

MOLECULAR CHARACTERIZATION OF POLYGALACTURONASE PRODUCING BACTERIAL STRAINS COLLECTED FROM DIFFERENT SOURCES

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ABSTRACT

Seventy-two bacterial strains were isolated from twenty various sources including soil, water, rotten fruits, vegetables etc. Preliminary screening for pectinase producing bacterial strains was done by well plate method and twenty-six bacterial strains gave zones on Minimum Salt (MS) medium supplemented with 0.2% pectin. Most of the isolates belonged to plant origin, either rotten fruits or vegetables due to high pectin presence as compared to other samples. Amongst the bacterial isolates, ZP-F5 and ZP-F6 gave the largest zone sizes i.e., 45 mm and 40 mm respectively. All selected strains were subjected to cellular, morphological and biochemical characterization. Polygalacturonase production by the selected strains was measured quantitatively by DNS method. Strain ZP-F5 and ZP-F6 were producing maximum amount of enzymes i.e., 1.85 U/ml and 1.86 U/ml respectively. Identification of pectinase producing bacteria was confirmed by amplification and sequencing of their 16S rDNA gene. The sequences obtained were BLAST which revealed that ZP-F5, ZP-F6, ZP-F14, ZP-F24 and ZP-F25 were found homologous to *Bacillus*. Whereas, ZP-F10, ZP-F16 and ZP-F18 were homologous to *Klebsiella variicola*, *Brevibacillus laterosporus*, *Ewingella americana* respectively. In the present study, other than *Bacillus* species some of the novel strains obtained like *Klebsiella variicola*, *Brevibacillus laterosporus*, *Ewingella americana*, *Providencia vermicola* and *Klebsiella oxytoca* demonstrated significant levels of pectinolytic enzymes capable of hydrolyzing pectin.

Key words. DNS method; *Bacillus*; Pectinase; Polygalacturonase; Pectin.

INTRODUCTION

The increasing demand of energy has now focused on the use of renewable energy resources like cellulose, starch, lignin, xylan and pectin due to their abundance throughout the environment. Most of the microbes which utilize them as their energy and carbon source have the tremendous potential for their production in various environmental conditions (Pedrolli *et al.*, 2009). The enzymes that catalyze pectic substances (fiber-shaped colloids) by degradation reactions through depolymerization (hydrolases and lyases) and de esterification (esterases) reactions are known as pectinolytic enzymes or pectinases. Various plants and microbes like bacteria, fungi and yeast are responsible for their production (Pires and Finardi-Filho, 2005). The enzyme production is primarily reported in various fungal species however, some bacterial strains have also been found capable of producing pectinases (Jayani *et al.*, 2010).

Significant improvement in the product yield from papaya, banana, pear and apple is reported due to use of pectinolytic enzymes along with other cell wall degrading enzymes like cellulases and hemicellulases (Soares *et al.*, 2001). Classification of Pectic enzymes is

based upon the mode of attack on their specific structural element of the pectin molecule (Benen *et al.*, 2002).

Fungi especially *Aspergillus niger* is widely involved in production of acid pectinases which are used in extraction, clarification and removal of pectin in fruit juices, in maceration of vegetables to produce pastes and purees and in wine making. The enzymatic reactions not only improve yields but also improve juice flavor, nutrition, texture and color (Sun and Cheng, 2002). Alkaline pectinases are generally produced by bacteria, particularly species of *Bacillus*, but are also made by some filamentous fungi and yeasts (Kapoor *et al.*, 2001). They may be used in the pretreatment of waste water from vegetable food processing that contains pectin residues; the processing of textile fibers such as flax, jute and hemp, coffee and tea fermentation, vegetable-oil extraction and the treatment of paper pulp (Zhang *et al.*, 2000).

The main thrust idea of the present study is concerned with the isolation and selection of over producing pectinases strains to fulfill the national demand. There is a dreadful need to uplift the beneficial microorganisms on new and novel lines to meet with the national needs required for different industries. For large scales, commercial production of pectinase enzymes, improvement of strains responsible for the production of pectinases enzyme is the only solution to overcome these

problems by new and novel techniques. These efforts will greatly increase the availability of enzymes for different industries. Improvement of strains by genetic engineering for enzymes production is the reliable solution to overcome these problems.

