Indian Journal of Experimental Biology
Vol. 54, August 2016, pp. 509-517

Improvement of *Bacillus* strains by mutation for overproduction of exopolygalacturonases

Hira Muzzamal & Zakia Latif*

Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore-54590, Pakistan

Received 12 June 2014; revised 01 April 2015

Pectinases, produced by microorganisms, have wide range application in food industry, textile processing, paper making, coffee and tea fermentation, etc. It accounts for 10% of the global industrial enzymes produced. The most important and widely used commercial pectinase polygalacturonase is produced by alkalophilic strains of *Bacillus* sp. and *Streptomyces* sp. Here, we explored 29 bacterial strains isolated from rotten mango samples for polygalacturonase production and selected 16 strains through preliminary screening by well-plate method for enzyme activity. The maximum zone of inhibition of pectin was observed up to 28 mm in diameter but one strain ZM11 was exhibiting no activity. Quantitative dinitrisalicylic acid (DNS) assay for polygalacturonase enzyme was also performed for the selected bacterial isolates. All the strains bestowed significant enzyme activity with the highest activity of 2.4 U/µL exhibited by strain ZM3 ($P \leq 0.05$). Characterization of the isolates was performed using different biochemical tests which also confirmed the isolates as members of the genus *Bacillus.* Mutation was induced to the selected strains by UV light and acridine orange for different periods of time. Qualitative and quantitative assays of the mutant bacterial isolates showed that the enzyme activity increased to 4.62 U/µL which clearly indicated that induced mutation enhanced the ability of *Bacillus* strains to produce more polygalacturonase enzyme up to 3-fold as compared to the wild strains ($P \leq 0.05$). Molecular characterization by 16S rRNA sequences further confirmed that the bacterial isolates belong to *Bacillus subtilis* and *B. amyloliquefaciens.*

Keywords: 16S rRNA sequencing, Acridine orange, Dinitrisalicylic acid, Pectinases, Polygalacturonase (PG) activity, UV mutagenesis

The living cells exclusively produce enzymes or biocatalysts and no life, in any form, could exist without enzymes. The enzymes hydrolyzing pectic substances are broadly known as pectinases or pectinolytic enzymes. Pectinases are a group of enzymes produced by variety of microorganisms that catalyze the breakdown of glycosidic bonds of the long chains of galacturonic acid residues of pectic substances, which are the structural polysaccharides of plant cells. Pectinases are polygalacturonase, pectin esterases and pectin lyases[1,2] according to their mode of action. The most important and widely used commercial pectinase is polygalacturonase. Pectinases have great significance in the current biotechnological area with wide range application in fruit processing industry (extraction and clarification of juice), vegetable oil extraction, processing of alcoholic beverages, a variety of applications in food industry, textile processing, paper making, coffee and tea fermentation, etc.[1-4]. Several microorganisms such as bacteria[5,6], yeast[7], fungi[3,4,8] and actinomycetes[9,10] are known capable of producing different kinds of pectin degrading enzymes. Pectinases account for 10% of the global industrial enzymes produced[11].

At present almost all pectinolytic enzymes used for industrial applications are produced by fungi. Most commercially available pectinases used in food industry are derived from *Aspergillus niger*. *A. niger* is generally recognized as a safe microorganism but is a slow growing fungus. Fungi and yeasts produce acidic polygalacturonases but bacteria produce mainly alkaline pectinases. The highest polygalacturonase production was reported by alkalophilis strains of *Bacillus* sp. and *Streptomyces* sp.[12]. Anuradha *et al.*[3], who explored various nutrients for polygalacturonase production by *Aspergillus awamori* MTCC 9166 using Plackett-Burman design showed that orange peel, casein hydrolase and NaCl to be the best pectin source, nitrogen source and mineral, respectively for maximum production. Wikiera *et al.*[13] achieved optimal polygalacturonase production efficiency with initial medium mass of 19.9 g and humidity 59.9% after 77.7 h of incubation at 28.9°C using *A. niger* 377-4. Dara *et al.*[4] advocated pomelo peels as substrate and use of shallow tray system for effective production using *A. niger* LFP-1. A study by Heerd *et al.*[14] showed that treatment of fungal strains by physical

*Correspondence:
E-mail: zakia.mmg@pu.edu.pk

and chemical mutagens enhanced production of polygalacturonase upto 2.4-fold as compared to their wild type strains. In another study, Sekar *et al.*[15] applied different auxins externally for enhanced production of polygalacturonase from paddy.

Wide application of pectinase enzymes in different industries and its successive increased demand encouraged researchers to develop techniques, including genetic makeup of the bacteria, for fast growing bacteria to boost mass production of pectinases. As a result, mutagenesis to develop hyper producing strains gained importance. Sources such as UV light and chemical mutagens were used to alter the genetic structure of the bacteria to enable increased production of the enzyme. Akbar *et al.*[16] used induced mutagenesis in *Aspergillus tamarii* for enhanced pectinase production. In this study, we used UV and acridine orange treated *Bacillus* strains for possible increased production of polygalacturonase.

