

Characterization of Pectin lyase and Polygalacturonase from Novel *Bacillus cereus* GS-2 isolated from Chittoor and Vellore Fruit Industrial Dump Sites by SEM, 16SrRNA Sequencing, Ion-exchange, SDS and HPLC Analysis

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The current novel research work emphasis on the production and characterization of the pectin lyase (PL) and polygalacturonase (PG) from *Bacillus cereus* GS-2 by utilizing the agro-industrial pectin rich substrates i.e. orange, mango, apple, papaya peels. The morphological characterization of potential isolate was carried out with Scanning Electron Microscope (SEM) which shows the shape and morphology similar to *Bacillus sp.* Further, molecular identification was carried out using 16SrRNA sequencing and the sequence was successfully submitted in NCBI (GenBank) under the Accession No: KC571175. The characterization of pectic substrates i.e. fruit peels by Fourier Transform Infrared Spectroscopy (FTIR) revealed the presence of pectic acids, amine, carboxyl, carbonyl groups that assist in the pectinase enzyme production. Maximum pectin lyase and polygalacturonase activity of about 12.66 U mL⁻¹ and 11.00 U mL⁻¹ was achieved utilizing an orange peel as major carbon source compared with other sources. Peptone and Urea acts as best nitrogen source for maximum PL and PG activity. The enzyme was partially purified by using ammonium sulphate precipitation, the pectinase activity (28.22 U mL⁻¹). Further, it was purified by adopting novel technology like ion exchange chromatography, the yield significantly improved up to 2-fold (40.13 U mL⁻¹) and it was later analyzed by SDS-PAGE and High performance Liquid Chromatography (HPLC), the size of pectinase was found to be 36Kda. HPLC results show a retention time of about (1.93 min) compared to control (2.518 min). This study has proven that agricultural waste like orange peels could be used as promising substrate for both pectin lyase and polygalacturonase production from novel *Bacillus sp.*

Keywords: Pectin lyase, Polygalacturonase, *B.cereus*, pectin, fruit peels.

The utilization of renewable agricultural and industrial waste has become worldwide attention due to an increase in energy demand¹. The agro-industrial wastes like fruit peels consist of an enormous amount of pectin which functions as an inducer and as well as support, so it can be utilized as the substrate for the microbial pectinase production. Pectinases are bio-catalysts which are responsible for the breakdown of long and complex molecules of pectin which are present as structural polysaccharides in the primary

cell walls and middle lamella of young plant cells². Pectinases were broadly classified into polygalacturonase, pectin esterase and pectin lyase. Polygalacturonase (PG) acts on the pectin chain by adding a water molecule to break the linkage between two galacturonan molecules; Pectin lyase (PL) acts on the galacturonan chain by removing a water molecule from the linkage to break it and to release its products; whereas, Pectin esterase esterifies the pectin by removing methoxy esters³. In recent years, there has been more interest towards food processing wastes for microbial polygalacturonase production⁴⁻⁶. Pectin lyases are significant compared to that of PG and PE in capability to undergo β -elimination mechanism to

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degrade highly esterified pectins (present in fruits) into small molecules without producing methanol⁷. At present times, mango peels are discarded as waste and it is not commercially utilized by the industries. Thus, it causes pollution and its waste treatment becomes a cost-effective⁸. Apple pomace creates considerable problems in disposal in apple processing industries⁹. Orange baggase is a great agro-industrial byproduct from the citrus juice industries in Brazil. As, it is composed of seed, peel, pulp and it has been utilized as animal feed. The production of dried animal feed consumes enough amount of heat which is costlier than the selling price of the products. Citrus peel waste can be utilized for the production of galacturonic acid as it considered being a pectin-rich agro waste¹⁰. Pectinases were used largely in the food industry for the extraction and clarification of fruit and beverage juice. It is also used for different industrial processes like oil extraction, coffee and tea fermentation, treatment of wastewater and degumming fibre etc.,¹¹. Thus, strains which can yield high PL and PG by utilizing the cheap substrates have to be developed to minimize the production cost of an enzyme and in eco-friendly manner.

