International Journal of Advanced Information Science and Technology (IJAIST) ISSN: 2319:2682
Vol.5, No.1, January 2016 DOI:10.15693/ijaist/2016.v5i1.127-138

Enhanced production of cellulase free xylanase from UV irradiated improved strain of *Bacillus altitudinis* Kd_1 (M) and its scale up in a stirred tank bioreactor

Dr Nisha Sharma
Microbiology Research Laboratory
Department of Basic Science
Dr. Y S Parmar University of Horticulture and Forestry
Nauni, Solan, HP, India- 173230

Dr Nivedita Sharma
Microbiology Research Laboratory
Department of Basic Science
Dr. Y S Parmar University of Horticulture and Forestry
Nauni, Solan, HP, India- 173230

***Abstract*— In this study, enhanced production of cellulase free xylanase by a stable mutant of *B. altitudinis* Kd_1 (M) (UV irradiated) by optimizing various process parameters and media components has been done. The UV irradiation mutated strain of *Bacillus altitudinis* Kd1 (M) has shown an increased xylanase production of 61% from the wild type strain Kd_1 (W). A good stability until 8 generations has also been observed. Optimization of process parameters by OFAT for enhanced enzyme production yielded 96.25 IU/ ml of enzyme on TGY medium at 5.5 pH with 12.5% inoculum following 72 h of incubation time. Further scale up of enzyme production in a 7.5 L stirred tank bioreactor, utilizing the optimized conditions yielded 96.00 IU/ml after 8 h of fermentation thus proving the efficiency of a stirred tank bioreactor in enzyme production with higher volumes in short time.**

Keywords: *Bacillus altitudinis*, xylanase, cellulase, mutation, bioreactor

I. INTRODUCTION

Xylanases (E.C.3.2.1.8) are key enzymes, which play an important role in the breakdown of xylan. Xylan, a major component of hemicellulose, is a heterogeneous polysaccharide consisting of ß-1,4 linked D-xylosyl residues. Enzymatic hydrolysis of xylan is catalysed by different xylanolytic enzymes such as endo-1,4-ß-xylanase, ß-xylosidase, ά-glucuronidase, ά-arabinofuranosidase, and esterase. Among these endo-1,4-β-xylanase (E.C. 3.2.1.8) and ß-xylosidase are the most important enzymes where the first attacks the main internal chain linkages, and the second releases xylosyl residues by endwise attack of xylo-oligosaccharides [1]. Cellulase-free xylanase is mainly considered for pulping and bleaching processes due to the potential industrial advantages such as the reduction in usage of bleaching agents, being consequently more environmental friendly.

Microbial xylanases have the significant feature of being able to be produced in large quantities by established and optimized fermentation techniques. Enzyme production is closely controlled in microorganisms and therefore, to improve its productivity these controls can be exploited and modified. To establish a successful fermentation process it is

necessary to make the environmental and nutritional conditions favourable for the microorganism for over-production of the desired metabolite. An intensive investigation is therefore, required to establish the optimum conditions to scale up enzyme production under SmF in an individual fermentation process [2].

Xylanases have gained notable importance due to their potential applications in industrial processes such as in bio bleaching, wine industry, improving the digestibility of animal feed stocks, bioconversion of lingo cellulosic material and agro-wastes to fermentable products and pre-bleaching of paper pulps. Cellulase free xylanases have a great demand in paper and pulp industry**.** Members of the genus *Bacillus* are used extensively in industrial fermentation since they secrete most of their enzymes into extracellular medium. Bacterial xylanases range from acidic to alkaline depending upon the type of the organism.

The production of xylanase by wild type strains is very low, whereas commercialization of these enzymes demands microorganisms with improved activity and better resistance to product inhibition**.** The improvement in the activities and desirable enzyme traits can be obtained through random/ sites directed mutagenesis and selection. Strain improvement for xylanase production via mutagenic agent including ultraviolet (UV), X-rays, gamma radiations, ethyl methane sulfonate (EMS) and N-methyl- N-nitrosoguanidine (NTG) has been demonstrated for strain improvement [3, 4, 5 ,6 ,7].

In this paper production of cellulase free xylanase from *B. altitudinis* Kd_1 UV irradiated stable mutant, optimization of parameters for enhanced production of xylanase finally scale up of enzyme in a stirred tank bioreactor has been reported. The results presented here suggest strongly that this enzyme could be a potential choice for industrial interest.

II. MATERIALS AND METHODS

2.1 Sample collection and isolation

Isolations of microorganisms were done from soil after its enrichment by adding 2% xylan powder and incubating the plates at 35±2 ^{0}C for 5 days following serial dilutions on Riviere's medium [8]. The pure line cultures were obtained by streaking each microbial colony on the respective medium at 35±2 ^{0}C and were preserved in refrigerator at 4 ^{0}C.

2.2 Screening of hyperxylanolytic isolates

Isolated microorganisms were screened on the basis of their xylanase production efficiency. Following qualitative and quantitative tests were used for the screening of the xylanolytic bacteria isolated from soil:

2.2.1Qualitative Screening of Hyperxylanolytic Isolates: Isolated microorganisms were screened based on their xylanase production efficiency by qualitative test on xylan agar medium [9]. The inoculated plates were incubated for 5 days at 30 ^{0}C. The colonies forming clear zones after flooding with congo red were declared to be xylanase producers. The plates were then washed with 1M NaCl. Potential xylanase producing isolates having maximum zone of inhibition was selected for further studies.

