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# Isolation, Screening, and Molecular Identification of Pectinase Producing Microorganism

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***Abstract*** **- Pectinase is one of the most popular industrial enzymes, utilized in the major processes of clarification of fruit juices, textile processing and biofuel and food. Novel pectinase producing bacteria could be isolated using pectin-rich agro-industrial wastes like fruit and vegetable peels, which are cheap waste. Bacterial species were isolated from fruit and vegetable waste samples collected from Haldwani, Nainital district, India and screened for their pectinase activity by a pectin-agar clearance-zone assay. Eight isolates were cultured, of which six were pectinase positive. Isolate B1B that formed the largest hydrolysis zone (18 mm) and exhibited the highest pectinase activity (157 IU/GDS) was chosen for molecular characterization. After the 16S rRNA gene was amplified and sequenced, isolate B1B was determined to be *Bacillus cereus* strain SH-02 by BLAST search and phylogenetic tree construction. This strain was closely linked to the reference strains of *B. cereus* HB45, EM10, and Y3. The results presented here show that *Bacillus cereus* SH-02 is a new bacterium for use in industry as a pectinase producer and can be used as a validated strain in downstream fermentation and optimization studies.**

***Keywords - Pectinase; bacterial isolation; agro-industrial waste; gene sequencing of 16S rRNA; phylogenetic analysis***

## I. INTRODUCTION

The microbial enzymes are utilized in various food processing, pharmaceutical, textile, detergents, leather, waste management, biofuels, and agriculture industry and Pectinase has a prominent role among these enzymes and it is used in commercial food, textile, and beverage industries [1]. The use of pectinase simplifies the process, decreases the amount of chemicals needed, and can utilize agro-industrial wastes as a substrate, which decreases processing cost and waste.

A common and effective procedure used for bacterial isolation from agro-industrial wastes is to obtain strains able to produce pectinase. The bacteria can be obtained from rotten fruits, vegetable market waste, fruit peels, and soil of waste dumps and a pectin containing agar plate is used for primary screening, and the pectinase producers can be identified by the clear hydrolysis areas around the colonies [2]. The promising isolates are then subjected to biochemical and molecular characterization to further ascertain the pectinase activity and to determine the bacterial species. The 16S rRNA gene sequencing is the most commonly accepted method for bacterial species level identification and has been successfully employed to identify a number of pectinase producing bacteria, such as *Bacillus spp.* obtained from marine sediment and coffee pulp waste material [2], [3].

Therefore, the present study was conducted to isolate and screen certain bacteria isolated from fruit and vegetable waste for pectinolytic activity and to identify the most promising isolate using 16S rRNA gene sequencing and phylogenetic analysis and further validate the isolate for process optimization and fermentation studies.

## II. MATERIALS AND METHODS

### *A. Chemicals*

Culture media constituents, buffer components, mineral salts and organic solvents were purchased from HiMedia Laboratories (India), Sisco Research Laboratories Pvt. Ltd. (SRL, India) and Sigma-Aldrich Chemicals Private Limited (India).

### *B. Sample Collection*

To isolate the bacterial strains producing pectinase five different samples from different locations of Haldwani town in Nainital district of India were collected with fruit and vegetable wastes and agricultural waste. Waste samples of various fruit and vegetable wastes were collected in sterile sampling poly-bags and led to the Biochemical Engineering Department, B. T. K. I. T. Dwarahat, Almora, Uttarakhand, India for further analysis using nitrile gloves.

### *C. Screening and Isolation of Bacteria for Pectinase Production*

The samples of waste were serially diluted in saline (0.85%) and then transferred on pectinase screening agar medium (PSAM) which contains (g/L): $K_2HPO_4$ 1, KCl 0.5, $NaNO_3$ 2, $MgSO_4$ 0.5, tryptone 0.5, agar 20 and pectin 10. The medium was adjusted to pH 7.0 ± 0.5. Plates were then cultured for 24 h at 37 °C. The cultures were subsequently isolated by streak plate to obtain pure cultures which could be distinguished by colony morphology. The strains that were able to use pectin as only carbon source to grow on PSAM were selected as the positive cultures. These were subcultured and preserved at 4 °C on fresh slants using the same base medium with agar. The colonies were isolated on sterile PSAM plates and flooded with iodine solution, the presence of hydrolysis zone after iodine treatment suggested the presence of pectin degrading bacteria and the diameter of the clearance zone was noted for each isolate [4].