The pectinolytic sp. *Bacillus cereus* GS-2 isolated from fruit industrial dump site effectively produces pectin lyase (PL) and Polygalacturonase (PG). In this current study, fruit peels were employed as the major carbon source for the production of pectinase. The media composition and culture conditions have been optimized. We also purified the enzyme by ammonium sulphate precipitation and ion exchange chromatography. The molecular weight of an enzyme was determined by using SDS- PAGE. The purity of the enzyme was evaluated by high-performance liquid chromatography (HPLC). Hence, this study is the foremost to report the production and partial purification of PL and PG utilizing different fruit peels as substrate by novel *Bacillus* species isolated from fruit industrial dump site.

MATERIALS AND METHODS

Collection of soil sample

Soil samples were collected from different industrial dump sites from Chittoor, Andhra Pradesh, and fruit waste yard in Vellore district,

Tamilnadu India. The author states clearly that no specific permissions were required for these locations/activities. The latitude and longitude of the dump sites 13.0828°N, 79.1192°E, 13.2000°N, 79.1167°E have been provided. The authors were not legally authorized to remediate these areas. The authors confirmed that the field studies did not involve endangered or protected species.

Chemicals and media

All the chemicals used were of analytical grade and procured from Hi media Laboratories, Bombay, India. Polygalacturonic acid (PG) and d-galacturonic acid were supplied by Sigma, USA.

Culture conditions

Soil samples were serially diluted and culture were inoculated in 250mL flask containing a isolation medium: pectin 10 g L⁻¹, tryptone 3 g L⁻¹, yeast Extract 2 g L⁻¹, potassium chloride 0.5 g L⁻¹, magnesium sulphate heptahydrate 0.5 g L⁻¹, manganese sulphate 0.5 g L⁻¹, ammonium sulphate 2 g L⁻¹, copper sulphate pentahydrate 0.4 g L⁻¹, sodium molybdate 0.8 g L⁻¹, zinc sulphate 8 g L⁻¹, sodium borate 0.04 g L⁻¹. The flasks were incubated at 37°C on a rotary shaker (100 rev/min) for 24 h interval. The culture was centrifuged at 10000×g for 10mins at 4°C and cell-free supernatant was assayed for pectinase activity. The potential isolate GS-2 strain produced a pectinase enzyme of about (15.81 U mL⁻¹) was taken for further study.

Characterization and Identification of Potential Isolate

Scanning Electron Microscope (SEM)

The morphological characterization of potential isolate was carried out using Scanning Electron microscope (ZEISS EVO 18 RESEARCH) equipped with an Oxford X-ray probe operated using retro-disperse electrons under low vacuum conditions at 10 KV. Biochemical characteristics of potential isolate were identified by standard biochemical tests.

Molecular Identification of potential isolate:

The DNA was isolated using AMpureB Bacterial gDNA Mini Spin Kit. Amplification of the Internal Transcribed spacer region was done with the forward primer: 27F-5'AGAGTTTGATCC TGGCTCAG-3' and the reverse primer: 1492R-5'TACGGYTACCTTGTTACGACTT3'. Sanger di- deoxy sequencing method was followed in which the primer: 518F-5'CCAGCAGCCGCGGTAAT ACG3' and

800R-5'TACCAGGGTATCTAATCC3' was used for amplification process. A Bio-Edit Sequence Alignment Editor Version 7.1.3.0 program was run for the processing of sequence assembly. The homo-similarity search was performed using the online tools in NCBI BLAST (Link: <http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were aligned using the CLUSTAL W program¹². For the neighbor-joining analysis distances between the sequences were calculated using kimura's two-parameter model^{13, 14}. The sequence was successfully submitted to (Genbank) Nucleotide sequence database with Accession No: KC571175. The Phylogenetic tree was constructed by running CLUSTAL W program with all the rRNA sequences having the most similarity value C93% with the unknown sequence.

Gravimetric Method

The fruit peel powder was treated by gravimetric method¹⁵ to estimate the pectin concentration in fruit peels. The precipitate was then transferred onto a glass plate. Then, it was air-dried at 100°C and weighed.