2.2.2 Quantitative Screening of Hyperxylanolytic Isolates: Enzyme production:

5 ml of the inoculum was added to each 45 ml of Riviere's broth containing 1% xylan in 250 ml Erlenmeyer flasks and the flasks were incubated at 35±2 ^{0}C for 5 days at 120 rpm. The samples were withdrawn at specific intervals, were centrifuged at 10,000 rpm for 15 min at 4 ^{0}C and the supernatant was used for enzyme assay.

Enzyme assay: To 0.5 ml of xylan solution centrifuged and clear supernatant was added along with 0.3ml citrate buffer (pH 5) and 0.2ml of enzyme. The control was run with all components except the enzyme. The reaction mixture was incubated at 45°C for 10 min and then 3ml of DNSA reagent was added and the mixture was then heated on boiling water bath for 15min, after cooling down at room temperature, absorbance of reaction mixture was read at 540nm[10].

2.3Quantitative Assay for Cellulase activity: Filter Paperase (FPase) assay [11]

Enzyme production: 5 ml of the inoculum was added to 45 ml of Riviere's broth containing 1% cellulose in 250 ml Erlenmeyer

flasks and the flasks were incubated at 35±2 ^{0}C for 5 days at 120 rpm. The samples were withdrawn at specific intervals, were centrifuged at 10,000 rpm for 15 min at 4 ^{0}C and the supernatant was used for enzyme assay.

Procedure: To 50 mg of filter paper strips (Whatman no. 1), 0.5 ml of citrate buffer (0.055M, pH-5) and 0.5ml of culture supernatant was added. Reaction mixture was incubated at 50°C for 30 min. After incubation 3ml of DNSA reagent was added. Tubes were immersed in boiling water bath and removed after 15 min when colour development was complete. Control was run with all the components except the enzyme. Tubes were cooled at room temperature and O.D was read at 540nm in spectrophotometer against a reagent blank i.e. 1ml of distilled water and 3ml of DNSA reagent. The standard curve was prepared from the stock solution of glucose (0.4mg/ml). The enzyme activity was expressed in terms of International Unit (IU) and Specific Activity (SA).

2.4Strain improvement of the selected cellulase free xylanase producer strain by mutation

The selected bacterial isolates were subjected to mutation by the physical mutagen i.e., ultraviolet irradiation (UV) and chemical mutagen i.e., ethidium bromide (EtBr) with an apparent aim to get mutants yielding higher enzyme titres.

2.4.1Mutation with Ultraviolet irradiation (UV): UV light of laminar air flow [Phillips TUV 30W/G30.T8 Ultra volt] was used as a mutagen. 24 h old culture of bacterial isolate (10 ml) was transferred to a sterilized petriplate and each of the samples was exposed to UV light. Before exposing sample to the UV irradiation, 1 ml of sample was withdrawn for determining the initial population in terms of colony forming units on BSYEM medium containing 0.5% of xylan. The samples were withdrawn after 15, 30, 45, 60 and 75 min of UV exposure and subsequently plated on to the BSYEM medium in order to get the survival count. The UV dose given to the samples was calculated as given below:

Intensity = Volt (mW)/ cm^2

Voltage used = 30 W

Distance = 15 cm

UV dose =Intensity × time (seconds)

UV exposure time (min)	**Calculated dose (mJoules/cm^2)**
15	0.00012
30	0.00024
45	0.00036
60	0.00048
75	0.00064

2.4.2 Mutation with Ethidium Bromide (EtBr): One ml of culture was withdrawn from a 24 h old culture of bacteria grown in 10 ml of basal salt yeast extract in order to determine the initial population in terms of colony forming units. EtBr was mixed well with culture and incubated at room temperature. One ml sample was removed for plating after 60 min of exposure. Different concentrations of EtBr (0.1-2.0 mg/ml) were evaluated for selecting a concentration that would result in 1 percent survival. This concentration was used for carrying out mutation at 30, 60, 90 and 120 min interval in order to optimize the time period that would results in 3log kill of cells (0.1-1.0% survival).

2.4.3 Screening of mutants: Survivors were screened on the basis of their capacity to produce enhanced enzyme with respect to their parent strain. Some isolates showed decrease while others showed an increase in enzyme production after mutation. Among them Kd_1 (M) showed an increase in xylanase production compared to its parent strain and thus was selected finally for further enzyme production studies.

2.4.4 Hereditary stability studies of mutants: The selected mutant Kd_1 (M) was studied for their stability for cellulase free xylanase production consecutively for 8 generations. The mutants after every generation were inoculated on PYC and TGY media and used for

inoculating next fermentation for respective enzyme assays.

2.5 *Morphological and physiological characteristics of kd_1 (w) and kd_1 (m)*

Morphological, cultural and biochemical characterization of pure cultures of wild and mutant strains i.e. Kd_1(W) and Kd_1(M) was done by applying standard techniques mentioned in the Bergey's Manual of Determinative Bacteriology [12].

2.5.1 Molecular identification of hyperxylanase producer bacterial isolates kd_1 (w) and kd_1 (m): Bacterial genomic DNA was isolated by using DNA purification kit (Banglore Genei, make). 16S rRNA gene was amplified by PCR using 16S universal primers 8F: 5' AGAGTTTGATCCTGGCTCAG 3' and 1492R: 5' ACGGCTACCTTGTTACGACTT 3'. PCR reactions were performed under the following conditions: Initial denaturation at 94°C for 1 min, followed by 35 cycles of 92°C for 1 min, annealing at 55°C for 1 min 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min. The presence and yield of PCR product was determined on 1% agarose gel electrophoresis at 200 V for 30 min in 1X Tris-acetate-EDTA buffer and stained with ethidium bromide.