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*D. Quantitative Detection of Pectinase Produced by Bacteria*

The pectinase-positive isolates of bacteria were utilized for quantitative screening for pectinase production under submerged culture in Erlenmeyer flasks of 250 mL occupied with 50 mL PSAM media (without agar) at pH 7.0 ± 0.5. After inoculation with overnight grown culture at 1% level, the flasks were incubated at 200 rpm for 48 hours at 37 °C in an orbital shaker. After centrifuging the culture for 15 minutes at 10,000 rpm at 4 °C, the pectinase activity was measured in the clear culture supernatants.

The assay for pectinase was carried out using a dinitrosalicylic acid (DNS) reagent technique to quantify the amount of reducing sugars that the enzyme releases from pectin [5]. The culture supernatant (100 µL) was used to extract the enzyme. The quantity of pectinase required to generate 1 µmol of D-galacturonic acid in one minute under the prescribed environments was used to estimate the pectinase activity. The amount of D-galacturonic acid released in experiment was quantified by relating the amount to a calibration curve and expressed as IU/GDS (unit per gram dry substrate).

*E. Identification of Bacteria*

The bacterial strain that gave maximum zone of hydrolysis (hydrolysis test) and also maximum IU/GDS in (quantitative test) was chosen for further characterization. The Gram staining was employed to determine the morphology of the isolate for the first time. Genotypic characterization was completed at the Triyat Scientific Co., Nagpur, India and molecular identification was done by sequencing of 16S rRNA gene.

*F. Isolation of DNA and PCR Amplification*

The bacterial sample was taken in a microfuge tube of 2 mL comprising 1 mL of extraction buffer and homogenized in a mortar. After adding and thoroughly mixing an equal volume of chloroform: phenol: isoamyl alcohol (24:25:1), the mixture was spun in centrifuge at room temperature for 15 minutes at 14,000 rpm. The aqueous phase was collected, combined with an equal volume of isoamyl alcohol: chloroform (1:24), and centrifuged once more at 14,000 rpm for ten minutes. The DNA was then precipitated by combining the aqueous phase with 0.7 ml of isopropanol and 0.1 ml of 3 M sodium acetate of 7.0 pH. After a 15 minute incubation at ambient temperature, the samples were spun at 14,000 rpm in centrifuge for 15 min at 4 °C to pellet the DNA. The pellet was rinsed two times with 70% ethanol, given a quick final rinse with absolute ethanol, and left to air dry before being redissolved in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). Residual RNA was removed by treating the sample with 5 µL of 10 mg/mL RNase A (DNase-free), yielding highly pure genomic DNA. DNA integrity was evaluated by agarose gel electrophoresis, and measurement of concentration was performed using a NanoDrop Lite spectrophotometer.

*G. DNA Sequencing and subsequent Phylogenetic Analysis*

PCR was used to purify and amplify the genomic DNA using universal bacterial primers of the 16S rRNA gene. The amplified product was sequenced and the sequence was blasted against the nucleotide database of the NCBI to find the closest match. The sequences were aligned and used to generate a phylogenetic tree by Weighted Neighbor-Joining algorithm in the MEGA 12 software, which revealed the evolutionary relationship between the isolate and reference strains, with a bootstrap analysis to determine the robustness of the clustering pattern.