Materials and Methods

Isolation and purification of bacterial strains

Rotten mango samples from local market of University of the Punjab, Lahore were collected and stored at 4°C in air tight bags. Three serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) from rotten mango pulp were prepared in LB broth[17] and 50 µL from each dilution was spread on LB agar plates. The inoculated plates were incubated in incubator set at 37°C. After 24 h inoculation, colonies with different morphological characteristics (e.g., colony shape, colony colour, and colony texture, etc.) were restreaked on new LB agar plates and incubated under the same conditions as before for further purification. All purified bacterial strains were subjected to preliminary screening of the pectinase production.

Preliminary screening of bacterial isolates for polygalacturonase (PG) activity

Preliminary screening of bacterial strains capable of producing polygalacturonase enzyme was done by well-plate method[15]. Broth culture was prepared by inoculating tubes of LB broth with 30 µL overnight starter cultures having optical density adjusted to 0.1 at 600 nm and incubated at 37°C on rotary shaker at 150 rpm. After 24 h incubation, the culture was centrifuged at 12000 rpm for 15 min to separate the cell mass and 50 µL cell free supernatant was added to each well made in the plates containing MS medium supplemented with 0.2% pectin (KH_2PO_4 3g/L, Na_2HPO_4 6g/L, NH_4Cl 2g/L, NaCl 5g/L, $MgSO_4.7H_2O$ 0.1g/L, Agar 15g/L**,** Pectin 2 g/L). The plates were left on the table for ½ h to diffuse the cultures through wells in medium and then the plates were incubated at 37°C for 18-24 h. Diameter of inhibition of pectin zone was measured in mm and recorded after flooding over the plates with iodine solution as it make the hallow zone more visible.

Quantification of polygalacturonase activity by DNS method

Selected bacterial strains capable of hydrolyzing pectin by well-plate method were further screened by quantification of pectinase by DNS (3,5-dinitri-salicylic acid) method using D-galacturonic acid for standard curve[18,19]. Bacterial isolates having relatively same number of cells were inoculated in 100 mL of LB broth medium and incubated at 37°C for 24 h on continuous shaking at 150 rpm. Bacterial cultures were centrifuged at 10000 rpm for 5 min to get cell free extract to quantify the polygalacturonase. In the tubes containing 0.5 mL of culture filtrates, 0.5 mL of 0.9% polygalacturonic acid prepared in 0.1M acetate buffer was added and incubated at 45°C for 30 min in water bath. About 1.5 mL of DNS solution (Sodium sulfite 0.05 g, NaOH 1 g, DNS 1 g in 100 mL distilled water) was added in the tube after cooling and boiled at 100°C for 10 min. A tube having only LB broth was taken as control. After cooling, optical density at 530 nm was observed and concentrations of enzyme units were quantified by standard curve.

Biochemical characterization of the selected bacterial isolates

Different biochemical tests were performed for biochemical characterization of selected bacterial isolates by following the methods of Gerhardt *et al.*, [17] and Cappucino & Sherman[20].

UV mutagenesis in bacteria

The mutagenesis of the bacterial culture was done adopting the method of Sudi *et al.*[21] with a little modified medium. The selected bacterial strains were grown overnight at 37°C in 5 mL of LB-broth and optical cell density was adjusted to 0.1 at 600 nm. The cells were harvested by centrifugation at 5000 rpm for 15 min and washed twice in cold, sterile 0.9% NaCl solution. Portions of cell suspensions (1.5 mL aliquots) was transferred to sterile Petri dishes and exposed under UV-light (254 nm) for 4 different periods (15, 30, 45 and 60 min). Each irradiated sample was centrifuged at 5000 rpm for 15 min and re-suspended in 1 mL LB-broth and incubated at 37°C for 3-4 h. Four dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of each culture were prepared in L-broth and 50 µL of

each dilution was spread on LB-agar plates. After incubation at 37°C for 24 h, morphologically different colonies from parental colonies were picked and restreaked on LB-agar plates.

Chemical (Acridine orange) mutagenesis

Bacterial cultures of selected strains were prepared by growing bacteria overnight in LB-broth in three flasks. Their optical density was adjusted to 0.1 at 600 nm. Acridine orange solution was added to each flask with the final concentrations of 0.5, 1.0 and 2.0 mg/mL, respectively under sterile conditions. The flasks were placed on shaker at 37°C at 150 rpm. After every 15, 30, 45 and 60 min, 1 mL was drawn out from each concentration and washed twice with LB-broth to remove the extra acridine orange particles by centrifugation. A control without acridine orange was also preceded. Final volume was made up to 1 mL with LB-broth medium and 50 µL from each treatment was spread on the LB-agar plates. After overnight incubation at 37°C, morphologically different colonies expected to be mutated were selected for further restreaking.

Statistical analysis

The data obtained from polygalacturonase activities of all strains were analyzed with 3 replicates and were expressed in mean±SD of each replicate. All the data were evaluated through statistical analysis by Analysis of variance (ANOVA) and means were compared by Duncan's Multiple Range Test (DMRT) at probability level 0.05 using IBMM SPSS Statistics 20 software.

Growth kinetics of selected bacterial strains (ZM1 and ZM1-15)

Growth kinetics of the selected bacterial strains was performed to check the growth patterns of the strains with the increase in incubation period. The highest polygalacturonase producing strain treated with UV light for 15 min (ZM1-U15) along with its wild type strain (ZM1) were selected for this study. LB broth (50 mL) was inoculated with overnight starter culture (50 µL) with optical density adjusted to 0.1 at 600 nm and incubated at 37°C at 150 rpm. Optical density was observed after every 4 h of incubation at 600 nm up to 48 h of incubation and graph was plotted to determine the growth pattern.