The PCR products were purified using QIA quick gel extraction kit (Qiagen, USA) and sequenced from the 50 end.

Nucleotide sequencing: The PCR amplicon obtained by amplifying PCR product was diluted in Tris buffer (10 mM, pH 8.5). Dilution used was 1:1000 in order to obtain the DNA concentration required for sequencing (30 mg/ µl), the sequencing required 8µl DNA. The primer used in sequencing reaction was 16SF (5'AGAGTTTGATCCTGGTCAG3') at a concentration of 3 µM. Sequencing was then performed using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA).

Phylogenetic tree construction: All the sequences were compared with 16S rRNA gene sequences available in the GenBank databases by BLAST search. Multiple sequence alignments of partial 16S rRNA gene sequences were aligned using CLUSTAL W, version 1.8. Tree generated was analyzed with the TREEVIEW program. Sequences obtained were submitted to NCBI for accession number.

2.6 Optimization of process parameters under SmF

Cellulase free xylanase from selected mutant Kd_1 (M) was optimized using conventional one factor at a time (OFAT) approach by varying different fermentation variables i.e. medium, incubation time, pH, temperature, inoculum size, different carbon source and concentration of carbon sources etc. using selected lignocellulosic biomass as substrate.

2.6.1 Effect of media, inoculum size and incubation period: Various inoculum sizes ranging from 2.5%, 5.0 %....17.5 % were tested for enhanced cellulase free xylanase production on different media PYE Media [13], **B**asal Salt Medium [14], Modified Basal Salt Medium [15], Okoshi *et al*. Medium [16], Li & Gao Medium [17] **,** Mandel and Reese Media[18] at varied incubation periods (24h, 48h…..144h). The quantitative tests were performed with the supernatant.

2.6.2 Effect of temperature and pH on enzyme production : The effect of different temperature (25, 30,...., 50°C) and at pH (5.0, 5.5.....9.0) on cellulase free xylanase production was studied. The organisms were incubated and cellulase free xylanase production and protein were determined in the supernatant.

2.6.3 Effect of carbon source and its concentration: Various carbon sources viz. xylose, maltose, xylan, arabinose, ribose and dextrose were used for hyperxylanase production at a concentration ranging from 0.5% to 3.0%.

2.7 scale up of xylanase production by B. altitudinis kd_1 (m) in a stirred tank bioreactor

Enzyme thus produced with optimized process parameters will be scaled up in a stirred tank bioreactor for enhanced cellulase free xylanase production.

2.7.1 Inoculum Preparation: *B. altitudinis* Kd_1 (M) was grown in 300 ml of nutrient broth and incubated at 30±2°C for 24 h. As soon as the substantial growth of the strain was observed in the broth, the optical density was set to 1.0 using nutrient broth as blank.

2.7.2 Batch Fermentation: Batch cultivation for the production of xylanase was carried out in a 7.5 L stirred tank bioreactor (New Brunswick Scientific, New Jersey USA) with a 3.0 L working volume at 30± 2°C and pH 5.5. Carbon and nitrogen sources and other conditions which were optimized were utilized for scale up of enzyme production. Foaming was controlled with addition of a few drops of polypropylene glycol. The medium was sterilized in situ for 20 min at 121°C. The bioreactor was inoculated with 10% inoculum under aseptic conditions. The agitation speed 200 rpm and agitation rate 1.0 vvm respectively used for batch cultivation in bioreactor. The sample was withdrawn regularly at different intervals ranging from 2, 4……..10 h.

III. RESULTS AND DISCUSSION

3.1 Isolation and screening of xylanase producing isolate

In total, 84 bacterial isolates were obtained from soil samples in PYC (Peptone Yeast Extract) medium [13] having pH 6.8. Maximum xylanase activity was exhibited by Kd_1 (31.72 IU) followed by SL_8 (31.54 IU) and the least xylanase activity was noted in SL_7 (2.930). Three isolates with highest enzyme activity i.e. N_{11} (29.64 IU), Kd_1 (31.72 IU) and SL_8 (31.54 IU) were thus selected as potential xylanase producing strains for further studies. None of the strains showed considerable cellulase production efficiency, thus the enzyme was regarded as cellulase free xylanase.

3.2 Strain improvement of Kd_1 (W) isolate for enhanced xylanase production by inducing mutation

Mutagenesis is a well recognized approach to enhance the expression of enzymes. A mutagen leads to mutation and alters many of basic characters of a strain by inducing lesions or modification in base sequences of DNA that remains unrepaired [19]**.** UV rays have been considered important inducers for strain mutations. The pyrimidines are especially sensitive to modifications by UV rays absorption which results in the production of thymine dimers that distort the DNA helix and block future replication [20].

Thus an attempt was made in the present study to enhance the cellulase free xylanase production in the screened wild strains by inducing mutation by using EtBr and UV irradiation. The isolates were exposed to UV dose of 0.00012 mJ/cm^2 for 15 min, 0.00024 mJ/cm^2 for 30 min, 0.00036 mJ/cm^2 for 45 min, 0.00048 mJ/cm^{2}for 60 min and 0.00064 mJ/cm^2 for 75 min in an open petriplate. The survival count at each interval was determined on TGY medium containing 0.5% xylan. Among the UV irradiated isolates, Kd_1 (M) and N_{11} (M) showed an increase in xylanase activity from 31.72 IU to 51.32 IU and 29.05 IU to 31.30 IU respectively, while SL_8 (M) showed a decrease in enzyme activity. Among the EtBr treated strains Kd1 (M) and N_{11} (M) showed an increased xylanase production i.e. 40.84 IU and 41.76 IU respectively, while SL_8 (M) showed a decreased activity of 24.63 IU.