MATERIALS AND METHODS

Samples from different locations of the Lahore, Pakistan were collected that included soil, water, rotten fruits and vegetables, fermented yoghurt, fermented flour etc. which were serially diluted and spread on L-agar plate for 24 hours at 37°C to isolate bacterial colonies which were further purified on L-agar plates under the same conditions as described above.

Preliminary screening of purified bacterial isolates for pectin degradation was done by using the plate assay method as described by Hannan, *et al.*, (2009). The isolates were stabbed on MS medium supplemented with 0.2% pectin as a carbon source and incubated for 24 hours at 37°C. After incubation, plates were flooded with Iodine solution to detect the clear zones around the colonies which is indicative of positive pectinase activity.

Purified strains were further screened qualitatively for pectin depolymerization by “well plate assay” (Hannan *et al.* 2009; Janani *et al.*, 2011). The same MS medium petriplates with wells of 5 mm in diameter were filled with 25 µl of cell supernatant and incubated for 24 hours at 37°C which were further flooded with Iodine solution (Janani *et al.*, 2011). After incubation, strains giving maximum clear zones were selected and preserved as glycerol stocks at - 70°C (Hegler, *et al.* 2010).

Identification of Microorganisms: Selected strains with significant pectinolytic activity were characterized by determining their morphological (colony morphology as well as cell morphology) and biochemical characterization (Cappuccino and Sherman, 2007). Their identification was further confirmed by 16S rRNA ribotyping.

Protein Estimation by Bradford Assay (Quantitative analysis): A series of protein standards of known BSA concentration (0, 250, 500, 750 and 1500 µg/ml) were prepared to quantify the protein contents of the bacterial strains by the method of Bradford (1976). Graph was plotted for absorbance at 595 nm of the known protein of each standard which was further used to evaluate the unknown protein concentration made by each bacterial isolate.

QUANTITATIVE ANALYSIS OF PECTINASE ACTIVITY

Standard Curve of Galacturonic Acid: Different dilutions of D-galacturonic acid (0, 0.1, 0.2, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0 µM) were prepared in 3ml autoclaved distilled water. About 3 ml DNS reagent was added in each test tube containing dilutions. The mixture was boiled for 10 mins at 100°C on water bath and then cooled at room temperature. Absorbance was measured at 530 nm on spectrophotometer. A graph was plotted between different concentrations of D-galacturonic acid and absorbance. The slope of variable was calculated and used for the measurement of reducing sugars released by the action of pectinolytic enzymes.

Polygalacturonase Activity: Spectrometric method of Miller *et al.*, (1959) was used to assay the exo-polygalacturonase activity. Substrate solution (0.5% pectin) was prepared in autoclaved deionized water and its 100 µl was incubated with 100 µl of overnight cell free culture filtrate at 40°C for 1 hour under static conditions. DNS reagent of 400 µl was added in the mixture and then boiled for 15 minutes. Mixture was diluted to 5 ml with deionized water and change in absorbance as compare to control was measured at 530 nm. One unit of enzyme is equal to the amount of enzyme which catalyzes the formation of 1 µM of galacturonic acid.

16S rDNA sequencing: DNA was isolated from selected bacterial strains varying in polygalacturonase activity by CTAB (Cetyltrimethylammonium bromide) method (Ausubel *et al.*, 1994). 16S rDNA amplification was performed according to the method described by Hasnain and Thomas (1996). The DNA fragment of 16s rRNA gene was amplified using forward primer (5'-AAACTCAAATGAATTGACGG-3') and reverse primer (5' ACGGGCGGTGTGTAC-3').

The PCR products were purified manually (Sambrook and Russell, 2001) and sent to Centre for Advance Molecular Biology (CAMB), University of Punjab Lahore for sequence. Sequences were analyzed with the help of chromaPro version 1.34 (Technelysium Pty Ltd) and results were compared to other sequences in databases using the BLAST package at <http://www.ncbi.nlm.nih.gov/blast/>. MEGA6 software was used for multiple sequence alignment and phylogenetic tree was constructed for this alignment and submitted to GenBank.