Molecular characterization of selected bacterial isolates (16S rRNA)

Bacterial isolates giving promising results for higher pectinolytic activity by well-plate method and polygalacturonase production after mutation with UV radiation and acridine orange were selected for molecular characterization through 16S rRNA sequencing. Genomic DNA isolation of the bacterial isolates was done following the protocol of Ausubel *et al.*[22]. Polymerase chain reaction (PCR) was performed using the universal 16S rRNA primers including forward Rs1:5-AAACTCAAATGAATTGACGG-3 and reverse Rs3:5-ACGGGCGGTGTGAC-3 Lane *et al.*[23]. PCR reaction mixture was prepared by adding 5 µL of 10X PCR buffer, 3 µL of 25 mM $MgCl_2$, 4 µL of 2mM dNTPs, 5 µL of 10 pM forward primer, 5 µL of 10 pM reverse primer, 1 µL Taq polymerase enzyme, 2 µL of DNA and 25 µL double distill water in a total reaction mixture of 50 µL. Amplification was performed in automated thermocycler Bioer xp cycler with the amplification profile as described by Awais & Rehman[24]. The amplified product was run on 1% agarose gel to confirm the amplification. Gene sequencing of the amplified product (0.5 kb) was done by the sequencing facility provided at Centre for Applied Molecular Biology (CAMB), Ministry of Science and Technology, Lahore, Pakistan. The sequence data obtained was analyzed using advance BLAST search program at the NCBI website: http://www.ncbi.nlm.mih.gov/BLAST/ and submitted to GenBank for accession numbers.

Results

Out of 32 bacterial strains having pectinolytic activity, 16 were identified as *Bacillus* (Table 1) by morphological and biochemical tests[17,20]. The names as ZM1, ZM2… ZM16 were assigned to the selected bacterial isolates and preserved in 30% glycerol stock at −80°C.

Qualitative and quantitative assays for polygalacturonase production by bacterial isolates

It was observed that all the selected bacterial strains showed varying levels of polygalacturonase activity in plate assay (Fig. 1) except one strain ZM11 that did not show any activity. Diameter of inhibition of pectin zone ranged 20-28 mm as the highest activity was shown by strains ZM15 and ZM16 producing zones of 28 mm diameter (Fig. 2A). In the quantitative assay of polygalacturonase activity, ennzyme units were calculated from the standard curve of D-galacturonic acid.[25]. One unit of poly-galacturonase is defined as the amount of enzyme that liberates one µmol of galacturonic acid per minute.

Table 1—Morphological and biochemical characterization of isolated bacterial strains

Strains	Shape	Spore	Catalase	Oxidase	Starch	Indole	MR	VP	Citrate	Identification
ZM1	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM2	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM3	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM4	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM5	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM6	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM7	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM8	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM9	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM10	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM11	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM12	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM13	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM14	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM15	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*
ZM16	Gm+Rods	+	+	-	+	-	-	-	-	*Bacillus*

Key: +, positive; -, negative; Gm, Gram; MR, Methyl Red test; and VP, Vogous Proskeur test

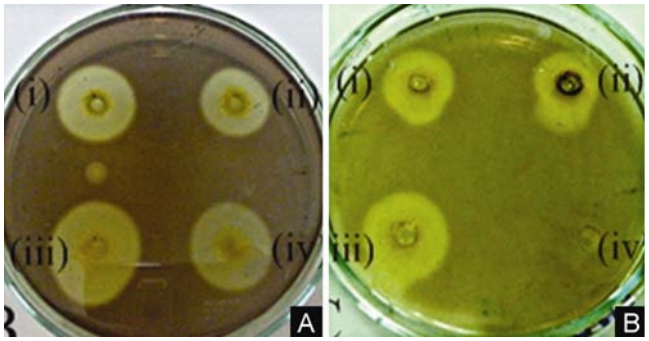

Fig. I—Qualitative assay (well plate assay) of isolated bacterial strains. A (i)-(iv), ZM1, ZM2, ZM3, ZM8; and B(i)-(iv), ZM6, ZM12, ZM13, and ZM11, respectively.

In the quantitative assay of polygalacturonase activity, the concentration of polygalacturonase ranged 0.93-2.4 U/µL. The highest concentration of 2.4 U/µL was shown by strain ZM3 while some bacterial strains also secrete valuable concentration of enzyme. The data is significant as analyzed by ANOVA and DMRT ($P \leq 0.05$). Zone of inhibition in diameter and enzyme production (U/µL) by bacterial strains are represented in Fig. 2 (A and B).

Mutagenesis and selection of mutants of Bacillus species

Seven *Bacillus* strains (ZM1, ZM2, ZM3, ZM4, ZM5, ZM6 and ZM11) were selected for UV treatment and only single strain (ZM1) for acridine orange treatment. UV and acridine orange treated *Bacillus* strains plated on LB agar plate were resulted in only 2-3 fast growing colonies per plate.

