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Isolation, Screening and Optimization of Pectinase-Producing Bacteria from Fruit Dump Yard Soil and Identification of Bacteria by 16s Rrna Gene Sequencing

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Abstract: Microbial Pectinases are an industrially important enzyme that has many applications and is used in the food industry, textile industry, and water treatment plant. In the present study, Bacteria were isolated from fruit dump yard soil and vermicompost and screened for Pectinase production. The best isolate was selected for optimization of various parameters like incubation time, pH, and temperature for pectinase production by growing the bacterium in a pectin-containing minimal medium and the total protein was estimated by the Lowry et al method. Natural substrates like orange peel, Cassava extract, Banana peel, and Rice bran powder were used for pectinase production by the bacterium. The optimum incubation time, pH, temperature, and substrate were determined as 24 hours, 6, 35°C, and orange peel powder. The pectinase enzyme activity of the bacterium with optimized cultural parameters was found to be 1.568U/ml. The pectinase enzyme was partially purified by Ammonium sulfate precipitation and dialysis and separated by SDS-PAGE. The Molecular weight of the partially purified pectinase enzyme was determined as 55KD. The bacterium was identified as Pseudomonas otitidis based on 16s rRNA Gene sequencing.

Keywords: Pectinase, Optimization, Pectin, Polygalacturonic acid, 16srRNA Gene sequencing, orange peel, Cassava extract, Pseudomonas otitidis

1.0 Introduction

Microbial enzymes are supplied, well-standardized, and marketed by a few competing companies. Among these industrially important enzymes, pectinases have a special significance due to their multiple uses in important sectors such as food, textile, beverages, pulp, paper, and biofuel industries. Microbial pectinases account for twenty-five percent of the worldwide food and industrial enzyme scale

and market increase from time to time (Rombouts et al., 1986) Pectinases are enzyme that breaks down pectin present in the cell walls of plants. These pectinolytic enzymes are produced by microorganisms like bacteria and fungi. Bacteria such as Bacillus subtilis and fungi such as Aspergillus niger produce pectinase enzyme (Tripathi et al., 2014).

The skin of the fruit is made of a pectin layer and this pectin can be hydrolyzed by microbial pectinases and thus used in food industries to extract the fruit juices quickly. The pectinolytic enzymes used for industrial application are produced by the fungi namely Aspergillus niger, Rhizopus, Alternaria, and Fusarium species (Beulah et al., 2015).

The microbial pectinases possess more advantages in plant-microbe symbiosis and decomposition of dead plant materials (Kumar et al., 2012). Pectinolytic enzymes are commonly used in processes involving the degradation of plant material and have a share of over 25% in the global scale of food enzymes. These enzymes have numerous applications in various types of industries like food industries, paper and pulp industry, and textile industry (Kumar et al., 2012).

Bacterial pectinases are advantageous over fungal pectinases as bacterial pectinases can tolerate high temperatures and alkaline pH. There are two types of pectinases – Acid and Alkaline pectinases. The major source of acidic pectinases is fungi. Alkaline. Pectinases are produced from alkalophilic bacteria, mainly Bacillus species. A mesophilic Bacillus produces alkalophilic and thermotolerant pectinases in higher amounts which are purified and used for commercial purposes (Kashyap et al., 2000).

Acid pectinase is widely used for the extraction and clarification, removal of pectin in juices and used for the maceration of vegetables to produce paste and purees and these pectinases are mainly produced by the fungi Aspergillus niger. Alkaline pectinases are widely used for treating the wastewater from vegetables, and food processing which contain a pectin residue and are mainly used in the textile industry and oil extraction. It is mainly produced by the bacteria, Bacillus species (Pedrolli et al., 2009).

The biotechnological applications of Microbial pectinases are the most important industrial enzymes. The biotechnological potential of pectinolytic enzymes from microorganisms has drawn a great deal of attention from various researchers worldwide as likely biological catalysts in a variety of industrial processes. Alkaline pectinases have significance in the current biotechnological arena with wide-ranging applications in textile processing, degumming of plant bast fibers,

treatment of pectic wastewaters, paper making, and coffee and tea fermentations (Pasha et al., 2013).

In the present study, an attempt was made to isolate and identify the effective pectinase-producing bacteria from fruit dump yards and vermicompost and optimize the strain for Pectinase enzyme production.