Statistical Analysis: Standard errors of the mean were calculated via Statistical Package for the Social Sciences (SPSS) software version 16.

RESULTS AND DISCUSSION

The present study was aimed to isolate and characterize the indigenous microflora associated with natural environmental and food. Out of all sources processed, seventy-two bacterial isolates capable of degrading pectin substrate were obtained. Most of the sources belonged to plant origin such as rotten mango, apple, bitter gourd, guava, grapes, potato, cucumber, banana, oranges, lemons, tomato, strawberries, spoiled butter, dates, fermented yoghurt, and fermented flour. Whereas environmental samples were soil samples, sewage water and juice factory fluid.

Bacterial strains isolated from different natural sources were preliminary screened out by using pectin as a sole substrate in the minimal salt medium by the “cup-plate assay”. Among the total seventy-two bacterial isolates, only twenty six strains produced colorless hydrolytic zones on MS medium supplemented with 0.2% pectin after flooding with Iodine solution (Figure 1).

In this study zones of clearance showed the utilization and depolymerization of pectin by the pectinase activity. Therefore, among the total isolates 36% bacteria were found to exhibit pectinolytic activity (Figure 2).



Figure 1. Well plate assay for pectinase activity for the bacterial strains, ZP-F7, ZP-F8, ZP-F14, ZP-F15, ZP-F16, ZP-F17, ZP-F18, ZP-F19, ZP-F24, ZP-F25, ZP-F26, ZP-F29

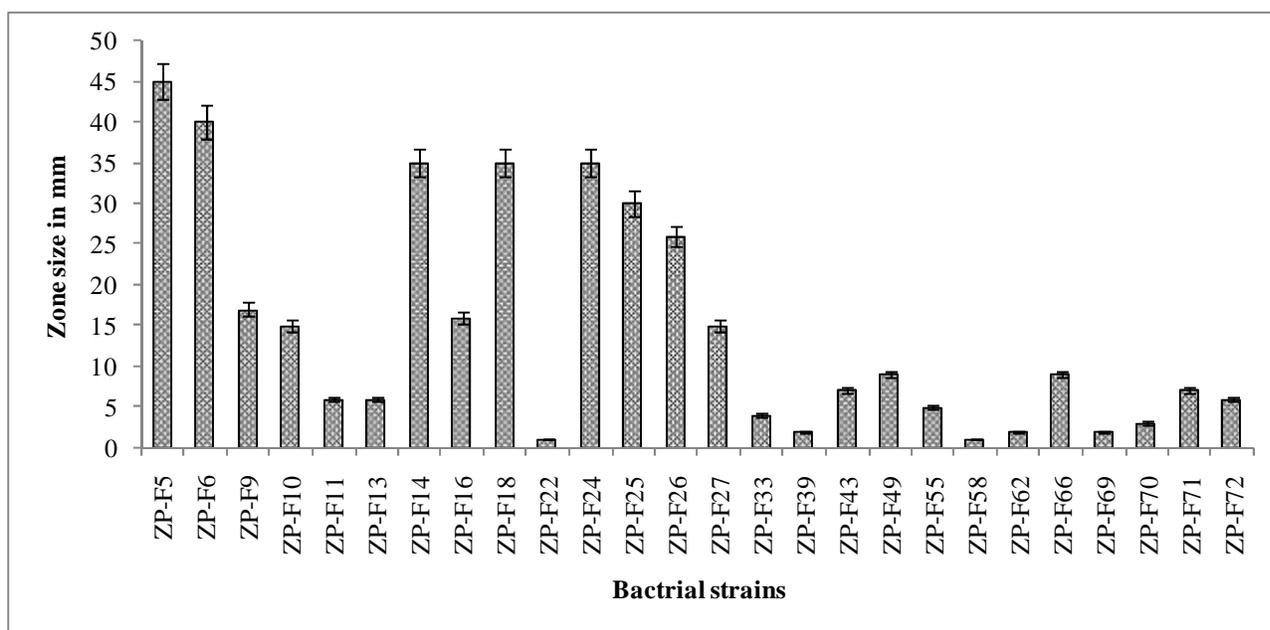


Figure 2. Qualitative assay to screen out bacterial strains by observing zone formation (mm) in well plate assay.