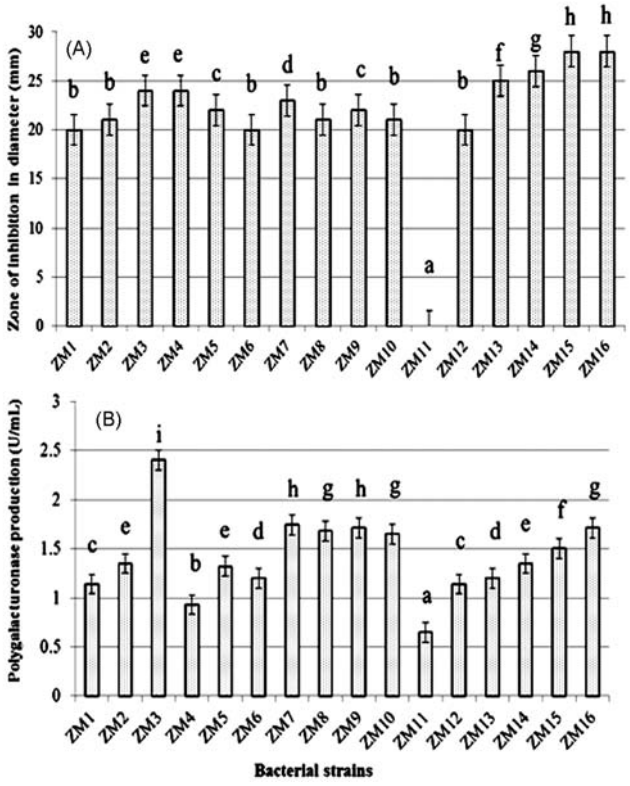

Fig. 2—Comparison of (A) zone of inhibition in diameter (mm); and (B) polygalacturonase production by different bacterial strains. [Bar represents mean ± SD of 3 replicates of each independent experiment. The P <0.05 was calculated by ANOVA and different letters indicate significant difference between means of each treatments calculated by Duncan's multiple range test (P=0.05)].

Qualitative and quantification assays of the mutant strains were performed for polygalacturonase activity. The protocols were same as done earlier with wild strains to check for the effect of the mutation to enhance the production of polygalacturonase. Since the exposure time for UV light was 15-60 min, the selected strains exhibited increased polygalacturonase activity as compared to their controls (wild types); but the results were variable.

The diameter of inhibition of pectin zone and polygalacturonase production varied 20-28 mm and 2.25-4.62 U/µL, respectively after mutagenesis with UV light. Effect of acridine orange on polygalacturonase production also varies in all bacterial strains exposed to different concentrations of acridine orange. Zone of inhibition in diameter (mm) of mutant strains showed increased polygalacturonase activity as compared to the parental strain (Fig. 3). Correlation between the zone size and enzyme production of mutant bacterial strains is represented in graphs (Fig. 4). Significance of the data was confirmed by ANOVA and DMRT ($P \leq 0.05$) at P = 0.05.

Enhancement of polygalacturonase production by ZM11

In case of bacterial strain ZM11, UV treatment significantly changed the ability for enzyme production as compared to parental strain. Wild type strain exhibiting no pectinolytic activity in well-plate assay synthesized only 0.65 U/µL of polygalacturonase. Very interesting findings were observed with ZM11 bacterial strain, supposedly non pectinolytic species. This strain showed more than 7-fold enhanced polygalacturonase production after mutation. The UV treatment enhanced enzyme production up to 4.50 U/µL at 30 min exposure and gave 28 mm of zone of inhibition in diameter in well-plate assay (Fig. 5). Again, the data was confirmed for significance by ANOVA and DMRT ($P \leq 0.05$). Correlation between zone size and enzyme concentrations of mutant strains are represented by graphs in Fig. 6.

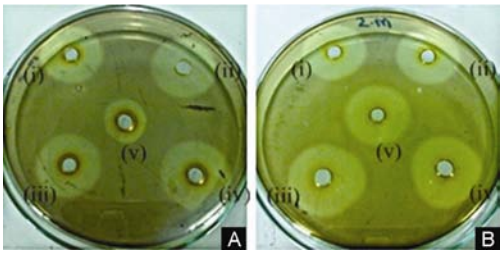

Fig. 3—Zone of inhibition of pectin in diameter (mm) by *Bacillus* strains (A) ZM2; and (B) ZM3 after UV light treatment for (i) 15 (U15), (ii) 30 (U30), (iii) 45 (U45), (iv) 60 min (U60); and (v) Control (non UV treated).

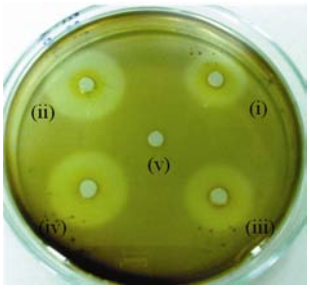

Fig. 5—Zone of inhibition of pectin in diameter (mm) by *Bacillus* strains ZM11 after UV light treatment for (i) 15 (U15), (ii) 30 (U30), (iii) 45 (U45), (iv) 60 min (U60); and (v) Control (not treated with UV).

