International Journal of Scientific Research in Engineering and Management (IJSREM)
Volume: 05 Issue: 07 | July - 2021 ISSN: 2582-3930

Screening of Pectinase producing bacteria from Orange waste

Saranya.A[1], Vaishali.V[2], Dr.M.Thenmozhi[3], MS. Simi Mathew[4]

[1]*Department of Biotechnology, Prathyusha engineering College, Chennai*
[2]*Department of Biotechnology, Prathyusha engineering College, Chennai*
[3]*Department of Biotechnology, Prathyusha engineering College, Chennai*
[4]*Junior Researcher, Biozone Research Technologies Pvt.Ltd., Chennai*

Abstract – Pectin is one among the nonstarch polysaccharides, which constitutes the main fraction of the plant cell membrane in association and substitutes with other polysaccharides, and they cover an excellent sort of biological functions and chemical structures. Naturally occurring polysaccharide pectin, the methylated ester of polygalacturonic acid, is extremely importance in both scientific and commercial world thanks to its biodegradability. An outsized group of pectinase enzymes causing breakdown of pectin polysaccharides of plants and fruit are utilized in industrial sector to extend the yield and clarity of fruit juices. During this study the bacterial strain was isolated using dilutions of 10-1 and 10-7 of rotten oranges. Isolated organisms were identified supported staining and biochemical tests. the pectinolytic activity decided using pectin containing pectinase screening agar well diffusion method at the temperature is 35_+ 2 degree C. Supported gram staining and biochemical tests, the bacterial strain was isolated and identified as *Bacillus cereus*. The strain showed different pectinolytic zones counting on the concentration of inoculum and therefore, the largest pectinolytic zones was observed by the strain. This strain was efficient and have potential to be implicated commercially to extend the clarity and quality of fruit juices.

KEYWORDS – Pectinolytic activity, pectin, pectinase screening agar medium, *Bacillus cereus*, polygalacturonic acid.

1. INTRODUCTION

Most of the critical life processes are established on the functions of enzymes in which Pectinase enzymes are mostly used in processes involving the degradation of plant materials, commonly referred to as pectic enzymes.The biotechnological significance of pectinolytic enzymes from microbial sources has pulled in the consideration of numerous scientists because of its various applications in different industrial processes.These enzymes are one of the most important groups of biological catalysts that have been established for usage in increasing juice yield and clarification in fruits and vegetable industries. It is used in the treatment and degumming of natural fibers used in the paper and textile industry.

Pectinase plays a significant role in coffee and tea fermentations. They are used in animal feed production for reducing feed viscosity, increasing nutrients absorption, releasing nutrients by hydrolysis of non-biodegradable fibers or by releasing nutrients blocked by these fibers and reducing the amount of nutrients in faeces.Microbial enzymes are produced either through submerged fermentation (SmF) or solid state fermentation (SSF) techniques.Medium is used for the detection of pectinolytic microorganisms especially those producing polygalacturonase.

Several types of conditions and media have been used to detect organisms capable of producing soft rots and degrading pectin.This general medium has been designated as MP medium. Murashige and Skoog Medium (MS) provide all the essential macroelements and microelements.Amongst citrus wastes, the waste of Citrus reticulata is a well-studied substrate for the enzyme and ethanol production under solid-state and submerged fermentation Pectin is a type of water-soluble fiber found inside plant cells. Many vegetables are excellent sources of pectin. Some fruits and vegetables are more pectin-rich than others. For example, apples, carrots, oranges, grapefruits, and lemons contain more pectin than cherries, grapes, and other small berries with citrus fruits containing the most pectin.The potential isolate was further identified using the molecular tool of 16S rRNA sequencing. In this method, a DNA purification kit was used for the extraction of genomic DNA of the selected isolate. The sequencing results were then processed using BioEdit software.The amplified PCR products were purified and sequenced. The obtained sequence data were blasted in the NCBI database and the likely microorganisms (with lower E value, higher identity percentage and maximum total score) were identified.

2. Materials and methods:

Sample collection:

For the isolation of pectinase producing bacterial strains, partially decayed fruit (oranges) were taken from the local market of Pallavaram, Chennai. The sample was collected aseptically using sterilized polythene bags. The physical characterization of the sample was analysed.