Pectin degrading bacterial isolates were further classified on the basis of their sizes of pectin depolymerization zones. Strains ZP-F5 and ZP-F6 exhibited maximum pectin degradative properties by

producing zones of 45 and 40 mm sizes respectively as shown in Figure 2. However, in other strains zones of pectinase activity were within the range of 1 to 40 mm depending on the production capabilities of the bacterial strains and the subsequent proliferation of the enzyme in medium were recorded.

Bacterial strains capable of hydrolyzing pectin were preliminary identified by their morphological and biochemical characteristics. Various aspects of colony morphology including colony color, shape, size, margins, elevation, texture and luminous behavior were recorded. Cell morphology of the selected strains was evaluated by Gram staining reaction which revealed the particular shape and type of bacterial cells. However, biochemical characteristics helped out to determine the metabolic behaviors of the bacterial strains in order to identify them. Morphological and biochemical characterization of the selected pectinolytic bacterial strains revealed that most of the isolates were *Bacillus sp.* the most potent pectin degrading bacteria present in the natural food as well as environmental sources. Other bacteria like *Exiguobacterium sp.*, *Klebsiella sp.*, *Providencia sp.*, *Ewingella sp.*, *Staphylococcus sp.* and *Corynebacterium sp.* were also identified.

Total protein content of these pectinase producing bacterial strains was estimated by Bradford assay. It revealed minimum protein concentration of 0.21 g/ml found in the isolates ZP-F22, ZP-F39 and ZP-F58. These isolates were found among those having minimum pectin degradative properties as indicated by the zone sizes. Whereas highly protein producer isolates, ZP-F5, ZP-F6, ZP-F14, ZP-F18, ZP-F24 and ZP-F25 were

recognized as maximum pectin degraders also indicated by their qualitative assessment (Figure 3).

Bacterial strains capable of pectin degradation were assessed quantitatively by DNS method in order to estimate the released reducing sugars as a result of polygalacturonase activity. Therefore, quantitative evaluation of the enzyme activity from the standard curve of galacturonic acid displayed the maximum enzyme activity among the two strains of *Bacillus i.e.*, ZP-F5 and ZP-F6 as shown in Figure 4. Both of these strains produced maximum quantities of polygalacturonase *i.e.*, 1.85 U/ml and 1.86 U/ml respectively.

Precise identification of the selected bacterial strains capable of high pectin degradation potential was done by conducting their genetic analysis of 16S rDNA sequencing. Molecular techniques are the most reliable, authentic and widely used methods to identify organisms in a single-step reaction. Eleven strains ZP-F5, ZP-F6, ZP-F9, ZP-F10, ZP-F14, ZP-F16, ZP-F18, ZP-F24, ZP-F25, ZP-F26 and ZP-F27 were processed for molecular identification and these strains were selected on the basis of their maximum enzyme activity confirmed by both "cup-plate assay" (qualitative method) and DNS method (quantitative assay). CTAB method was employed for DNA isolation (Ausubel *et al.*, 2002). This method was more helpful for maximum genomic DNA isolation than the other methods applied. Amplified and purified 16S rDNA fragments of 500 bp (Figure 5) were sequenced by the courtesy of CAMB, University of the Punjab, Lahore-Pakistan. Sequenced files were exported to NCBI for nucleotide BLAST which gave the maximum strain homology with the already submitted strains to the GenBank data.