AIM

To isolate and identify the efficient Pectinase producing bacteria from the fruit dump yard and vermicompost and optimization of substrate, pH, Temperature and incubation time for pectinase production and separation of enzyme by SDS-PAGE.

4.0 Materials and Methodology

4.1. Collection of Samples:

Vermicompost samples were collected from Anna Nagar and fruit dump yard soil from Ambattur.

4.2. Isolation of Bacteria:

One gram of soil sample was weighed and suspended in 10 ml of sterile distilled water and this gives the dilution of 10^{-1} and the range of dilution is 10^{-1} to10^{-9}. The Nutrient agar medium was prepared, sterilized, and then poured into the sterile Petri plates. After the solidification of the medium, the plates were labelled and the nutrient agar plates were inoculated with 0.1 ml of a suspension of different dilutions (10^{-4}, 10^{-5}, 10^{-6}) separately and spread on the surface of the agar using L - rod and the plates were incubated at 37°c for 24 hours. After 24 hours of incubation, the isolated colonies were picked and streaked on nutrient agar plates to obtain a pure culture.

4.3. Screening of bacterial isolates for pectinase

Pectin containing minimal essential medium was prepared, sterilized, and poured into sterile Petri plates. The isolates were streaked and incubated at 37° C for 48 hours. After incubation, the colonies were flooded with 1% cetyltrimethylammonium bromide (CTAB).

4.4. Optimization of various parameters for Pectinase

Commercially available Polygalacturonic acid was used as a pectin substrate. The Bacterial isolate that gave the best result in the primary screening for pectinase was optimized for various parameters such as incubation time, temperature, and pH.

4.4.2. Optimization of incubation time:

100 ml of sterile pectin containing minimal broth media was prepared, and sterilized. 5 ml inoculum (0.5 MacFarland standard) of the selected bacterial strain was added and incubated at 37° C with shaking (Rotary shaker) for 150 rpm. The samples were collected in a centrifuge tube and centrifugation was carried out at 5000 rpm for 20 mins. The supernatant was taken for the assay. As pectin was used as a substrate in the medium, it specifically produces pectinase The pectinase production was estimated by estimating the total protein by the Lowry et al method at regular time intervals (0, 12, 24, 48, 72 hours).

4.4.3 Optimization of temperature:

Temperature plays an important role in pectinase production. The optimum temperature for pectinase production by the selected bacterial strain was studied by inoculating 5 ml of the bacterial culture in 100 ml of sterile Pectin containing minimal broth in different conical flasks. Then the flasks are incubated at various temperatures (25 ° C, 30 ° C, 35 ° C, 40 ° C, 45 ° C, 50 ° C, and 60° C). The samples were collected in a centrifuge tube and centrifugation was carried out at 5000 rpm for 20 mins. The supernatant was taken for the assay. The pectinase production in the broth incubated at different temperatures was estimated by estimating the total protein by the Lowry et al method.

4.4.4 Optimization of pH:

The optimum pH for pectinase production by the selected Bacterial strain was determined by inoculating and incubating the bacterial culture in the Pectin containing minimal broth with different pH. The experiment was carried out individually at various pH (5, 6, 7, 8, 9). The Pectinase production was estimated by the Lowry et al method.

4.4.5 Optimization of Carbon Source

Cassava peel, rice bran, and orange peel were used for the study. The extract was prepared separately from these by boiling in 250ml of distilled water. The extract was oven-dried at 37°C for 48 hours and then the powder was used as a substrate for pectinase production. The total protein was estimated by Lowry et al method

4.4.6 Pectinase activity:

The Pectinase enzyme activity was determined for the selected Bacterial strain which was grown in a medium containing optimized substrate, optimized pH, and

incubated with optimized temperature and incubation time. Pectinase enzyme activity was carried out by DNS method (Miller, 1959). 1.5 ml of freshly grown culture was taken and centrifuged at 10,000 rpm for 5 minutes. 0.1 ml of the supernatant act as source of pectinase enzyme. The substrate was prepared by mixing 0.5% pectin in 0.1M of pH 7.5 Phosphate buffer. Then, 0.9 ml of substrate was added to three clean test tubes. First tube act as enzyme blank, second tube act as reagent blank in which 0.1 ml of distilled water was added and third tube for enzyme in which 0.1 ml of crude enzyme was added. All the three test tubes were kept in the water bath at 50 c for 10 minutes. After incubation, 2 ml of Dinitro salicylic acid reagent (DNSA) was added to all the test tubes. Then all the test tubes are kept in a boiling water bath for 10 minutes. After cooling the test tubes, Optical density was measured using Spectrophotometer at 540 nm and enzyme activity was measured against enzyme blank and reagent blank. The enzyme unit was defined as the amount of enzyme that catalyzes µmol of galacturonic acid per minute under the assay conditions.