Fig 1: Rotten Orange Sample

Isolation of bacterial strain:

The 1g of rotten orange sample was crushed into pre-sterile mortar and pestle with distilled water to form suspension, 1 ml of rotten pulp was added in 0.9% saline as a diluent and made 7 fold serial dilutions (10-1 to 10-7).100µl of the diluted samples from 10-3, 10-5, and 10-7 was plated onto nutrient agar plates by spread plate method. The plates were observed for colonies after an incubation of 24 hours at 37°C in the incubator. The colonies (P1, P2, P3) was chosen and maintained as pure culture in a rotatory shaker at 150rpm and 37°C.

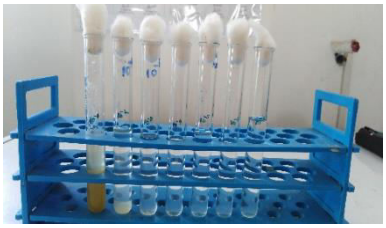


Fig 2: Serial dilution

Screening of pectinolytic bacteria:

Screening the bacterial isolates for pectinase activity was carried out by inoculating the cultures in pectinase screening agar medium (PSAM) and observing the zone of clearance.The pH of the medium is 7.0. The plates with PSAM were prepared and a loopful of bacterial cultures was inoculated at the respective wells of the plate and incubated at 37°C. After 48 hours, the plate was flooded with gram's iodine solution for 10 minutes and then washed with distilled water. A clear halo zone around the well indicates pectinase enzyme activity. The zone diameter was measured. The colony, which forms a large clear zone diameter, was used for further biochemical test and molecular identification processes.

Identification of pectinolytic bacteria:

Identification of strains was done on the basis of gram staining and biochemical testing (catalase, IMViC-Indole, Methyl Red, Voges Proskauer and Citrate).

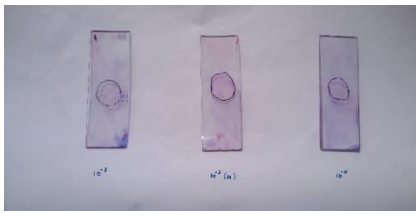


Fig 3: Gram Strained Slides

Molecular identification of pectinolytic strain:

Genomic DNA was extracted from screened strain (P2) by ethanol precipitation method.

After extraction, 2 µl of DNA fragments was mixed with Novel Juice dye contains 3 pursuit dyes like bromophenol blue, resolvent Cyanol FF and Orange 5.The gel was then electrophoresed at 100 volts for 45 minutes and it absolutely was determined in an exceedingly gel documentation system.

PCR was employed to amplify the DNA sequence in between two known sequences (forward primer 27F (5′-(AGA GTT TGA TCM TGG CTC AG)-3′) and reverse primer 1492R (5′-(CGG TTA CCT TGT TAC GAC TT)-3′) which are complementary to the known 16S rDNA sequences, were used).

The PCR sequence products were purified and sequenced. The obtained sequence data was compared with known sequences within the GenBank using the Basic local alignment search tool (BLAST) of the national center for biotechnology information (NCBI). Species that were identified supported the shared similarity with the known species sequences within the database.

3. Results and discussion:

From the various observations recorded during experimentation, the relevant results are being compiled and represented below.

Isolation of bacteria:

The orange sample was partially decayed. The top part of the orange was rotten, it was appeared as soft brown coloured texture with sour smell.

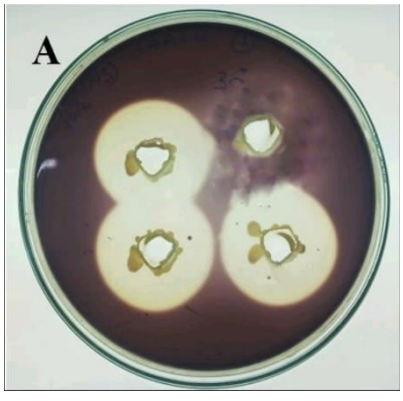
Fig 4: Crushed orange paste

To isolate microorganisms from the collected rotten orange sample, serial dilution (Fig 2), Spread plating (Fig 5) isolation techniques were used. Subsequently, the selected isolates (P1, P2, P3) was subcultured into nutrient medium until pure culture (Fig 6) were isolated .In total, seven (7) white isolates were identified from the 1gm of rotten orange sample. From seven isolated white colonies, Three colonies were selected namely P1, P2, P3 for screening of pectinase activity.