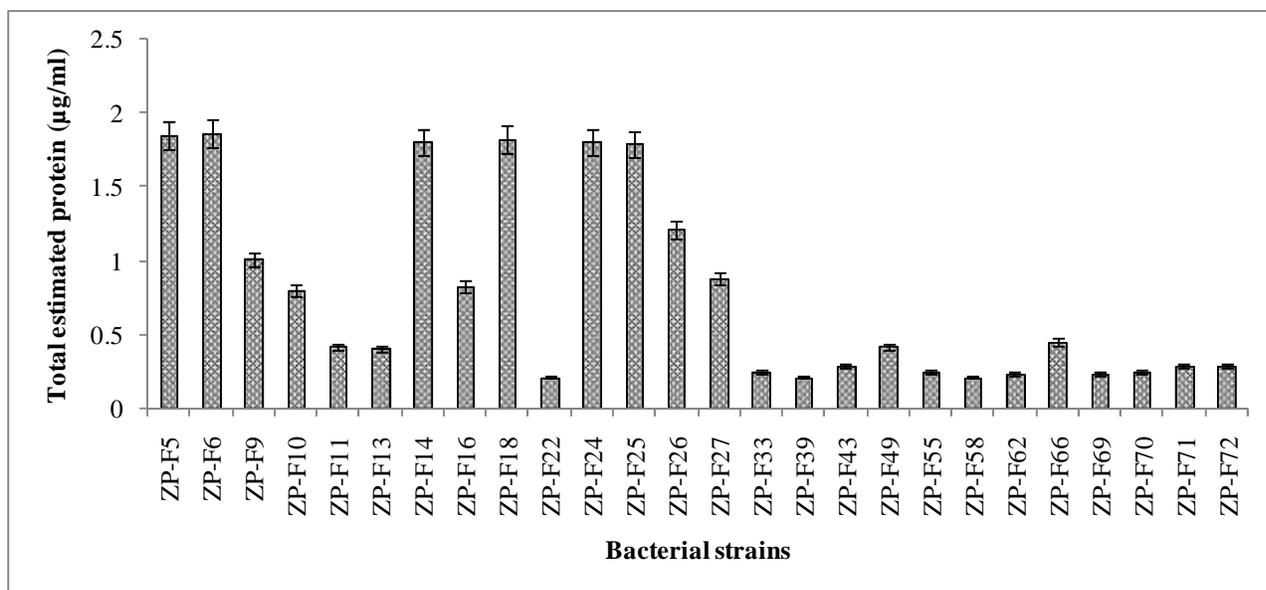


Figure 3. Total Protein estimation of selected bacterial strains. Error bars in the graph indicate standard error (SE).

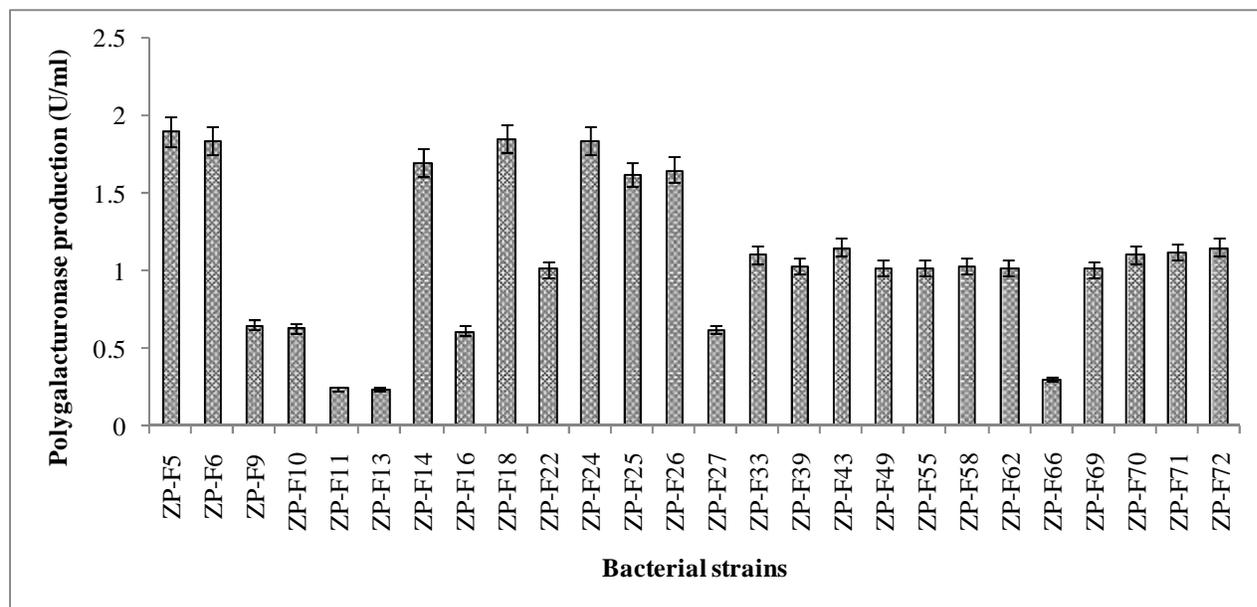


Figure 4. Polygalacturonase production (U/ml) by different strains of bacteria. Error bars in the graph indicate standard error (SE).