FTIR analysis

The characterization of fruit peels were carried out by using FT-IR (Model: ALPHA-T, Bruker) spectroscopy. Total 32 no. of scans were performed where the dry precipitates were blended prior to the addition of 99% FT-IR grade potassium bromide (Sigma, USA). Commercial pectin (d-galacturonic acid, Sigma, USA) was used as a standard for the analysis.

Growth media

The potential pectinolytic strain *Bacillus cereus* GS-2 was inoculated into modified yeast extract pectin broth contains yeast extract 10.0 g L⁻¹; pectin 2.5 g L⁻¹ and instead of pectin, fruit peel powder after gravimetric and FTIR analysis were taken in 250mL (pH 7.0) conical flasks. The flasks were incubated at 37°C on a rotary shaker (100 rev/min) for 24 h interval. The culture was centrifuged at 10000×g for 10mins at 4°C and cell-free supernatant was assayed for pectinase activity and further investigations.

Nitrogen sources

Single Optimization study was carried out for the hyper production of PL and PG activity. Various nitrogen sources (urea, yeast extract, beef extract, peptone, ammonium chloride, ammonium nitrate; Himedia, India) were chosen. This study of

nitrogen sources was carried out with optimized RPM, temperature, pH, pectin concentration and incubation time.

Enzyme assays

Pectin lyase (PL) activity was assayed by conducting the enzymatic reaction carried out in the following conditions 40°C; 6pH; length of light is 1cm and optical density at 235nm. Two eppendorf tubes were named as blank and test. In both tubes, 20µl of phosphate buffer (pH 6) and 180 µL of pectin (5%) was taken. To the blank 50 µL of (1%) BSA and 50 µL of freshly prepared culture filtrate to the test were taken followed by 5mins incubation and absorbance was measured. The Unit (U) is the term used to express enzyme activity. One unit of enzyme activity is defined as the quantity of enzyme that catalyzes the release of 1µmol of product per unit volume of culture filtrate per unit time at standard assay conditions¹⁶.

$$\text{Activity } U/ml = \frac{(\text{Absorbance of Test} - \text{Absorbance of blank})(2.5)(\text{Dilution factor})}{(5)(\text{Volume of enzyme used})}$$

From, the above formula 2.5 refers to the total volume used in the assay in mL, 5 refers change in absorbance per minute.

$$\text{Activity } (U) = \left(\frac{\text{absorbance}}{212.2} \right) \times (1000) \times \left(\frac{1}{20} \right) \times \left(\frac{1}{0.086} \right)$$

Polygalacturonase (PG) activity was assayed by the procedure followed by¹⁷. In this, instead of pectin, Polygalacturonic acid (PG) was used as substrate (2.4 g/L, 6.6 pH and 26°C). The amount of enzyme and substrate 0.086 and 0.4 cm³ were used. The PG activity was calculated as:

Protein concentration

Bovine serum albumin was taken as the standard and the protein concentration was determined by the Lowry method¹⁸.

Partial purification of pectinase

Ammonium sulphate precipitation

Pectinase from *Bacillus cereus* GS-2 was purified from 1000mL of pectin enriched broth of growth conditions (37°C, 12 hrs). The cell-free supernatant was saturated with (NH₄)₂SO₄ to cut offs (0±55%). The precipitates were dissolved and it was dialyzed against Tris-Hcl buffer (0.01M, pH 7.5).

Ion exchange chromatography

DEAE - Sephacel (anion exchanger) was packed into a glass column (20× 0.75cm, 15mL-bed volume). The column was equilibrated with Tris-Hcl buffer of (10mM, pH 7.5) and 4mL of sample was loaded on to the column. The column

was repeatedly washed with same buffer containing 0, 50, 100 and 150mM NaCl concentrations. 2.5mL volume of fractions was collected. The protein content and pectinase activity were measured for all the fractions spectrophotometrically at 280nm and 235nm followed by the same method as described earlier. The fractions showing higher pectinase activity were pooled, concentrated and saved for further purification processes.

SDS- Polyacrylamide gel electrophoresis (PAGE)

2% SDS-PAGE was performed to determine the molecular weight of partially purified pectinase-positive sample by the method followed by ¹⁹ using Bio-Rad electrophoresis apparatus. The gel was constantly run on a voltage of 50V.