Fig 5: Spread Plate **Fig 6: Pure colony**

PLATE ASSAY METHOD FOR PECTINASE QUALITATIVE ASSAY:

Subsequent to isolation and purification, the selected isolates (P1, P2, P3) were assessed for pectinase activity using pectinase screening agar medium (PSAM).



‘

Fig 7: PSAM plate showing zone of inhibition

Table 1: Zone Measurement

S.No	Colony	Diameter(in cm)
1.	Control	-
2.	P1	1.5
3.	P2	1.8
4.	P3	1.6

The control did not show any clear zone while the well-defined clear zones are visible around well (colonies) having pectinase activity. The diameter of the clear zone is directly proportional to the pectinase activity. It means that colonies with large clear zones possess high pectinase quantity while those with low diameter clear zone have low activity. Therefore, the P2 colony shows larger clear zone of 1.8cm and it possess higher pectinase activity when compared to other two colonies (P1 & P3).

Biochemical tests:

GRAM STRAINING

The Gram strained slides were observed under microscope. The screened bacteria (P2) was viewed as purple colored colonies. It indicates the isolated colonies were Gram Positive Bacteria (Fig 8).

P1 **P2** **P3**

Fig 8: Gram Strained Colonies under Microscope (Purple colored colonies)

IMViC TEST

INDOLE TEST:

After adding Kovac’s reagent to the culture broth,formation of a red color ring in the reagent layer on top of the medium within seconds of adding the reagent indicated the positive result (Fig 9).

Fig 9: Indole Test

(Red ring formation)

METHYL RED (MV) TEST:

After the addition of methyl red indicator, the culture broth turns red because of a pH at or below 4.4 from the fermentation of glucose. This indicates the positive result of methyl red test (Fig 10).

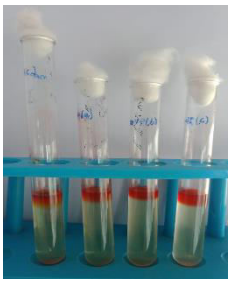


Fig 10: Methyl Red Test
(Red color formed)

VOGES-PROSKAUER (VP) TEST:

After the addition of Barritt's reagent A and Barritt's reagent B. The culture broth turns Pink red color on the surface.This indicates the positive result of Voges-Proskauer test (Fig 11).

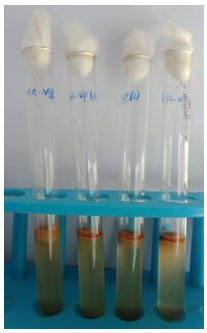


Fig 11: Voges-Proskauer Test
(Pink red color formed)

CITRATE TEST:

The results of citrate test are indicated by a change in color in the medium.The medium was appeared as growth with a color change from green to intense blue along the slant.This shows the positive result in citrate test (Fig 12).

Fig 12: Citrate Test

(Blue color changes)

Table 2: IMViC Test Results

Colonies	Indole	Methyl Red	Voges-Proskauer	Citrate
Control	+ve	+ve	-ve	-ve
1	+ve	+ve	+ve	+ve
2	+ve	+ve	+ve	+ve
3	+ve	+ve	+ve	+ve

CATALASE TEST

The immediate appearance of bubbles in the slide demonstrated the positive result in catalase test (Fig 13).

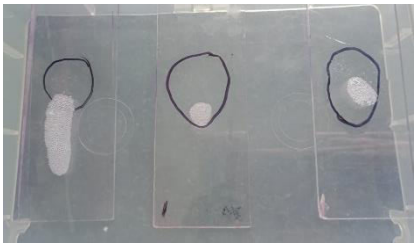


Fig 13: Catalase Test
(Bubbles Formed)

The P2 strain was identified as Large, irregular and colonies with Higher clear zone diameter appeared on pectinase screening agar medium (PSAM). Gram-positive, rod-shaped on Gram staining, arranged in short chains. The strain was catalase, indole, methyl red, Voges-Proskauer, and citrate tests positive (Table 3).Although, the P2 strain shows positive results in biochemical and staining test but 16S rRNA sequence analysis is required for confirmation. The P2 strains was further analysed for molecular characterization.