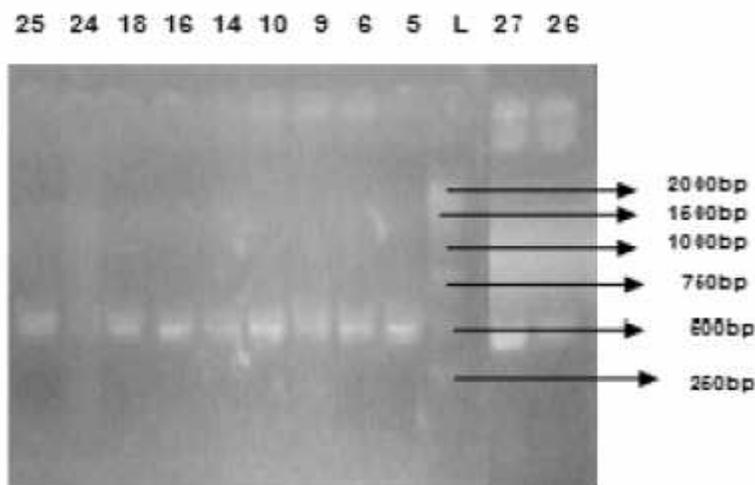


Figure 5. 16S rDNA fragments of the isolates (from right to left) ZP-F26, ZP-F27, 1kD DNA ladder (L), ZP-F5, ZP-F6, ZP-F9, ZP-F10, ZP-F14, ZP-F16, ZP-F18, ZP-F24, ZP-F25

16S rRNA gene sequence data of the reference strains with their accession numbers were taken from NCBI gene bank and phylogenetic tree for all the eleven bacterial strains was constructed by MEGA6 (Figure 6). The BLAST query revealed that the bacterial strains ZP-F5, ZP-F6, ZP-F14, Z-FK-24 and ZP-F25 were homologous to *Bacillus* sp. (KP886822.1, KT462731.3, KP670228.1 and KT462750.1) and *Bacillus subtilis* (KT580625.1, KP876486.1, KP941575.1). The bacterial strains also showed similarity in their 16S rDNA sequences among themselves. From the dendrogram, maximum similarities and differences between species were observed. Some of the selected strains were

homologous to more than one species level e.g., ZP-F9 displayed 99% sequence homology with *Exiguobacterium* sp. (KM058082.1) and 97% homology with *Bacillus subtilis* (Figure 6). ZP-F10 and ZP-F27 have 99% homology with *Klebsiella variicola* (KR558704.1) and *Klebsiella* sp. (KR232918.1 and KT327644.1). ZP-16 showed 99% sequence similarity with *Bravibacillus laterosporus* (JX129168.1 and KR919625.1). ZP-F18 displayed 99% homology not only with *Ewingella americana* (JX129169.1) but also with *Rehnelia* sp. (KT580643.1). Similarly ZP-F26 showed 98% sequence homology with *Providencia vermicola* (KP790056.1 and KP772083.1).