Xylanase activity showed a considerable increase at the dose of 0.00024 mJ/cm^2 for 30 min in UV irradiated Kd_1 from 31.72 IU in wild to 51.32 IU in mutant Kd_1 (M). Kd_1 (M) UV irradiated was thus selected for further optimization studies.

3.2.1 Hereditary stability studies of mutants: The selected mutants i.e. Kd_1 (M) for cellulase free xylanase production were found to be stable for 8 generations. Figure 1 showed xylanase activities of the mutant upto 8 generations. Mutants generally are not stable with respect to their activity for continued generations, compared to the parent strain. However, Kd_1 (M) has shown a good stability by retraining the xylanase production efficiency till 8 generations.

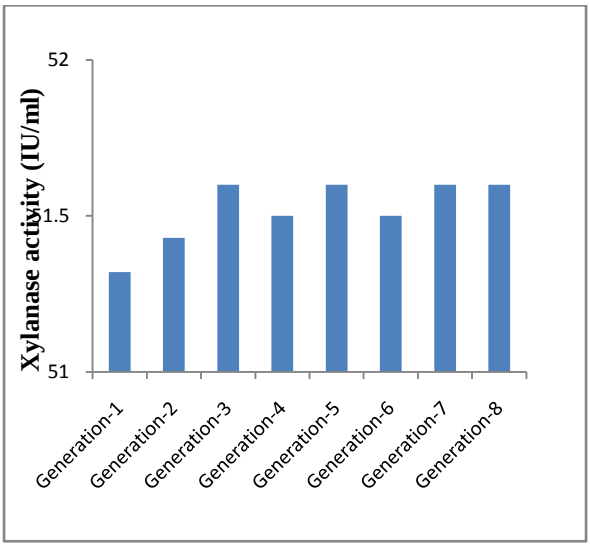

Fig. 1 Cellulase free xylanase activity in terms of stability of UV irradiated *Bacillus altitudinis* Kd_1 (M)

3.3 Phenotypic and genotypic identification of screened hyper xylanolytic isolate kd_1 (w) and kd_1 (m)

3.3.1 Phenotypic Identification of Bacterial Isolates : On the basis of phenotypic and biochemical characters Kd_1 (W) and Kd_1 (M) was placed under the group of Genus *Bacillus* as per Bergey's Manual of Determinative Bacteriology, 7th Edn.

3.3.2 Genotypic Characterization: Kd_1 (W) and Kd_1 (M) were then identified at genomic level by using 16S rRNA gene technique. Genomic DNA of the isolate was isolated by using DNA purification kit (Banglore Genei, make). The DNA was quantified by using standard protocol [21]. The isolated genomic DNA was used in PCR to amplify small subunit of 16S rRNA using universal primer having product size of approximately, 1500 bp. The PCR product so obtained after amplification was visualized using ethidium bromide on 2% agarose gel (Plate 2). Amplified PCR products were purified and got sequenced by the services provided by Xcelris Labs Ltd, Ahmadabad- India to confirm the results.

Nucleotide Sequencing: Following sequences of isolates Kd_1 (W) and Kd_1 (M) were obtained after sequence analysis.

Sequences of Kd_1 (W): Kd_1 (W)_8F_S6980_F02_006 (Sequence with Forward primer:880 bp)

TGAGTATCACGTGGGTAACCTGCCTGTAAGACTGGG
ATAACTCCGGGAAACCGGAGCTAATACCGGATAGTT
CCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTT
CGGCTGTCACTTACAGATGGACCCGCGGCGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACG
GAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATC
GTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGA
GTAACTGCTTGCACCTTGACGGTACCTAACCAGAAA
GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGT
AAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGA
AAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAAC
TGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTC
CACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA
ACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACT
GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTG
CAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG
GTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG
CAACGCGAAGAACCTT

Kd_1 (W)_1492R_S6980_G02_004 (Sequence with Reverse primer:873 bp)

CTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCC
GGAACGTATTCACCGCGGCATGCTGATCCGCGATTA
TAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTG
CGATCCGAACTGAGAACAGATTTGTGGGATTGGCTA
AACCTTGCGGTCTCGCAGCCCTTTGTTCTGTCCATTG
AGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATG
ATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACC
GGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCA
ACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAA
CCCAACATCTCACGACACGAGCTGACGACAACCATG
CACCACCTGTCACTCTGTCCCCGAAGGGAAAGCCCTA

TCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAA
GGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTT
CAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAA
TGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCT
AACACTTAGCACTCATCGTTTACGGCGTGGACTACCA
GGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTC
CTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCC
ACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCT
ACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGT
TTCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGG
GGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGC
CCTTTACGCCCAATAATTCCGGACAACGCT