$$\text{Pectinase activity (U/ml)} = \frac{\text{Change in Absorbance at 540 nm/ time}}{\text{VXC}}$$

Where V = final reaction volume in ml

C = Concentration of the substrate (pectin) in the final reaction mixture (in mg/ml)

4.5.0 Isolation of Genomic DNA, Amplication of 16s rRNA by PCR and Identification of bacteria based on 16s rRNA gene sequence analysis

Chromosomal DNA was isolated from the isolate by the standard phenol/ chloroform method. The isolated genomic DNA was used as a template in PCR amplification of 16S rRNA. The Universal primers used for amplification of 16S rRNA are 16S-RS-F (forward) 5' CAGGCCTAACACATGCAAGTC-3' and 16S-RS-R (reverse) 5'-GGGCGGWGTGTACAAGGC-3'. PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100 Tris HCl, pH-8.3; 500mM KCl), 0.2mM each dNTP (dATP, dGTP, dCTP, and dTTP), 2.5mM MgCl2, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5 pm of forward and reverse primers and template DNA. The amplification reactions were performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) that was programmed as follows: initial denaturation at 95°C for 5 mins; 35 cycles of denaturation of 95°C for 30 sec, annealing at 60°C for 40 sec, extension

at 72°C for 60 sec and final extension at 72°C for 7 mins. The PCR products were stored at −4°C for further use. The amplified products were quantified in 1.2% agarose gel electrophoresis. The PCR product (5 μL) and 1 μL loading dye were loaded along with 5 μL of 1 kb ladder into the separate wells. The gel was run at 100 volts for 1 hour and the amplified products were visualized under UV transilluminator and photographed. The amplified 16s rDNA gene from the isolate was directly sequenced after purification by precipitation with polyethylene glycol and Nacl.

4.5.1 Phylogenetic analysis

DNA sequences obtained from both strands were edited and contigs assembled using DNA Dragon - DNA Sequence Contig Assembler (Sequentix version, 1.6.0. – Digital DNA Processor, Germany. Partial sequencing of 16S rRNA for the bacterial strains was performed with the help of a DNA sequencing service, with universal primers, 16S-RS-F (forward) 5' CAGGCCTAACACATGCAAGTC-3' and 16S-RS-R (reverse) 5'-GGGCGGWGTGTACAAGGC-3'. The online program BLAST (NCBI-2012) was used in identifying the related sequences with known taxonomic information available at the databank of the National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA). A phylogenetic tree was constructed with the CLUSTAL X program (Thompson et al., 1997), which involved sequence alignment by the neighbor-joining method (Saitou and Nei, 1987) and maximum parsimony with the MEGA4 program (Kumar et al., 2001). Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

4.6 Production of enzyme

4.6.1 Extraction of enzyme

The selected bacterial strain was inoculated in the pectin containing minimal medium and incubated with optimized incubation time, temperature, and pH. The enzyme was extracted by centrifugation. The broth was taken at the end of incubation time and was centrifuged at 5000 rpm for 20 minutes. The supernatant was taken and treated as a crude extract.

4.7 Partial Purification of enzyme

4.7.1 Ammonium sulphate precipitation

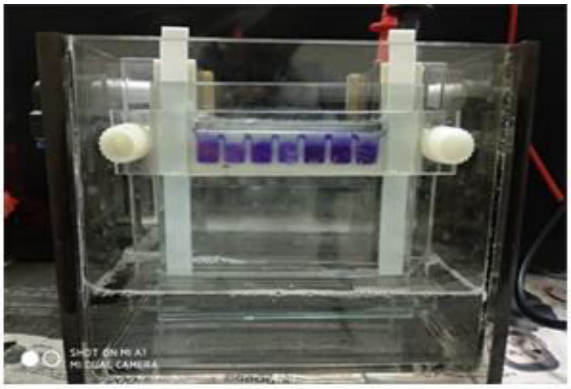
10g of Ammonium sulfate was added to 50 ml of crude sample and stirred slowly. The Process was carried out at a cooled condition for 45 min. Then the sample was

kept overnight at 4^0C. The sample was centrifuged at 10,000 rpm for 10 min. The pellet was collected and dissolved in 10 ml of 50 mM phosphate buffer.