HPLC

The purified pectinase which is recovered during different stages of purification was determined by HPLC (Waters 1525) equipped with C18 column with rheodyne manual injector and Waters 2487 dual λ absorbance detector with following conditions: flow rate of 1mL/min⁻¹ ;

temperature 50°C, acetonitrile and water were used as solvent in the ratio of 70:30 was measured with Perkin-Elmer model (Waltham, MA, United States of America).The retention time for each signal was recorded at a wavelength of 280nm and data was processed with Empower software (developed by waters).The commercial pectinase was used as standard. The standard peak and sample peak were analyzed.

RESULTS AND DISCUSSIONS

Our initial objective was to isolate an efficient pectinolytic isolate. However, production efficiency of *Bacillus cereus* GS-2 (15.81 U mL⁻¹) is more comparable to other *Bacillus* strains seen in earlier observations made by Klug ²⁰.The potential isolate was characterized and identified. Fig.1 shows the SEM images of potential isolate *Bacillus cereus* GS-2 at different magnifications: a) 10,000 x and b) 20,000 x. The images displayed the shape and morphological typical of *Bacillus*. Table.1 shows the morphological and biochemical characteristics of the isolate.

Table 1. Morphological and Biochemical Characteristics of the isolated *Bacillus cereus* GS-2

Test	Results	Test	Results
Morphological tests			
Margin	Entire	Elevation	Raised
Pigment	-	Opacity	Opaque
Surface	Flat	Gram's reaction	+
Size (μ m)	2-3	Shape	Rods
Motility	+		
Biochemical tests			
Methyl reductase test	+	Oxidase	-
Voges Proskauer test	-	Casein hydrolysis	+
Citrate Utilisation test	+	Urea hydrolysis	+
Catalase test	+	Nitrate reduction	+
Lactose	+	H ₂ S Formation	-

Table 2. Pectin Concentration of different fruit peels after Gravimetric analysis

S.No.	Fruit and Vegetable peels	Pectin Concentration (%)
1	Apple	35.0
2	Orange	24.0
3	Papaya	25.0
4	Mango	18.6

Based on the combined sequence of Internal Transcribed spacer (ITS) region and domain of rRNA gene, unknown strain (GS-2) has near same distance with *Bacillus cereus*. So, it was identified and named as *Bacillus cereus* GS-2 (Fig.2). The nucleotide sequence of *Bacillus cereus* GS-2 was successfully submitted to GENBANK with an accession no. KC571175.

Moreover, our main objective of this study was to produce pectin lyase (PL) and Polygalacturonase (PG) utilizing the cheap raw pectin substrates like fruit peels as a major carbon source. The pectin concentration of fruit peels were estimated using Gravimetric method followed by ²¹. Fruit peels were taken as a substrate for pectin lyase and polygalacturonase activity. These peels were considered to be carbon source for the enzyme production as it contains pectin. Pectin concentration was estimated and results were

shown in table 2. The structural characterization of pectin present in the fruit peels was done by IR spectroscopy. The functional groups present in the pectin exist in fruit peels were characterized and the results were shown in the (Fig.3a) Commercial pectin, (Fig.3b) papaya, (Fig.3c) orange, (Fig.3d) apple, (Fig. 3e) mango respectively. The major absorption peak at 3315.63 cm⁻¹, 3356.14 cm⁻¹, 3334.92 cm⁻¹ and 3332.99 cm⁻¹ of commercial pectin are attributed to stretching of hydroxyl groups mainly due to pectic acid and phenols. And, C=H stretching peak at 2941.44 cm⁻¹, 2939.52 cm⁻¹ and 2941.44 cm⁻¹ corresponds to O=CH₃ which is methyl ester of galacturonic acid. The C-H stretching peaks are absent in papaya and mango. The stretching peak at 1602.85 cm⁻¹, 1608.63 cm⁻¹, 1631.78 cm⁻¹, 1604.73 cm⁻¹ and 1658.78 cm⁻¹ of commercial pectin are due to the C=O stretching vibration of the ionic carboxyl groups^{22,23}. The