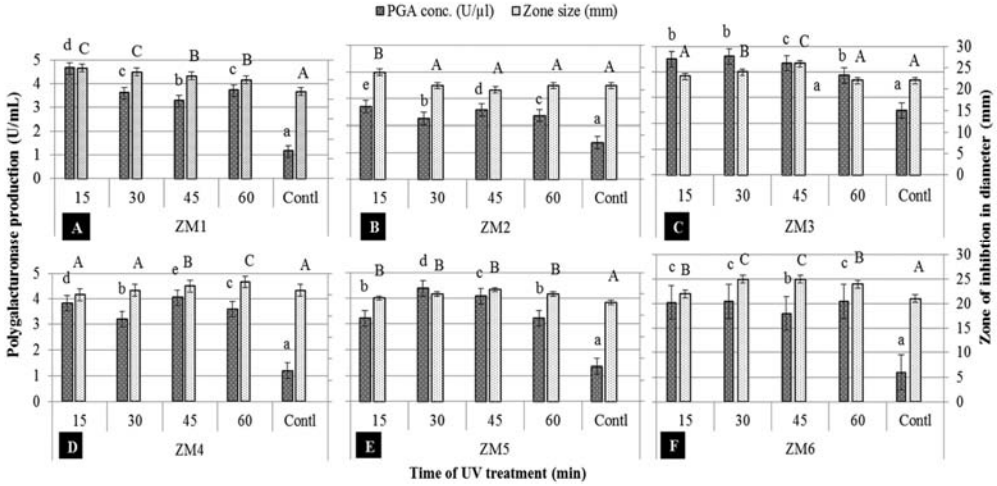

Fig. 4—Correlation between enzyme production and zone of inhibition in diameter by UV mutated bacterial strains. (A) ZM1, (B) ZM2, (C) ZM3, (D) ZM4, (E) ZM5 and (F) ZM6. [Bar represents mean ± SD of 3 replicates of each experiment and different letters on bars indicate significant difference between means of each treatment analyzed by DMRT ($P \leq 0.05$)]

Evaluation of ZM1 Bacillus strain treated with acridine orange

The bacterial strain treated with different concentrations of acridine orange showed similar behaviour as with UV light but the synthesis of polygalacturonase by the bacterial cells treated with acridine orange was less compared to the UV. Single bacterial strain ZM1 was selected for acridine orange mutagenesis on the basis of maximum size of inhibition zone in UV treatment. The behaviour of bacterial strains with different concentrations of acridine orange varied according to the treatment time (Fig. 7). Enhanced polygalacturonase production was observed with the acridine orange mutants exhibiting 3-fold increased activity as compared with the parental strains (Fig. 8). Comparatively, it was less than the UV mutants, which exhibited 4-fold increase. Fig. 8 represents correlation between zone of inhibition (mm) and enzyme production (U/µL) by acridine orange mutant bacterial strain ZM1.

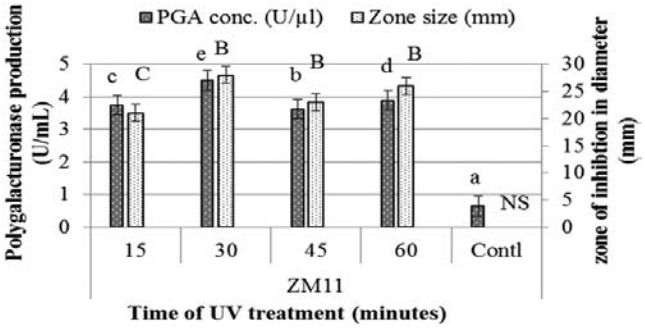

Fig. 6—Correlation between enzyme production and zone of inhibition in diameter by UV mutated bacterial strain ZM11 (increases in enzyme production). [Bar represents mean ± SD of 3 replicates of each treatment and different letters on bars indicate significant difference between means of each experiment by DMRT ($P \leq 0.05$). (NS= not significant)]

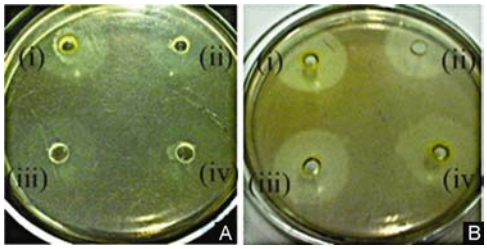

Fig. 7—Zone of inhibition of pectin in diameter (mm) by *Bacillus* strain ZM1 after treatment with different concentrations of acridine orange (A) 1.0 mg/mL; and (B) 2.0 mg/mL for (i) 15 (15), (ii) 30 (30), (iii) 45 (45), and (iv) 60 min (60).

Growth Kinetics of the selected bacterial isolates

Growth kinetics of mutant ZM1 (U15) and its wild type strain ZM1 was checked by observing optical density at 600 nm after every 4 h interval. The bacterial cell number was found increased in UV treated bacterial cells and was double as compared to the wild type strain; but the pattern of growth was almost same. There was increase in cell number up to 32 h of incubation for both the strains and then decreased with increasing time period (Fig. 9).