Table 3: Biochemical test results of screened strain (P2) with pectinolytic activity

Strain	Gram Staining	Indole	Methyl Red	Voges-Proskauer	Citrate	Catalase	Pectinase
P2	+	+	+	+	+	+	+

Molecular identification isolated bacterial strain:
Genomic DNA extraction methods was performed to isolate genomic DNA away from screened strain (P2). Finally, the DNA is isolated by ethanol precipitation.

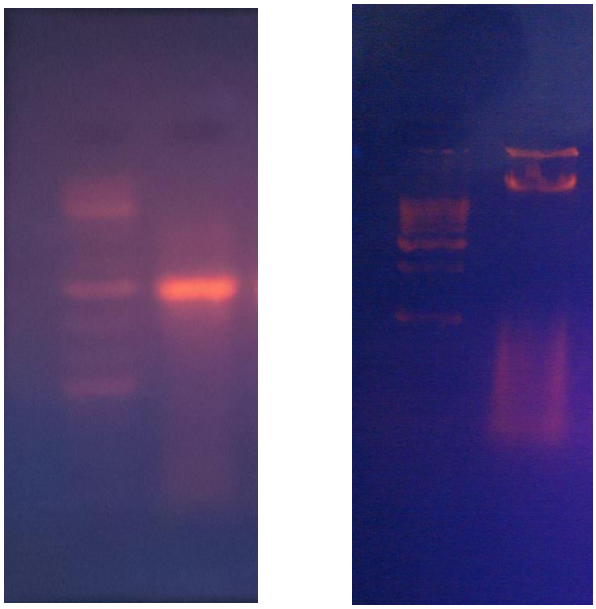
Agarose Gel Electrophoresis:

After separation, the resulting DNA fragments are visible as clearly defined bands after running in agarose gel electrophoresis. The DNA standard or ladder should be separated to a degree that allows for the useful determination of the sizes of sample bands (Fig 14).

Fig 14: Bands appeared on Agarose Gel Electrophoresis

PCR:
The isolated bacterial strain DNA was used to perform colony PCR. The strain was selected for 16S rRNA analysis and for this purpose colony PCR was done and the amplified product was run in agarose gel against ladder, both the bands (**a**) and (**b**) appeared on the same level in the agarose gel thus indicating the similarity of newly isolated bacterial strain (Fig 15).

(a) **(b)**



Lane 1 – 1KB DNA Ladder
Lane 2 – Bacterial genomic DNA

Lane 1 – Ladder
Lane 2 – Bacteria 16srRNA

Fig 15: PCR Bands

Sequencing:

Sequence:

>VS_Pec01
TGGGAGACTTGAGTGCAGAAGAGGAAAGTGGA
ATTCCATGTGTAGCGGTGAAATGCGTAGAGATA
TGGAGGAACACCAGTGGCGAAGGCGACTTTCTG
GTCTGTAACTGACACTGAGGCGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGTCCACGCC
GTAAACGATGAGTGCTAAGTGTTAGAGGGTTTC
CGCCCTTTAGTGCTGACCGCCTGGGGAGTACGG
CCGCAAGGCTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGAAGCAACGCGAAGAACCTTACCAGGTCTTG
ACATCCTCTGAAAACCCTAGAGATAGGGCTTCT
CCTTCGGGAGCAAAATGACAGGTGGTGCATGGT
TGTCCTCACCTCCTGTCGTGAAGTTGCCACCATT
AAATTGGGCACTCTAAGGTGACGGCCGGTGACA
AAACCAAGGAAGGGGGGGATGAACTCAAATCA
TAATGCCCCTTATAACCGGGGCTACACCATGCT
ACAATGGGCTACAAAACCCCCAGGGGGAGCTA
ATCTCATAAAACCTTCTCAGTTCGGATTGGAGG
CTGCCTCCCTACATGAAACTGAAAGCGTAGAAA
TCGAGAGAAGCATACCCCGCGGAAAAAGTTCC
CGGCTTTAACACAGCCGCACACACGGAAATTTA

AATCCCAAGAATGGGGGGACCTGTGGACCCAA
CGAAAATAGGAGGAAGGAAACGAACAGGAGG
AGAGAAG

Later on 16S rRNA sequencing confirmed the Bacillus status of the strain (P2). The 16S rRNA sequence was submitted Basic local alignment search tool (BLAST) of the national center for biotechnology information (NCBI).Colony PCR is an efficient way for identification of species (P2).