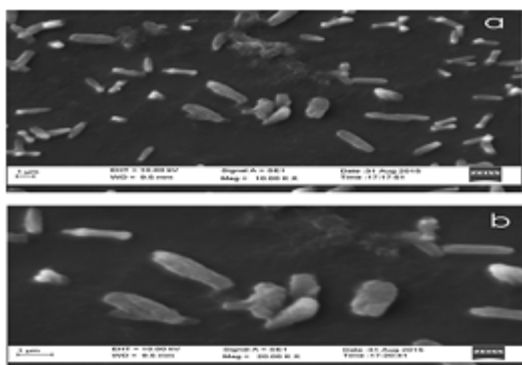


Fig. 1. SEM images of potent strain *Bacillus cereus* GS-2 at different magnifications (a) 10,000 x (b) 20,000 x

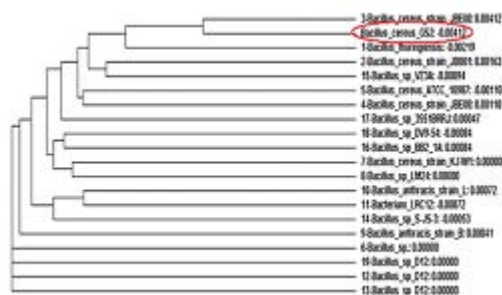


Fig. 2. Neighbor- joining tree of *Bacillus cereus* GS2 strain and related species for the Phylogenetic analysis. Evolutionary distances were calculated according to kimura (1980)

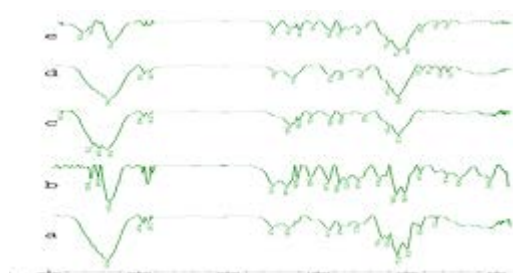


Fig. 3. FTIR transmittance spectra of (a) Standard pectin, Sigma, USA, Pectin obtained from (b) Papaya peel, (c) Orange peel (d) apple pomace and (e) Mango peel

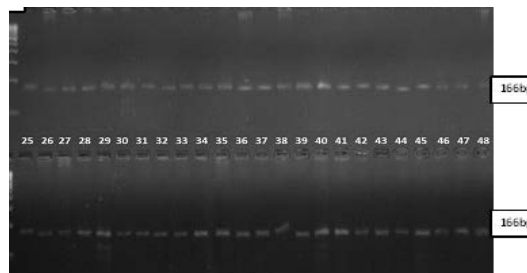


Fig. 4. Pectin Lyase activity on different substrates

asymmetric stretching vibration also present in around $1400-1450\text{ cm}^{-1}$ corresponds to the COO-group respectively²⁴. The weaker C=O stretching vibration absorption peak at 1741.72 cm^{-1} , 1737.86 cm^{-1} , 1745.58 cm^{-1} and 1747.51 cm^{-1} of commercial pectin corresponds to methyl esterified carboxyl groups which may be considered to be low methoxyl pectin²⁵. The C=O and C=C vibration were observed around $1000-1200\text{ cm}^{-1}$, were due to bands of glycosidic bonds and pyrenoid ring²⁶. Moreover, the FTIR spectrum appears in the range around $800-1300\text{ cm}^{-1}$, is considered as 'fingerprint' region of C-O-C stretching, OH bending and CH_3 plane deformation^{27,28}. Hence, the above characteristic peaks powerfully confirm the existence of pectin in the fruit peels and compared with commercial pectin. IR spectra at 3300 cm^{-1} confirm the presence of pectic acid.

The different pectin enriched substrates namely apple, orange, papaya, mango peels were selected to observe pectinlyase activity. Maximum

pectinlyase activity was observed in commercial pectin (22.02 U mL^{-1}) and it is compared with other substrates. From the (Fig. 4), we can infer that Orange peels shows more PL activity of about (12.66 U mL^{-1}) and apple peels shows PL activity of about (11.66 U mL^{-1}) Whereas, papaya peels shows minimum PL activity of about (9.33 U mL^{-1}). The same pectin enriched substrates were selected for polygalacturonase (PG) activity. Again, maximum PG activity was observed in commercial pectin of about (21.12 U mL^{-1}) and it is compared with other substrates. From the (Fig.5) maximum PG activity was observed in orange peel of about (11.00 U mL^{-1}) followed by mango peels of about (10.66 U mL^{-1}). The apple peels shows minimum PG activity of about (9.00 U mL^{-1}). The result may be due to the presence of an abundant amount of pectin present in the orange peel of about 14.4 ± 0.3 (w/w) was observed with earlier observations²⁹.