Molecular characterization of bacterial isolates (16S rRNA sequencing)

Seven bacterial isolates selected on the basis of qualitative and quantitative assays of polygalacturonase production were characterized through 16S rRNA

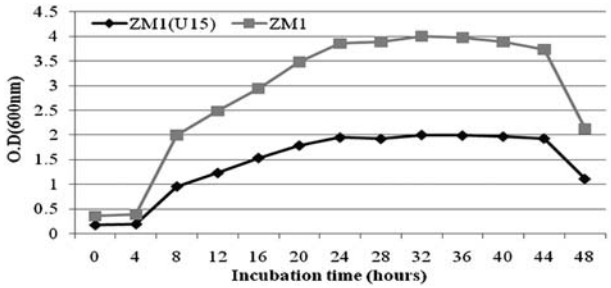

Fig. 9—Growth kinetics of *Bacillus* strain: (i) mutant ZM1 (U15); and (ii) wild type ZM1

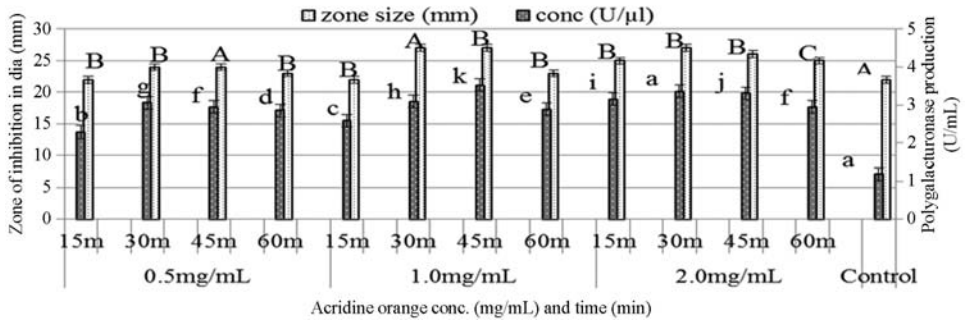

Fig. 8—Correlation between zone of inhibition and enzyme production by *Bacillus* strain ZM1 treated with different concentrations of acridine orange (mg/mL) for different time period (min=m). [Bar represents mean ± SD of three replicates of each experiment and different letters on bars indicate significant difference between means of each treatment by DMRT ($P \leq 0.05$)]

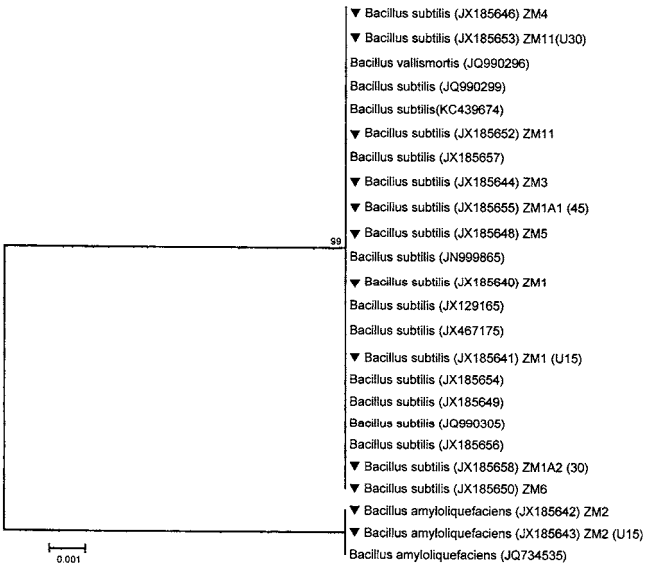

Fig. 10—Phylogenetic analysis showing the similarity with closely related members of *Bacillus*. [U15 and U30 =Bacterial strains exposed to UV light for 15 and 30 min, respectively. A1 (45) and A2 (30) = Treatment of bacterial strain with 1.0 and 2.0 mg/mL acridine orange for 45 and 30 min, respectively]

sequencing. The partially amplified (500 bp) 16S rRNA gene from local bacterial isolates was uploaded to the National Centre for Biotechnology Information website in order to search for similarity with the known sequences and to confirm the species of the isolated bacteria. The blast query revealed that the gene was 97-100% homologous to the already reported gene of *Bacillus subtilis* and 100% homologous to *Bacillus amyloliquefaciens*. It also revealed that random mutation did not change the most conserved region of the 16S rDNA region. Other close matches (Fig. 10) to *B. subtilis* (ZM1, ZM3, ZM4, ZM5, ZM6 and ZM11) included *B. subtilis* (JQ990299, KC439674, JX185657, etc.; 100% similarity; Fig. 10) and *B. vallismortis* (JQ990296; 100% similarity). Similarly, ZM2 (*Bacillus amyloliquefaciens*; JX185642) was found close to *Bacillus amyloliquefaciens* (JQ734535; 100% similarity). Phylogenetic analysis indicated (Fig. 10) the identity of the isolates as species of *Bacillus* and supported this grouping with high bootstrap value (0.001).

Discussion

Polygalacturonase enzymes hydrolysis the glycosidic bonds of polygalacturonan to give the end products α-1,4-D-galacturonide and galacturonic acid. Acidic polygalacturonase are produced by fungi and yeasts whereas alkaline pectinases are produce by bacteria at highest levels[9,12,26,27] which can be used in different industries[28].

In the present study, morphologically and biochemically characterized *Bacillus* strains capable of producing polygalacturonase enzyme were used to improve the strains to enhance the enzyme production by mutagenesis. Overproduction of pectinases by microorganisms is an important requisite because there is need to fulfill the increasing demand from different industries.