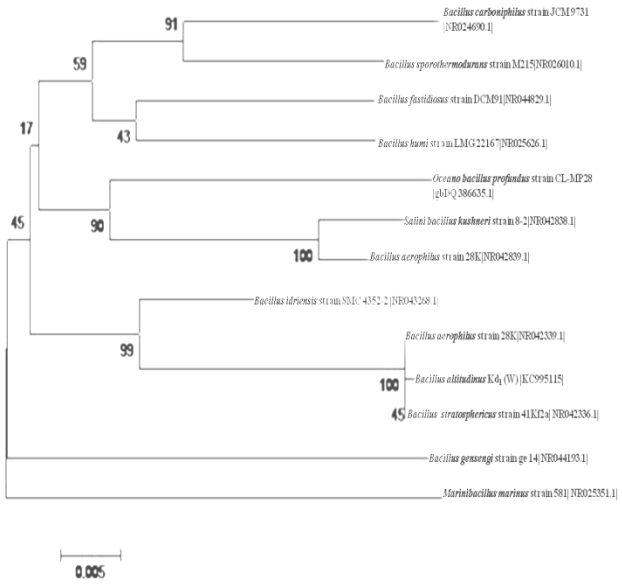

Fig 2. Neighbour- joining tree constructed in Clustal X showing phylogenetic relationship of *Bacillus altitudinis* Kd1 (W) |KC995115| among the genus *Bacillus* based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The number at the nodes show the bootstrap values obtained with 100 replicates.

Sequences of Kd_1 (M): Kd_1 (M)_8F_S6980_H02_002 (Sequence with Forward primer:919 bp)

CGGACGGCTGAGTAACACGTGGGTAACCTGCCTGTA
AGACTGGGATAACTCCGGGAAACCGGAGCTAATACC
GGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAA
GACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGC
GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG
CGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG
TCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTT
TCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAG
TGCAAGAGTAACTGCTTGCACCTTGACGGTACCTAAC
CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTG
GGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGAT
GTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGG
AAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGG
AATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTG
GAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTG
TAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGT
GCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGGTCGCAAGACTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATC
CTCTGACAACCCTAG

Kd_1 (M)_1492R_S6980_A03_031 (Sequence with Reverse primer:881 bp)

TGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACG
TATTCACCGCGGCATGCTGATCCGCGATTACTAGCGA
TTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCC
GAACTGAGAACAGATTTGTGGGATTGGCTAAACCTT
GCGGTCTCGCAGCCCTTTGTTCTGTCCATTGTAGCAC
GTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTG
ACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAG
TCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAA
GATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAA
CATCTCACGACACGAGCTGACGACAACCATGCACCA
CCTGTCACTCTGTCCCCGAAGGGAAAGCCCTATCTCT
AGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTC
TTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGC

TTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTC
TTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGT
TAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACAC
TTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTA
TCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGC
GTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGT
GTTCCTCCACATCTCTACGCATTTCACCGCTACACGT
GGAATTCCACTCTCCTCTTCTGCACTCAAGTTTCCCA
GTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTT
CACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTAC
GCCCAATAATTCCGGACAACGCTTGCCACCTACGTAT

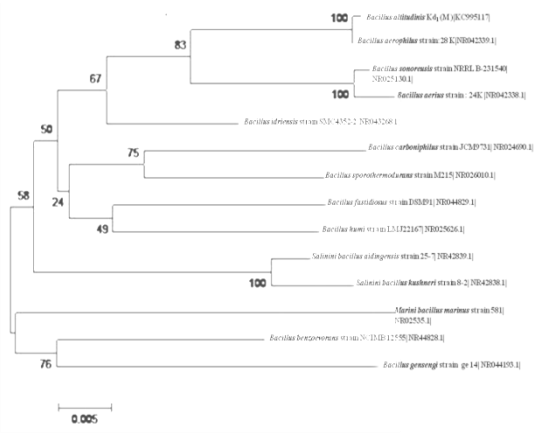

Fig3. Neighbour- joining tree constructed in Clustal X showing phylogenetic relationship of *Bacillus altitudinis* Kd1 (M) |KC995117| among the genus *Bacillus* based on a distance matrix analysis of 16S rRNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The number at the nodes show the bootstrap values obtained with 100 replicates.

Sequences of the isolates thus obtained were submitted to NCBI database and matched with already existing sequences. 16S rRNA sequences of these isolates have been registered to genebank databases. Isolate Kd_1 (Wild) showed 99% species specific alignment with *Bacillus altitudinis* strain MD02 and Kd_1 (Mutant) showed 99% species specific alignment with *Bacillus altitudinis* strain H34. These isolates have been registered with accession numbers as *Bacillus altitudinis* Kd_1 (W) [KC995115] and *Bacillus altitudinis* Kd_1 (M) [KC995117] with NCBI. Phylogenetic trees of *Bacillus altitudinis* Kd_1 (W), *Bacillus altitudinis* Kd_1 (M), with respect to other *Bacillus sp*. bacteria as inferred by neighbour joining method have been presented in Fig 2 and 3.

3.4 Optimization of process parameters for enhanced xylanase production by Bacillus altitudinis Kd_1 (M) by using one factor at a time (OFAT) approach

3.4.1 Effect of media: *B. altitudinis* Kd_1(M) showed maximum growth in TGY medium containing Yeast extract (0.5%), tryptone (0.5%), K_2HPO_4 (0.1%), glucose (1.0%). Enhanced enzyme production using defined medium may be due to the presence of nitrogen, carbohydrate and other compounds in appropriate concentration as per the physiological requirement of this organism that would have resulted in enhancing the ability of the cells to synthesize most of extracellular cellulase free xylanase [22]. Glucose present in medium must have served as a ready utilizable carbon source leading to higher growth of *B. altitudinis* Kd_1 (M) consequently causing more synthesis of extracellular xylanase. Besides, tryptone has stimulating effect causing highest production of xylanase as nitrogen sources play a significant role in promoting the extracellular release of enzyme.