## III. RESULTS AND DISCUSSION

*A. Isolation and Screening of Bacteria based on Pectinolytic Activity*

The streak-plate approach was used to purify the bacterial isolates up to 8 streaks, and eight different strains were obtained, which were distinguishable by colony morphology. It can be seen from the Table 1 that the most pectinolytic activity was exhibited by isolate B1B (18 mm) followed by B2A (15 mm) and B1C (12 mm). Isolates B1A, B2B and B2D had a smaller hydrolysis zone than B1B and B2A, and B1D and B2C exhibited no pectinolytic activity. The results suggest that B1B and B2A are the best candidates for further quantitative pectinase testing and possible commercial or biotechnological application.

*B. Quantitative Analysis of Pectinase Production by Bacterial Isolates*

The six bacterial isolates that produced pectinase are shown in Fig. 1, which represents the quantitative amount of pectinase in total units per gram dry substrate (IU/GDS). B1B exhibited the highest pectinase activity (157 IU/GDS) among all the isolates analysed indicating considerable enzymatic potential. Moderate pectinase synthesis was observed for B2A (105 IU/GDS) and B1C (91 IU/GDS). The activity of B1A was lower at 84 IU/GDS, and B2B and B2D were the lowest at 56 and 60 IU/GDS, respectively. The differences in activity among the isolates are due to the difference in activity in the degradation of pectin, where isolate B1B is good candidate for further development and industrial application.

This agrees with other studies which have found that there is a significant relationship between hydrolysis-zone size and enzyme productivity. Of the eight isolates, six were pectinase positive (75%) which is indicative of the richness of the environment sampled and the effectiveness of the isolation method.

TABLE 1. Bacterial isolate screening for pectinase production (pectin-agar clearance-zone assay).

| S. No. | Isolate | Hydrolysis zone (mm) | S. No. | Isolate | Hydrolysis zone (mm) |
|---|---|---|---|---|---|
| 1 | B1A | 10 | 5 | B2A | 15 |
| 2 | B1B | 18 | 6 | B2B | 9 |
| 3 | B1C | 12 | 7 | B2C | 00 |
| 4 | B1D | 00 | 8 | B2D | 5 |



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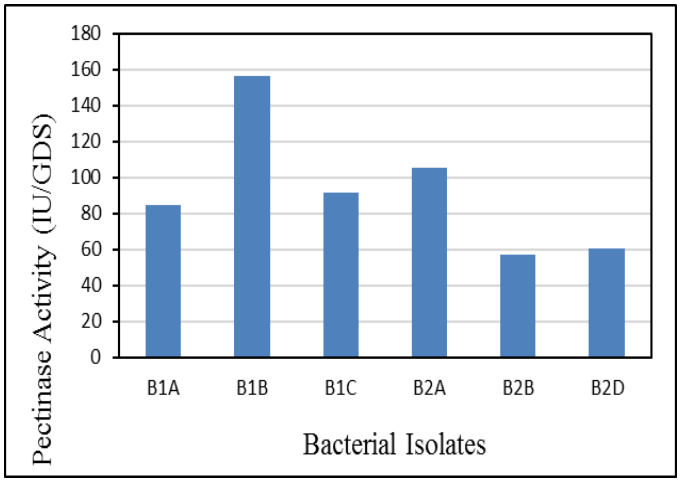



**Fig. 1.** Quantitative analysis of pectinase production from bacterial isolates (IU/GDS).

### *C. Molecular Characterization of the Bacterial Isolate With the Highest Pectinase Activity*

By using the bacterial genomic DNA to amplify the 16S rRNA gene, sequencing the PCR-amplified product, and performing a BLAST analysis of the 16S rDNA sequence at NCBI, the isolate B1B was determined to be *Bacillus cereus* strain SH-02, based on the combined molecular characterization and BLAST results. It is similar to the one used in microbial taxonomy where a compromise is found between the conserved and hypervariable regions of 16S rRNA gene to resolve species [6]–[9]. Other research has also focused on sequencing of 16S rRNA gene and phylogenetic examination to identify the microorganisms responsible for the pectinase activity found in the isolates. Senan et al. screened bacteria isolated from marine sediment for pectinase activity and identified a bacterium that produces pectinase as *Bacillus cereus* by phylogenetic investigation and 16S rRNA gene sequencing [3], while Oumer and Abate screened bacteria isolated from coffee-pulp for pectinase activity and used 16S rDNA sequencing to characterize the most active ones, finding that the most of the pectinase producing bacteria were members of the genus *Bacillus* [2]. These examples confirm the reliability of sequencing of 16S rDNA gene for pectinase producing bacterial identification.