4.7.2 Dialysis

The dialysis membrane was added to 100 ml of boiling water in a beaker and left for 10 minutes. Then 2% sodium hydrogen carbonate was added and boiled for another 10 minutes. Then the dialysis membrane was transferred into another beaker containing distilled water and boiled for 10 min Then the dialysis membrane was taken out with the help of forceps. One end of the membrane was tied and 10 ml of enzyme suspension was added into the dialysis membrane. Another side of the membrane was tied and placed in a beaker. Then Dialysis membrane is placed in another beaker containing phosphate buffer.

4.8 Separation of Enzyme by SDS PAGE



SDS PAGE

The Extracted partially purified pectinase enzyme was separated by Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE). The glass plates, comb and spacers are washed and dried. The plates were assembled in the Electrophoresis apparatus. Separating gel was prepared and poured through the gap between the glass plates to three – fourth of its length and the gel was allowed to polymerize for 30 minutes. The separating gel was overlaid with the stacking gel upto the rim of the notched plate and immediately inserted a clean Teflon comb into the stacking gel and the gel was allowed to polymerize for 20 minutes. After polymerization, the comb was removed carefully and the spacer was removed from the bottom and the glass plate was fixed in the electrophoresis apparatus filled with running gel buffer. 10µl of sample solubilizing buffer was added to the 10 µl of sample and boiled at 100°c for 2 minutes. 10 µl of the sample and protein marker were loaded to the separate lane and was run with 50v until the dye front reaches the

bottom of the gel. Then the gel was stained for 30 minutes and destained overnight. The protein bands were observed on the gel.

5.0 Result

Isolation of bacteria

fig 1: Plates showing isolates from fruit dump Vermicompost

fig 2: Plate showing isolates from Yard soil

Nine bacterial isolates were obtained from two different samples. Five bacterial isolates (named A, B, C, D, and E) were obtained from vermicompost (fig 1), and four isolates (named F, G, H, I) were obtained from fruit dump yards soil (fig 2).

Screening of Pectinase producing bacteria

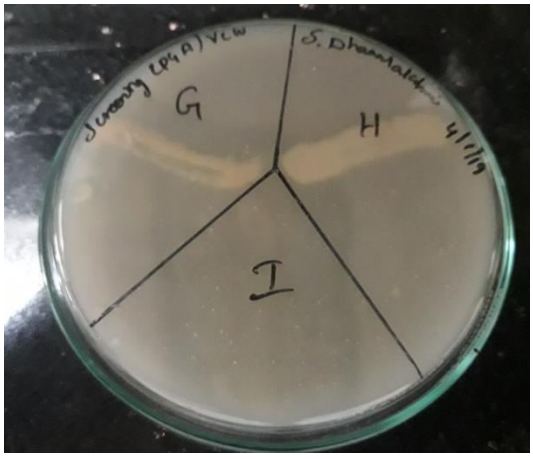


fig 3: Screening of isolates from dump yard soil

The bacterial isolates were screened for pectinase on Polygalacturonic acid agar. Out of nine isolates, the strain I showed the maximum zone around the growth and was selected for further study (Fig 3)

fig 4: Pure culture of Pectinase-producing isolate (I strain)

Optimization of incubation time for Pectinase production

S.No	Incubation time	Total estimation of protein
1	12 hours	40 µg/ml
2	24 hours	52 µg/ml
3	48 hours	45 µg/ml
4	72 hours	30 µg/ml