3.4.2 Effect of pH: The highest xylanase titres 59.09 IU were observed at acidic pH 5.5 for this strain thus exhibiting acidophilic nature of this mutant. Since pH of the medium influences the growth of microorganisms and hence the enzyme production. Each microorganism possesses a specific pH range for its growth and activity. The extracellular pH has a strong influence on the pathways of metabolism and product formation by microorganism. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the organism thus lowering their overall metabolic activity [23].

3.4.3 Effect of temperature: Incubation temperature is the most important physical factor which affects enzyme production dramatically and their stability. *B. altitudinis* Kd_1 (M) exhibited optimum temperature of 30°C (71.33 IU) for maximum xylanase production which is significantly higher than others. Minimum xylanase was produced at 50°C (31.46

IU). Microorganisms grow slowly at a temperature below or above the normal temperature because of reduced rate of cellular production. The reduction in enzyme activity at higher temperature could be due to denaturation of enzymes. Maximum enzyme activity at optimum temperature may be due to the faster metabolic activity and increase in protein content and extracellular enzyme production in culture supernatant. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins thus lowering enzyme activity [23].

3.4.4 Effect of Inoculum Size: Different inoculum sizes studied for enhanced enzyme production were 2.5%, 5.0 %, 7.5%, 10.0%, 12.5%, 15.0% and 17.5% (v/v). Highest xylanase production from *B. altitudinis* Kd_1 (M) i.e. 73.44 IU was obtained at 12.5% of inoculum size having statistically significant differences over other inoculum sizes. In the present experiment, inoculum size ranging from 2.5% to 7.5 % probably was low in turn resulting in lower number of cells in production medium thus causing lesser production of enzyme. A further increase in the amount of the inoculum i.e. 12.5% had improved the titres of enzyme reaching to maximum. Beyond this concentration i.e. @ 15% enzymatic activity had dipped due to an overload in the number of cells causing nutrient starvation. Enzyme activity is maximum at optimal level because at this point equilibrium is maintained between inoculum size and availability of substrates while the decline in enzyme yield at larger inoculum size might be due to formation of thick suspensions and improper mixing of substrates in shake flasks.

3.4.5 Effect of Incubation Time: Enzyme activity was measured at regular intervals up to periods of 24 to 120h and maximum xylanase production was noticed at 48 h i.e. 74.45IU/ml. At 48 h, the cell population in the culture reaches at its peak which leads to a stable microbial association with the substrate and resulted in maximum enzyme production. The decline in the activity afterwards could be due to various reasons. A decline in the enzyme afterwards may be because of proteolysis or due to depletion of nutrients available to the strain, causing a stressed microbial physiology resulting in an in activation of enzyme [24]. Another reason could be the product inhibition i.e. the increase in the production of reducing sugars, which after a certain period of growth could exhibit inhibitory effect on enzyme production.

3.4.6 Effect of carbon source: With various sources of carbon viz. xylose, maltose, xylan, arabinose, ribose and dextrose used for xylanase production, enzyme titres varied from 18.55 to 75.16 IU in *B. altitudinis* Kd_1 (M). Maximum xylanase activity of 75.16 IU was observed with xylose.

3.4.7 Effect of substrate concentration: *B. altitudinis* Kd_1 (M) exhibited highest xylanase activity of 95.25 IU with supplementation of 2.5% of xylose. There were statistically significant variations among the different substrate concentrations used for xylanase production ranging from 0.5-3.0%. Very low substrate concentration fails to trigger enzyme production to desirable level because most of the inoculum remains without substrate and hence resulting in minimum secretion of enzymes. Optimum substrate concentration normally results in an increase in the yield and reaction rate of the hydrolysis [25]. However, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme production [26, 27].

Classical approach for one factor at a time (OFAT) used in the present enzyme optimization study from *Bacillus altitudinis* Kd_1(M) has resulted in statistically significant and an impressive increase (Fig. 4) in the production of xylanase proving the direct utility of this technique in increasing enzyme titres. A considerable increase of 85.60 % has been noticed in cellulase free xylanase production after optimizing different process parameters and medium components as shown in Fig 4. Finally all these optimized conditions were employed for the further study.

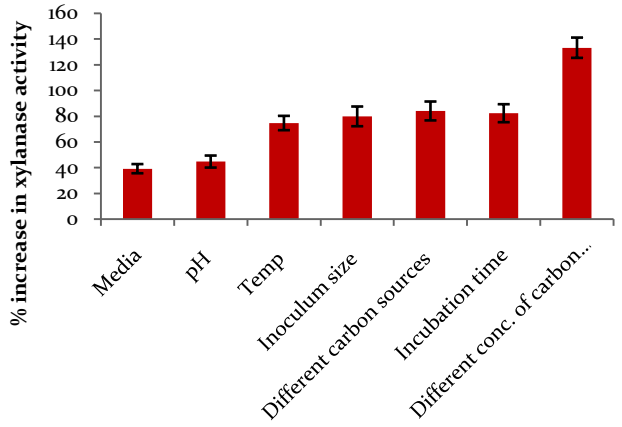

Fig 4. An overview of percent increase in xylanase activity from *B. altitudinis* Kd1 (M) after optimization of different parameters under SmF

3.5 Scale up of cellulase free xylanase in a stirred tank bioreactor

The optimized conditions for xylanase production were shifted from shake flask studies to a batch in the bioreactor. The bioreactor was equipped with accessories and automatic control system for DO, pH, antifoam, impeller speed, aeration rate and temperature. Production of xylanase by *B. altitudinis* Kd_1 (M) was carried out in a 7.5 L stirred tank bioreactor with a 3.0 L working volume, utilizing the conditions optimized at shake flask level. Foaming was controlled with addition of 2-3 drops of polypropylene glycol. 12.5% inoculum was added to the bioreactor under sterilized conditions. The agitation speed 200 rpm and agitation rate 1.0 vvm respectively used for batch cultivation in bioreactor. The sample was withdrawn regularly at different intervals ranging from 2, 4……..10h. Fig.5 shows the physiological behaviour of *B. altitudinis* Kd_1 (M) in the stirred tank bioreactor during scale up process.

