cycles of UV-irradiation and there was no increase in the third cycle. Heat shock and EMS treatments, did not significantly improve the xylanase production.

Acknowledgement

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