Selected strains of *Bacillus* were subjected to UV and acridine orange mutagenesis. To induce the mutations, cells were exposed to UV light for different time periods and different concentrations of acridine orange to observe the more effective conditions. General morphology of mutants indicated that the colonies of selected strains after mutation appeared to be larger than the wild type. The increase in colony size is either due to the change in growth pattern of bacterial strains or change in the genetic makeup that enables the bacterial strain to survive the harsh conditions like higher dose of UV light and higher concentrations of acridine orange. Although the exact mechanism of this growth pattern is unknown, still there is clear change in the morphology. Moreover, mutagens such as UV and acridine orange have lethal effect on bacteria but sublethal doses of mutagens can allow the bacteria to survive and resist the effect of mutagens. Maximum dose of UV has been reported as lethal along with its effect on production of other enzymes such as laccasesas[29].

Qualitative and quantitative assays of selected mutant strains were performed to determine the overproduction of pectinases by mutant strains. Although enhanced enzyme production was observed by all the bacterial strains exposed for different time periods to UV light and acridine orange, higher efficiency was observed in UV induced mutants. It was observed that the maximum zone of inhibition was 28 mm at 15 min of UV treatment exhibited by strain ZM1 and the highest concentration of enzyme was obtained at 15 min of UV treatment that was 4.7 U/μL of enzyme. A remarkable behaviour was observed in case of bacterial strain ZM11. In qualitative assay, ZM11 showed 0 mm zone of inhibition of pectin and only 0.65 U/μL poly-galacturonase was observed in wild type strain. Activity was enhanced by UV treatment for 30 min up to the level of 28 mm of zone of inhibition of pectin and 4.50 U/μL polygalacturonase.

All the data was evaluated statistically by ANOVA and DMRT using ($P \leq 0.05$) which confirmed that our data was significant. These results clearly showed that the ability of strain ZM11 to produce polygalacturonase enzyme has increased approximately up to 7-folds by UV induced mutation. Although the pattern of zone of inhibition of pectin and production of enzyme concentration were not in relationship to each other with respect to time period of UV treatment, still there is clear evidence that the UV treatment has augmented the ability to produce polygalacturonase enzyme in the strain that initially have little ability to produce enzyme. Our findings are strongly in consistent with observations of Koboyashi *et al*.[30] and Sandana-Mala *et al*.[31]. The findings of Heerd *et al.*[14] also indicated that the physical and chemical mutagens improved fungal culture for enhanced polygalacturonase production upto 1.7-fold in solid state culture. Similar behaviour was also observed in our study. When *Bacillus* strains were exposed to UV treatment their activity increased by 7-fold. Moreover, when ZM1 was exposed to acridine orange similar pattern was also observed but the quantified level of polygalacturonase was low.

Genetic development of bacterial strains for pectinase production using the methods of mutagenesis or protoplast fusion has also been successfully used previously. Cao *et al*.[32] used the value of P/C ratio (ratio of the diameter of zones of clearance [P] to the diameter of colonies [C]) on plates to differentiate hyperproducing mutants of pectinases. The use of rifampin to isolate natural mutants of the alkalophilic *Bacillus* sp. NTT33 allowed the obtaining of two mutants that produce polygalacturonase activities up to 82.4% higher than the maximum achieved for the wild type[12].

Growth kinetics of the wild type bacterial strain ZM1 and mutant ZM1 (U15) was also determined. It was seen that the bacterial cell number increased with the passage of time up to 32 h for both the strains and then decreased with increased time. Molecular characterization of 7 morphologically and biochemically characterized bacterial strains was done by 16S rRNA gene sequencing and identified as *Bacillus subtilis* and *B. amyloliquefaciens*.

Conclusion

The present study indicates that the *Bacillus* strains have great potential to improve its genome by mutagenesis for maximum production of polygalacturonase enzyme and can be used as a model for mutation to obtain the required target of overproducing enzyme. Use of these mutant bacteria on pilot scale can be definitely a great help on large scale production of polygalacturonase enzyme in meeting the demands of fruit and other industries.

References

1 Alkorta I, Gabirsu C, Lhama MJ & Serra JL, Industrial applications of pectic enzymes: a review. *Process Biochem*, 33 (1998) 21.

2 Abbasi IH & Fazaelipoor MH, Pectinase production in a defined medium using surface culture fermentation. *Int J Indust Chem*, 1 (2010) 5.

3 Anuradha, K, Padma, P Naga, Venkateshwar, S & Reddy, G, Selection of nutrients for polygalacturonase production by *Aspergillus awamori* MTCC 9166 using Plackett-Burman design. *Indian J Biotechnol*, 13 (2014) 502.

4 Darah I, Taufiq MMJ & Lim SH, Pectinase production by *Aspergillus niger* using pomelo peels as substrate: An optimization study using shallow tray system. *Indian J Biotechnol*, 14 (2015) 552.

5 Kapoor M, Beg QK, Bhushan B, Dadhich KS & Hoondal GS, Production and partial purification and characterization of a thermo-alkali stable polygalacturonase from *Bacillus* sp. MGcp-2. *Process Biochem*, 36 (2000) 467.

6 Jayani RS, Shuklaaxena SK & Gupta R, Screening of bacterial strains for polygalacturonase activity: Its production by *Bacillus sphaericus* (MTCC 7542). *Enz Res*, 2010 (2010) 1.