Maximum xylanase titres of 96.00 IU were obtained after 8 h of fermentation time followed by 79.540 IU after 10h. The minimum xylanase production 31.952 IU was found in 2 h of fermentation time. A short fermentation period with a greater volume in the stirred tank bioreactor has resulted in the production of similar titres of enzyme which has been obtained in 3 days in shake flask. Maximum production of xylanase achieved at a shorter fermentation time revealed the significance of scale up process. The scale up of enzyme production thus is a much sought after procedure due to comparatively short operation time and an easy recovery of the end product.

Table 1 Xylanase production by *B. altitudinis* Kd1 (M) in a stirred tank Bioreactor

Time (h)	Xylanase (IU)
2	31.952
4	51.368
6	74.436
8	96.00
10	74.540

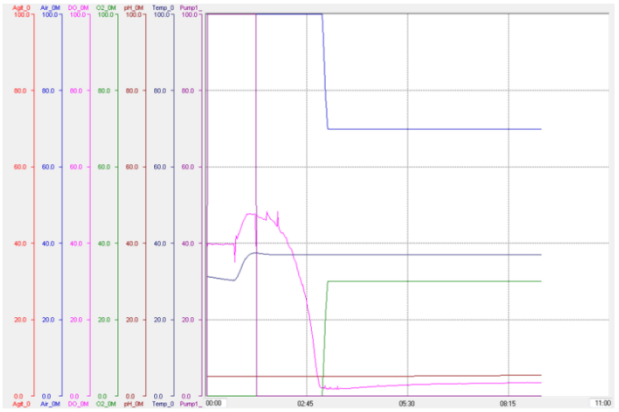

Fig 5 Physiological behaviour of *B. altitudinis* Kd1 (M) during xylanase production in a stirred tank bioreactor

IV. CONCLUSION

Members of the genus *Bacillus* produce a great variety of extracellular enzymes, of which xylanases are of significant industrial importance. Cellulase free xylanases are most sought after in paper and pulp industries in addition to food, animal feed, bioethanol production and pharmaceutical industries etc. The UV irradiation mutated strain of *Bacillus altitudinis* Kd1 (M) has shown a good stability until 8 generations thereby proving a significant role of mutation in strain improvement for enhanced enzyme production efficiency. *Bacillus altitudinis* Kd1 (M) showed an enhanced xylanase production upto 96.25 IU/ml at TGY media, 5.5 pH, 72 h of incubation period, 12.5% inoculum size, xylose as

carbon source @ 2.5% after xylanase production parameters were optimized by using OFAT approach. The scale up of the enzyme production in a stirred tank bioreactor with a working volume of 3 L has yielded 96.00 IU after a fermentation period of only 8h.

V. ACKNOWLEDGEMENTS

Authors acknowledge the financial help provided by DST GOI, New Delhi in the form of INSPIRE fellowship.