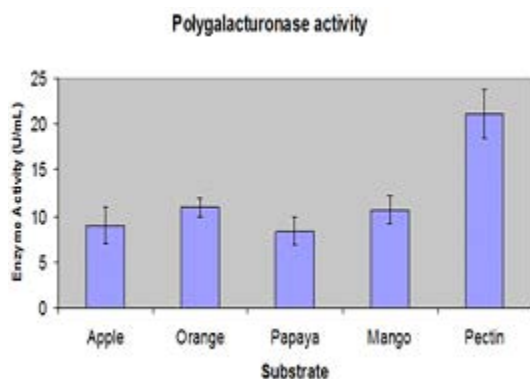


Fig. 5. Polygalacturonase activity on different substrates

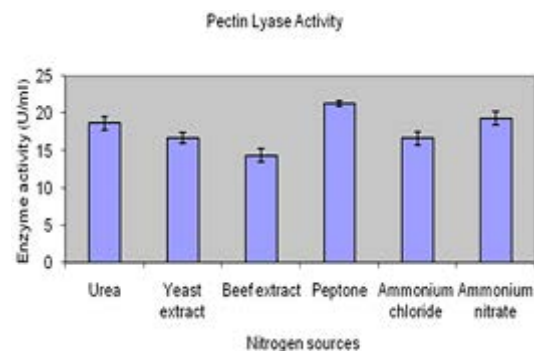


Fig. 6. Effect of nitrogen sources on Pectin lyase activity

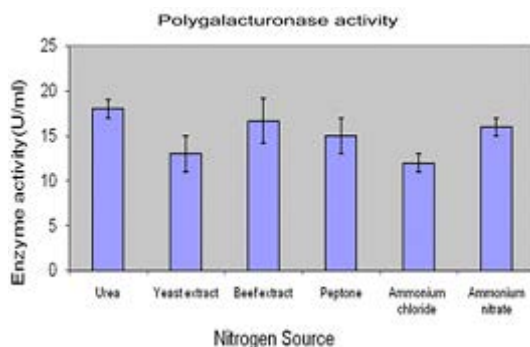


Fig. 7. Effect of nitrogen sources on Polygalacturonase activity



Fig. 8. Pectinase activity of various dialyzed fractions

The effect of various sources of nitrogen on production of pectinase by *B. cereus* was studied. Maximum Pectin lyase activity (PL) activity was observed in peptone (21.33 U mL⁻¹) followed by ammonium nitrate (19.33 U mL⁻¹). Least PL activity was observed with beef extract. These results show that Peptone was suited nitrogen source for PL activity (Fig.6). Peptone is a simple nitrogen source consists mostly of tryptophan, essential vitamins which may be responsible for improving pectin lyase production.

Hyper production of Polygalacturonase activity was observed in urea of about (18.03 U mL⁻¹) followed by beef extract (16.33 U mL⁻¹). Least PG activity was observed with ammonium chloride. This shows that Urea was suited best nitrogen source for PG activity (Fig.7). The nitrogen sources cause a remarkable effect on microbial pectinase production.

The pectinase present in the crude extract of *Bacillus cereus* GS-2 was partially purified by ammonium sulphate precipitation and dialysis, the pectinase activity was found higher in the 45% salt saturation fraction and have enzyme activity of 28.22 U mL⁻¹ (Fig.8). From (Fig. 9), using DEAE-sephacel the enzyme activity was increased up to 2-fold and it was found to be 40.13 U mL⁻¹ (Fig.10). This partially purified enzyme was electrophoresed on 2% SDS-Page, (Fig.11) shows a single band was observed and it was compared with standard protein markers the size of the enzyme was found to be 36 Kda.