BLAST:

The **BLAST** result was interpreted as

Fig 16: Tabular Representation

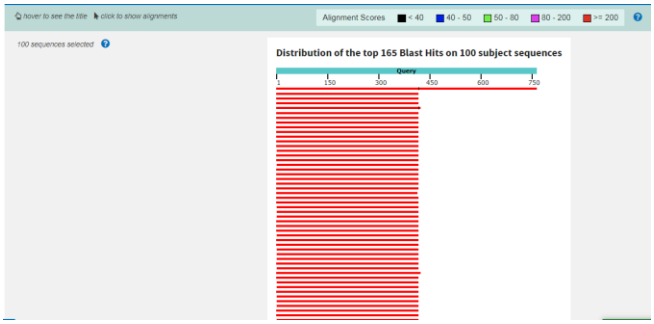


Fig 17: Graphical Representation

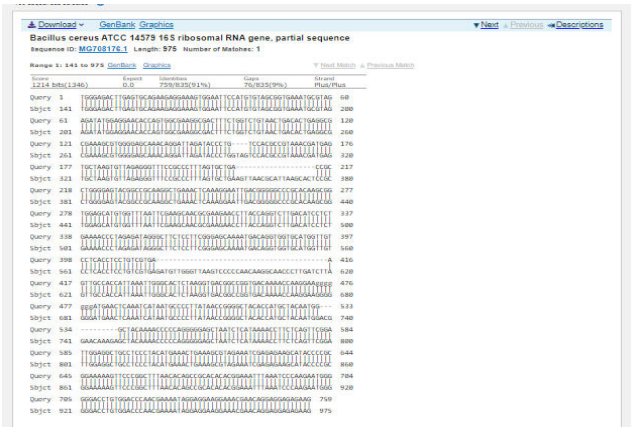


Fig 18: Alignment Representation

Table 4: BLAST Interpretation

Bacterial Strain	*Bacillus cereus* ATCC 14579
Max Score	660
Total Score	1241
Query Cover	100%
Percentage Identification	94.75%
Accession Length	975

The amplified PCR products were purified and sequenced. The obtained sequence data were blasted in NCBI database and the likely microorganisms (with lower *E* value, higher identity percentage and maximum total score) was identified as ***Bacillus cereus* ATCC 14579** with max score - 660, total score - 1241 and percentage identification - 94.75% (Table 4).

4. CONCLUSIONS

Bacillus cereus was isolated from the rotten fruit and was found as efficient pectinase producing bacterial strains which will be used for various industrial applications including extraction and clarification of fruit juices, processing of cotton fabric in textile industries, bleaching of paper, removal of pectic waste water and maceration of tea leaves. In future, the further studies to identify some other microorganisms to be used in the industries for fruit juice clarification process and perform a comparative study by performing a pairwise alignment to know their activity levels.

ACKNOWLEDGEMENT

We pay special thanks to Dr. M.THENMOZHI and MS. SIMI MATHEW for their full support to encourage and complete this study well.

6. REFERENCES

[1] Naidu G, Panda T. Production of pectolytic enzymes–a review.
Biopro Engg 1998; 19:355-61.
[2] Kanmani P, Dhivya E, Aravind J, Kumaresan K. Extraction and analysis of pectin from citrus peels: augmenting the yield from citrus lemon using statistical experimental design. Iranian J Energy Environ 2014; 5:303-312.
[3] Silva D, Tokuioshi K, da Silva Martins E, Da Silva R, Gomes E.
Production of pectinase by solid-state fermentation with Penicillium viridicatum RFC3. Proc Biochem 2005; 40:2885-9.

[4] Antier P, Minjares A, Roussos S, Viniegragonzalez G. New approach for selecting pectinase producing mutants of Aspergillus niger well adapted to solid state fermentation. Biotech Adv 1993; 11:429-40.
[5] Patil NP, Chaudhari BL. Production and purification of pectinase by soil isolate Penicillium sp. and search for better Agro residue for its SSF. Rec Res Sci Tech 2010; 2:36-42.
[6] Suneetha V, Prathyusha K. Bacterial pectinases and their potent biotechnological application in fruit processing/juice production industry: a review. J Phytol 2011; 3:16-19.
[7] Raju EVN, Divakar G. Screening and isolation of pectinase producing bacteria from various regions in Bangalore. Int J Res Pharma Biomed Sci 2013; 4:151-154.