VI. REFERENCES

1. U. Gupta, and R. Kar, "Optimization and scale up of cellulase free endo-xylanase production by solid state fermentation on corn cob and by immobilized cells of a thermolerant bacterial isolates", Jordan J. Biol. Sci., vol. 1, pp. 129-134, 2008.
2. P. F. Omojasola, and O.P. Jilani, "Cellulase production by *Trichoderma longibracheatum*, *Aspergillus niger* and *Saccharomyces cerevisiae* cultured on waste materials from orange", Pak. J. Biol Sci**.,** vol. 11, pp. 2382-2388, 2008.
3. J.G.S. Mala, N.R. Kamini, and R. Puvanakrishnan, "Strain improvement of *Aspergillus niger* for enhanced lipase production". J. Gen. Appl. Microbiol., vol. 47, pp. 181-186, 2001.
4. X.E. Chen, X.B. Feng, and W.S. Xia, "Strain improvement and optimization of the media composition of chitosananse-producing fungus *Aspergillus* sp. CJ 22-326", Afr. J. Biotechnol., vol. 7, pp. 2501-2508, 2008.
5. V. H. Vu, T.A. Pham, K. Kim, "Fungal strain improvement for cellulase production using repeated and sequential mutagenesis", Microbiol., vol. 37, pp. 267-271, 2009.
6. M.S. Abdel-Aziz, F.N. Talkhan, M. Fadel, A.A. AbouZied, and A.S. Abdel-Razik, "Improvement of xylanase production from *Streptomyces pseudogriseolus* via UV mutagenesis", Aust. J. Basic Appl. Sci., vol. 5, pp. 1045-1050, 2011.
7. H. Xu, S. Jia, J. Liu, "Development of a mutant strain of *Bacillus subtilis* showing enhanced production of acetoin", Afr. J. Biotechnol., vol. 10, pp. 779-788, 2011.
8. J. Riviere, "Isolement of purification des bacteries cellulolytiques aerobies du sol. Annales de L"institute Pasteur, 253-258, 1961.
9. N. Nakamura, R. Yamada, S. Katahira, T. Tanaka, H. Fukuda, and A. Kondo, "Effective xylose/cellobiose cofermentation and ethanol production by xylose-assimilating *S. Cerevisiae* via expression of β- glucosidase on its cell surface", Enzyme Microbial Technol., vol. 43, pp. 233-236, 2008.
10. G.L. Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugars", Anal. Chem., vol. 31, pp. 426-428, 1959.
11. E.T. Reese, and M. Mandels, " Enzymatic hydrolysis of cellulose and its derivatives", *In*: Methods Carbohydrate Chemistry (ed. Whistler R L) 3rd edn., Academic Press, London, pp. 139-143, 1963.
12. K.R. Aneja, "Experiments in microbiology, plant pathology and biotechnology", 4th rev. ed. (New Delhi: New Age International (P) Limited Publishers), 2003
13. J. Kim, S. Hur, and J. Hong, "Purification and characterization of alkaline cellulase from a newly isolated alkalophilic *Bacillus* sp. HSH-810", Biotechnol. Letters**,** vol. 27, pp. 313-316, 2005.
14. T. Maniatis, E.F. Fritsch, and J. Sambrock, "Molecular cloning: a laboratory manual", (New York: Cold Spring Harbour Laboratory), 1982.
15. N. Sharma, K.L. Bansal, and B. Neopaney, "Comparison of forest waste degradation under solid state fermentation and submerged fermentation by co-culture of *Bacillus coagulans* and *Bacillus licheniformis*" Asian J. Microbiol. Biotechnol. Environ. Sci., vol.7, pp. 51-54, 2005.
16. H. Okoshi, K. Ozaki, S. Shikate, S. Oshoni Kawai, and S. Ito, "Purification and characterization of multiple carboxymethyl cellulases from *Bacillus* sp. KSM-522", Agric. Biol. Chem., vol. 54, pp. 83-89, 1990.
17. X. Li, and P. Gao, "CMC-liquefying enzyme, a low molecular mass initial cellulose-decomposing cellulase responsible for fragmentation from *Streptomyces* sp. LX", Appl. Microbiol., vol. 83, pp. 59-66, 1997.
18. A. Vyas, D. Vyas, and K.M. Vyas, "Production and optimization of cellulases on pretreated groundnut shell by *Aspergillus terreus* AV49", J. Scient. Indust. Research, vol. 64, pp. 281-286, 2005.
19. M. Devehand, and D.I. Gwynne, "Expression of heterologous proteins in *Aspergillus*", J. Biotechnol, vol. 17, pp. 3-9, 1991.
20. A. Chavan, D. Chougale, R.Y. Lakshmikantha, and S.P.R. Satwadi, "Mutational study of *Bacillus* species for production, purification and characterization of lipase", Int. J. Pharma. Chem. Biol. Sci., vol. 2, pp. 545-551, 2012.
21. J. Sambrook, and D.W. Rusell, "*In*: Molecular cloning- A laboratory manual", 3rd edn. (New York: Cold spring harbor laboratory press, Cok Spring Harbor), 1989.
22. B. Barsar, M.M Shamzi, M. Rosfarizan, N.N.T. Puspaningsih, and A.B. Ariff, "Enhanced production of thermophilic xylanase by recombinant *Escherichia coli* DH5α through optimization of medium and dissolved oxygen level", Int. J. Agric. Biol., vol. 12, pp. 321-328, 2010.
23. J.M. Willey, L.M. Sherwood, and C.J. Woolverton, *In:* Prescott, Harley and Kleins Microbiology 7th Edn. McGraw Hill Co. Inc., Boston, ISBN, 978-007-126727-4, 2008.
24. M.E. Flores, R. Perez, and C. Huitron, C, "β-xylosidase and xylanase characterization and production by *Sreptomyces* species CH-M-1035", Letters Appl. Microbiol., vol. 24, pp. 410-416, 1997.
25. M. Regina, F. Broetto, S. Giovannozzi, R. Marabatini, and C. Peranni, "Influence of stationary and bioreactor cultivation on *Lentinula edodes* (bark) Pegler lignocellulolytic activity", Braz. Arch. Biol. Technol., vol. 51, pp. 223-233, 2008.
26. J. Liu, and J. Yang, "Cellulase production by *Trichoderma koningii* A83.4262 in solid state fermentation using lignocellulosic waste from the vinegar industry", Food Technol. Biotechnol., vol 45, pp. 420-425, 2007.
27. R.R. Singhania, R.K. Sukumaran, and A. Pandey, "Improved cellulase production by *Trichoderma reesei*

RUTC30 under SSF through process optimization", Appl. Biochem. Biotechnol., vol. 142, pp. 60-70, 2007.

Authors Profile

Dr. Nisha Sharma is recently passed out PhD scholar of Microbiology section, Department of Basic Sciences, Dr Y S Parmar university of Horticulture and Forestry Nauni, Solan (H.P.) –India. She has published more than 9 publications in International and National journals and Proceedings.

Dr Nivedita Sharma is a Professor in Microbiology section, Department of Basic Sciences, Dr Y S Parmar university of Horticulture and Forestry Nauni, Solan (H.P.) –India. She has 20 years of teaching and research experience. She has published more than 50 publications in International and National journals and Proceedings. She is an active organizing committee member for seminars, conferences, workshops of the department and university.