### *D. Molecular Characterization of the Bacterial Isolate With the Highest Pectinase Activity*

Sequencing data from the gene of 16S rRNA of isolate B1B is a key molecular fingerprint to assign isolate B1B to its taxonomic position. The gene of 16S rRNA is a much conserved portion of the bacterial genome with both conserved and variable regions, which are used to differentiate closely related species. In this study, the entire amplified sequence was analyzed by BLAST and found to be highly similar to the sequence of *Bacillus cereus* strain SH-02.

A BLAST search of the gene sequence of the 16S rRNA showed the isolate B1B was very similar to other strains of *Bacillus cereus*. The highest similarity obtained was with *Bacillus cereus* strain SH-02 (Accession No. OM992297.1) and *Bacillus cereus* strain EM10 (Accession No. KJ612536.1), indicating a close genetic similarity, and several other strains such as HB45, Y3, 18 and mv50b showed similarity of 99.76%, reflecting the limited number of nucleotide differences between this taxonomic cluster. Interestingly, the same high level of conservation of the 16S rRNA gene was observed among several *Bacillus sp.* isolates (Pm-L4, PR65, ISL1 and 92) which had 99.76% identity. The conservation is in line with previous reports on the high level of sequence similarity of group members of *Bacillus cereus*, of the order of 99–100% for 16S rRNA, which may hinder discrimination at the subgenetic level and is typically accompanied by other genetic markers (e.g., gyrB, 23S rRNA) useful to distinguish the domain of lower taxonomic levels within the group [10]–[12].

The results of BLAST analysis are summarized in Table 2, in which the closest matching reference sequences have been isolated to determine the identity of B1B, which was *Bacillus cereus* strain SH-02 and the close similarity with reference strains HB45, EM10 and Y3.

### *E. Building Phylogenetic Trees*

Fig. 2 presents the phylogenetic tree obtained from the gene sequences of 16S rRNA of culture B1B and reference strains from GenBank. The tree also confirmed the identification of B1B, as it was found to be closely related with known *B. cereus* strains, such as HB45, EM10 and Y3. The clustering supports low genetic divergence, as expected for the *B. cereus* group recognized to have a well-defined taxonomic structure and a high bootstrap value. The resolution of B1B into an accepted phylogenetic grouping is essential for downstream applications like comparative enzymology, strain improvement and molecular markers to monitor industrial bioprocesses. *Bacillus cereus* strains with high genetic similarity to the reference strains and high enzymatic [2], [3], as demonstrated by the strong BLAST alignment scores, along with the corresponding phylogenetic clustering, indicate that isolate B1B is part of the *B. cereus* strain SH-02. This genetic similarity to other members of the *Bacillus* group that produce pectinases also indicates potential industrial applications for B1B, especially in bioprocesses involving valorization of pectin rich waste.

## IV. CONCLUSION

A novel bacterium which produces pectinase enzyme was successfully isolated, screened, and identified using molecular biology techniques from the discarded vegetables and fruits in the present study. All the seven pectinolytic bacterial isolates purified from waste samples were screened for pectinase production and best culture was recognized by sequencing of 16S rRNA gene and phylogenetic investigation as *Bacillus cereus* strain SH-02, similar to the reference strains of *B. cereus*, HB45, EM10 and Y3 with maximum hydrolysis zone of 18 mm and highest quantitative activity (157 IU/GDS).