Table 1: Optimization of incubation time for Pectinase production

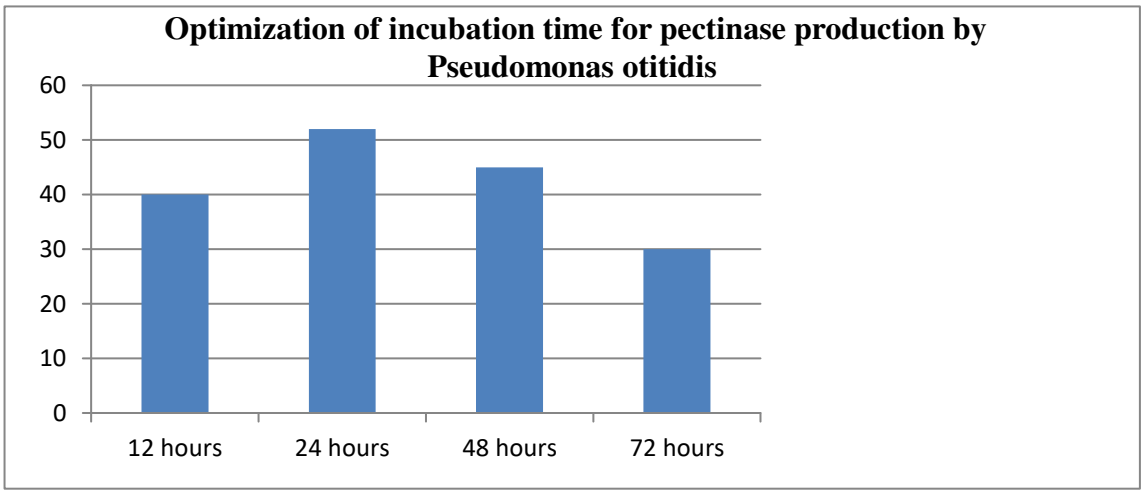


Chart 1: Optimization of incubation time for Pectinase production

Table 1 and Chart 1 show the effect of incubation time for pectinase production by the selected bacterial strain I. The maximum amount of pectinase was observed with 24 hours of incubation (52µg/ml) and the minimum amount was observed with 72 hours of incubation (30 µg/ml).

Optimization of pH for Pectinase production

S.No	pH	Total estimation of protein
1	5	90 µg/ml
2	6	125 µg/ml
3	7	105 µg/ml
4	8	25 µg/ml
5	9	10 µg/ml

Table 2: Effect of pH for pectinase production

Chart 2: Optimization of pH for Pectinase production

The bacterial isolate I was cultured in the pectin containing production media and adjusted with different Ph. The optimum pH was determined as 6 in which the maximum amount of protein was found to be 125 µg/ml and the amount of total protein was less (10 µg/ml) at pH 9 (Table 2 and chart 2)

Optimization of temperature for pectinase production

S.No	Temperature	Total estimation of protein
1	25°C	35 µg/ml
2	30°C	50 µg/ml
3	35°C	80 µg/ml
4	40°C	45 µg/ml
5	45°C	40 µg/ml