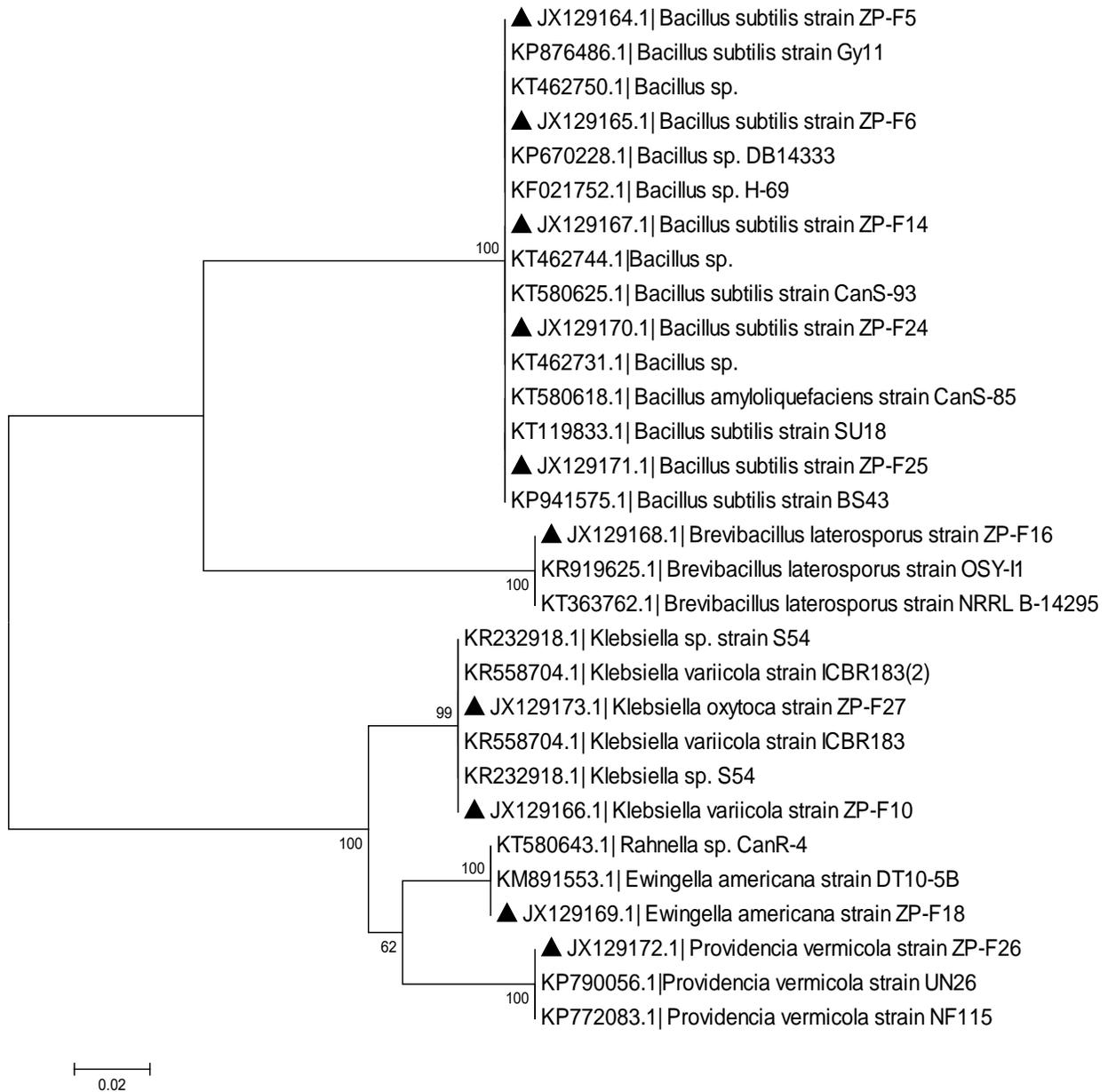


Figure 6. Constructed phylogenetic tree by MEGA6 software. The isolates ZP-F5, ZP-F6, ZP-F10, ZP-F14, ZP-F16, ZP-F18, ZP-F24, ZP-F25, ZP-F26 and ZP-F27 along with their bootstrap values are shown adjacent to the nodes that they support. Scale bar represents 0.02 changes per nucleotide.

Pectic enzymes are the major requirements in the fruit and vegetable juice manufacturing processes because pectin is the principle component of fruit and plant cell wall. Therefore, the present study indicated that the *Bacillus subtilis* strains ZP-F5 and ZP-F6 exhibited the significant pectinolytic activities among all microfloras extracted from the natural sources. Although, many other strains which were capable of producing pectinase were also isolated and are believed to be among the best sources of exopolysaccharidases (Kobayashi *et al.*, 2001). Therefore, both of the *Bacillus* strains along

with other natural producers of polygalacturonase may serve as significant enzyme producers in the industrial applications at commercial scale.