The general conclusion from all these results indicates that *Bacillus cereus* SH-02 is a new strain of bacterium which has potential to be a useful bacterium for the enzyme pectinase production utilizing waste of agro-industries. The validated strain is the basis for further statistical optimization of fermentation medium and process parameters (described in a companion paper) and for further development of the enzyme purification, structural characterization and evaluation in target industrial applications like clarification of fruit juice and textile processing.



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**Table 2.** Closest BLAST matches for the sequence of 16S rRNA gene of isolate B1B

| S. No. | Organism / gene | Accession No. | % Match |
|---|---|---|---|
| 1 | *Bacillus cereus* strain SH-02 16S rRNA gene | OM992297.1 | 99.84% |
| 2 | *Bacillus cereus* strain EM10 16S rRNA gene | KJ612536.1 | 99.84% |
| 3 | *Bacillus cereus* strain HB45 16S rRNA gene | KC311341.1 | 99.76% |
| 4 | *Bacillus cereus* strain Y3 16S rRNA gene | MW205818.1 | 99.76% |
| 5 | *Bacillus* sp. strain Pm-L4 16S rRNA gene | MT533980.1 | 99.76% |
| 6 | *Bacillus* sp. strain PR65 16S rRNA gene | MN232164.1 | 99.76% |
| 7 | *Bacillus* sp. strain ISL1 16S rRNA gene | MK999907.1 | 99.76% |
| 8 | *Bacillus* sp. strain 92 16S rRNA gene | MH399247.1 | 99.76% |
| 9 | *Bacillus cereus* strain 18 16S rRNA gene | MH399235.1 | 99.76% |
| 10 | *Bacillus cereus* strain mv50b 16S rRNA gene | KU230017.1 | 99.76% |

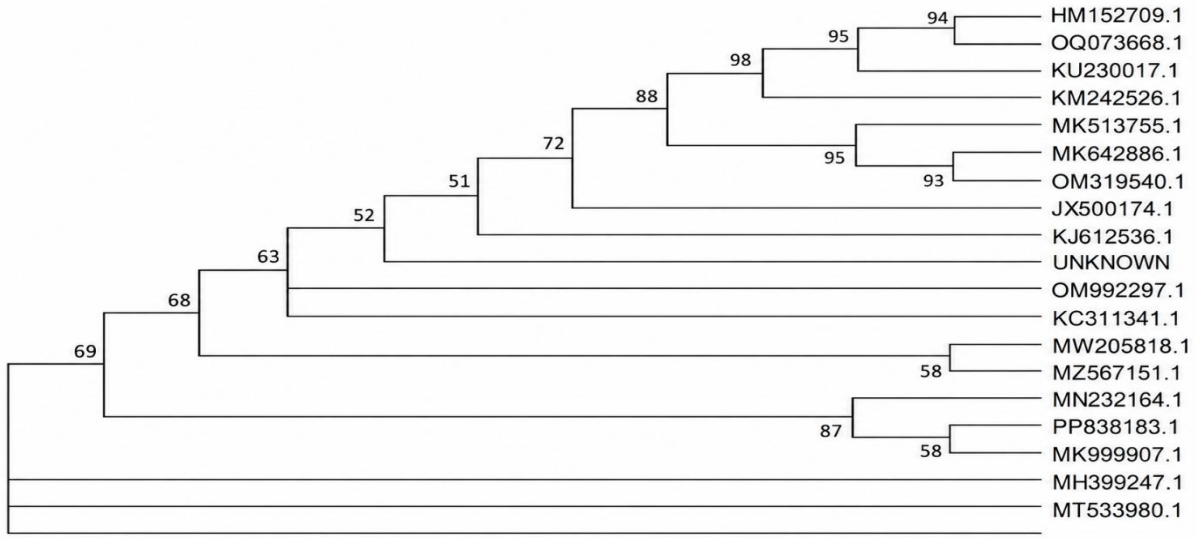



**Fig. 2.** Phylogenetic tree constructed (MEGA 12) for isolate B1B as unknown (*Bacillus cereus* strain SH-02).

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