7 Pathan AAK, Bhadra B, Begum Z & N Shivaji S, Diversity of yeasts from puddles in the vicinity of Midre Lovenbreen Glacier, Arctic and bioprospecting for enzymes and fatty acids. *Curr Micribiol*, 60 (2010) 307.

8 Marcela A, Marcela R, Gally MC & Levin GL, Pectinase enzyme production by *Colletotrichum truncatum*, causal agent of soybean anthracnose. *Rev Iberoam Micol*, 27 (2010) 186.

9 Kuhad RC, Kapoor M & Rustagi R, Enhanced production of an alkaline pectinase from *Streptomyces* sp. RCK-SC by whole-cell immobilization and solid-state cultivation. *World J Microbiol Biotechnol*, 20 (2004) 257.

10 Saxena A, Upadhyay R & Kango N, Isolation and identification of actinomycetes for production of novel extracellular glutaminase free L-asparaginase. *Indian J Exp Biol*, 52 (2014), 786.

11 Stutzenberger F, Pectinase Production. In: *Encyclopedia of Microbiology*, 3rd ed, (Ed. J Lederberg; Academy Press, New York), 1992, 327.

12 Torres EF, Sepúlveda TV & González GV, Hydrolytic depolymerising pectinases: review. *Food Technol Biotechnol*, 44 (2006) 221.

13 Wikiera A, Mika M, Janiszewska AS & Zyla K, The optimization of some extracellular enzymes biosynthesis by *Aspergillus niger*. *J Sci Ind Res*, 74 (2015) 145.

14 Heerd D, Tari C & Fernández-Lahore M, Microbial strain improvement for enhanced polygalacturonase production by *Aspergillus sojae*. *Appl Microbiol Biotechnol*, 98 (2014) 7471.

15 Sekar C, Prasad NN & Sundaram MD, Enhancement of polygalacturanse activity during auxin induced *para*

nodulation and endorhizophere colonization of *Azospirillum* in rice roots. *Indian J Exp Biol*, 80 (2000) 80.

16 Akbar S, Prasuna RG & Khanam R, Strain improvement by induction of mutagenesis for hyper production of pectinase using *Aspergillus tamarii*. *J Sci Ind Res*, 74 (2015) 160.

17 Gerhardt GG, Murray RGF, Wood WA & Krieg NR, Methods for general and molecular bacteriology. (American Society for Microbial, 1325. Massachuesett Ave., N. E. Washington, DC), 1994.

18 Jayani RS, Saxena S & Gupta R, Microbial pectinolytic enzymes: A review. *Process Biochem*, 40 (2005) 2931.

19 Miller LG, Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem*, 31 (1959) 426.

20 Cappucino JG & Sherman N, Microbiology, A Laboratory Manual, 4th edn, (Rockland Community College, Suffern, New York) (2007).

21 Sudi IY, De N & Ali-Dunkrah U, Mutagenesis and selection of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* for potential use as starter culture. *J Amer Sci*, 4 (2008) 80.

22 Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA & Struhl K, Short Protocols in Molecular Biology, vol 1, 5th edn (2002), Publisher: Current Protocols.

23 Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML & Pace NR, Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci*, 88 (1985) 6955.

24 Awais S & Rehman A, Isolation of arsenite-oxidizing bacteria from industrial effluents and their potential use in wastewater treatment. *World J Microbiol Biotechnol*, 27 (2011) 2435.

25 Hannan A, Bajwa R & Latif Z, Status of *Aspergillus niger* strains for pectinases production potential. *Pak J Phytopathol*, 21 (2009) 77.

26 Li Z, Bai Z, Zhang B, Xie H, Hu Q, Hao C, Xue W & Zhang H, Newly isolated *Bacillus gibsonii* S-2 capable of using sugar beet pulp for alkaline pectinase production. *World J Microbiol Biotechnol*, 21 (2005) 1483.

27 Sharma DC & Satyanarayana T, A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcsr1 in submerged fermentation by using statistical methods. *Bioresource Technol*, 97 (2006) 727.

28 Hoondal GS, Tiwari RP, Tewari R, Dahiya N & Beg QK, Microbial alkaline pectinases and their industrial applications: a review. *Appl Microbiol Biotechnol*, 59 (2002) 409.

29 Khanum R & Prasuna G, Strain improvement of white rot fungi *Pycnoporus cinnabarinus* with the influence of physical and chemical mutagens for enhancing Laccases production. *J Sci Ind Res*, 73 (2014) 331.

30 Kobayashi TN, Higaki N, Suzumatsu A, Sawada K, Hagihara H, Kawai S & Ito S, Purification and properties of a highmolecular-weight, alkaline exopolygalacturonase from a strain of *Bacillus*. *Enzyme Microb Technol*, 29 (2001) 70.

31 Sandana-Mala JG, Numbi RK & Rengrarajulu P, Strain improvement of *Aspergillus niger* for enhanced lipase production. *J Gen Appl Microbiol*, 47 (2001) 181.

32 Cao JW, Sun WH, Pan Y & Chen SY, High-producers of polygalacturonase selected from mutants resistant to rifampin in alkalophilic *Bacillus* sp. NTT33. *Enz Microb Technol*, 27 (2000) 545.