Conclusion: The present study indicates that the natural environmental and food sources play significant role in the production of polygalacturonases. Therefore, it was inferred that in addition to *Bacillus subtilis*, other bacterial strains like *Klebsiella variicola*, *Brevibacillus laterosporus*, *Ewingella americana*, *Klebsiella oxytoca* and *Providencia vermicola* also displayed characteristic

enzymatic activities. Use of these bacteria on large scale Polygalacturonase enzyme production will be helpful to fulfill the local demand of fruit and other industries. Production of enzyme on pilot scale will not only strengthen the economy of country by saving the foreign exchange but also promote the local strains to use in industry.

REFERENCES

- Ausubel F.M., R. Brant, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (1994). Current protocol in Molecular Biology. New York. John Willey and Sons Inc.
- Benen J.A.E., J.P. Vinklen and G.W.M. Alebeek (2002). Pectins and their manipulation Blackwell Publishing, Oxford. 174-180.
- Bradford M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72:248-254.
- Cappuccino J. and N. Sherman (2007). Microbiology: A Laboratory Manual, 7th ed. Pearson Education.
- Hannan A., R. Bajwa and Z. Latif (2009). Status of *Aspergillus niger* strains for pectinases production potential. Pakistan J. Phytopath. 21:77-82.
- Hasnain S. and C.M. Thomas (1996). Two related rolling circle replication plasmids from salt-tolerant bacteria. Plasmid. 36:191-199.
- Hegler F. and A. Kappler (2010). Cryopreservation of anoxygenic phototrophic Fe (II)-oxidizing bacteria. Cryobiology. 61:158-160.
- Janani L., G. Karthik, K.V. Kumar and B. Rao (2011). Screening of pectinase producing microorganisms from agricultural waste dump soil. A.J.B.P.R. 2:2231-2560.
- Jayani R.S., S.K. Shukla and R. Gupta (2010). Screening of bacterial strains for Polygalaturonase activity: its production by *Bacillus sphaericus* (MTCC 7542). Enzyme Res. 2010:1-5,doi: 10.4061/2010/306785.
- Kapoor M., Q.K. Beg, B. Bhushan, K. Singh, K.S. Dadhich and G.S. Hoondal (2001). Application of an Alkaline and thermostable polygalacturonase from *Bacillus* Sp. MG-cp-2 in degumming of ramie (*Boehemia nivea*) and sunn hemp (*Crotolaria juncea*) bast fibres. Process Biochem. 36:803-807.
- Kobayashi T., N. Higaki, A. Suzumatsu, K. Sawada, H. Hagihara, S. Kawai and S. Ito (2001). Purification and properties of a high molecular weight alkaline exo polygalacturonase from a strain of *Bacillus*. Enzyme Microb. Technol. 29:70-75.
- Miller G.H. (1959). Use of dinitrosalicylic acid reagent for determinatkon of reducing sugar. Anal. Chem. 31:426-429.
- Pedrollil D.B., A.C. Monteiro, E. Gome and E.C. Carmonal (2009). Pectin and pectinases: production, characterization and industrial application of microbial pectinolytic enzymes. Open Biotechnol. J. 3:9-18.
- Pires T.C.R. and F. Finardi-Filho (2005). Extraction and assay of peptic enzymes from Peruvian carrot (*Arracacia xanthoriza* Bancroft). Food Chem. 89:85-92.
- Sambrook J. and D.W. Russel (2001). Molecular Cloning: A Laboratory Manual, CSHL Press.
- Soares M.M.C.N., R. Da silva, E.C. Carmona and E. Gomes (2001). Pectinolytic enzyme production by *Bacillus species* and their potential application on juice extraction. J. Microbiol. Biotechnol. 17:79-82.
- Sun Y. and J. Cheng (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83:1-1.
- Zhang J., G. Henriksson and G. Johansson (2000). Polygalacturonase is the key component in the enzymatic retting of flax. J. Biotechnol. 81:85-89.