HPLC analysis of purified pectinase sample peak (Fig.12c) matched with that of standard pectinase (Fig.12a). The residence time for the standard pectinase was 2.518 min and that of partially purified pectinase after dialysis was found to be 1.665 and purified pectinase

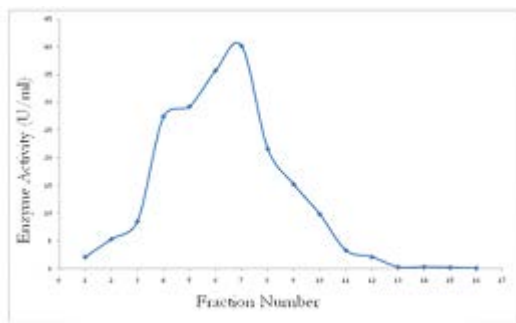


Fig. 9. Elution profile of partially purified protein on DEAE

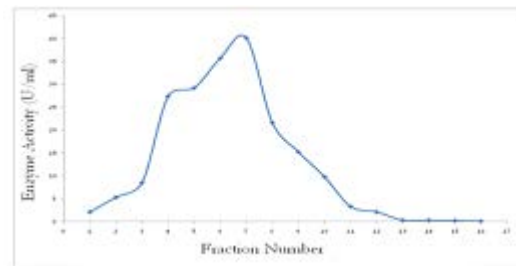


Fig.10. Pectinase activity for various fractions after DEAE

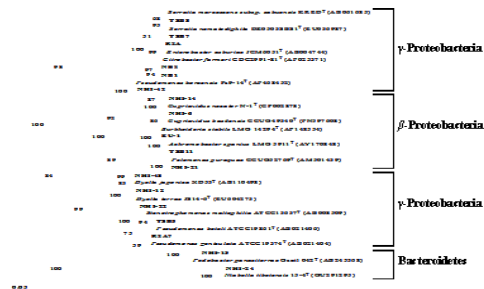


Fig.11. SDS PAGE of pectinase on 2% gel at 50 V. Lane M: molecular weight protein standards; Lane 1: ammonium sulphate precipitated protein; 2: DEAE-Sephacel elute; Lane3: Pure Pectinase

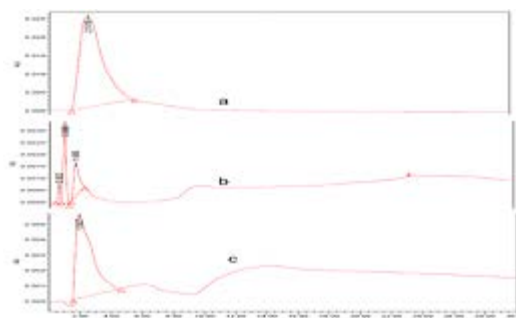


Fig. 12. HPLC chromatogram of standard pectinase from Sigma, USA (a) Dialyzed sample (b) and Purified pectinase obtained from *Bacillus cereus* GS-2 (c). Sample peak has matched with Standard pectinase

reveals a residence time of about 1.93 in the same experimental condition. Furthermore, no other significant peaks were found in the sample's chromatogram which clearly reveals its high purity.

There is no report on the production and partial purification of pectin lyase and polygalacturonase utilizing different fruit peels as substrate by *Bacillus cereus* so far, which has been confirmed by the analysis of 16SrRNA Sequencing. Future investigations like Zymography, MALDI analysis may confirm the purity of enzyme.

CONCLUSION

In this study, we successfully isolated a potential strain, *B.cereus* GS-2, which produces pectinase on various cheapest pectin sources (fruit peels). The isolated strain was morphologically characterized and identified by 16SrRNA sequencing. FTIR characterization of peels reveals the functional groups of pectin. Pectin from Orange peels gave enhanced pectin lyase and polygalacturonase production compared to other sources. Peptone and Urea gave enhanced pectinase production on various nitrogen sources. Further, Purification and characterization experiments revealed the purity of an enzyme. Because, of enhanced yield and the purity of an enzyme. We expected that *B.cereus* would be industrially exploited strain for the enhanced production of pectinase in an eco-friendly and cost-effective manner in mere future.

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