Table 3: Optimization of temperature for Pectinase production

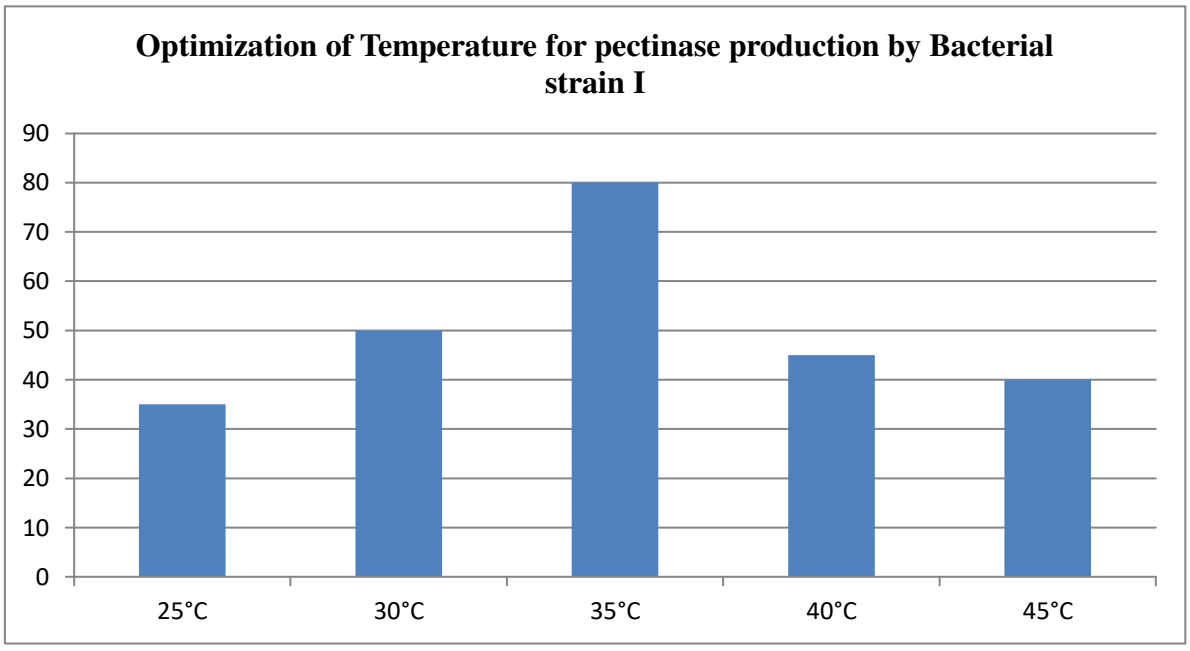


Chart 3: Optimization of temperature for pectinase production

The optimum temperature for pectinase production was found to be 35°C and the concentration of total protein was determined as 80 µg/ml (Table 3 and chart 3).

Optimization of Substrates for pectinase production

S.No	Substrate	Total Estimation of protein
1.	Orange peel powder	320µg/ml
2.	Cassava extract	200µg/ml
3.	Sweet potato extract	120µg/ml
4.	Rice bran powder	90µg/ml

Table 4: Optimization of substrate for Pectinase production

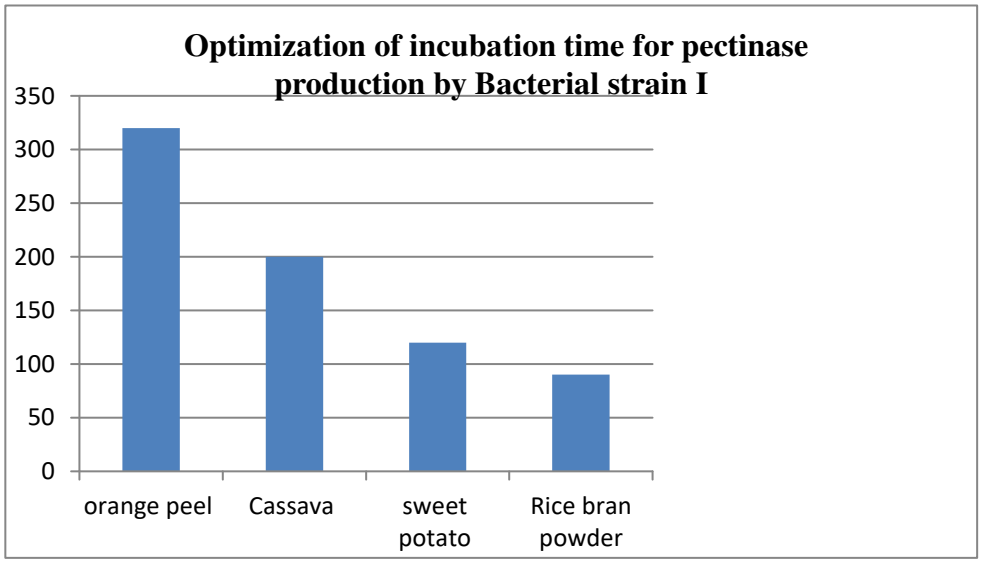


Chart 4: Optimization of Natural substrates for pectinase production

Various substrates like orange peel powder, sweet potato extract, cassava extract, and rice bran were used as substrates for pectinase production. The maximum amount of protein was recorded for orange peel extract (320µg/ml) and the minimum for Rice bran powder (90µg/ml) (Table 4 and Chart 4)

Pectinase Activity

The bacterial strain I was tested for pectinase activity which was grown in a broth containing natural substrate, orange peel powder adjusted with a pH of 6 and incubated at 35°C for 24 hours. The pectinase activity for the bacterial strain I was determined as 1.568 U/ml for 5% inoculum.

Identification of bacteria

Identification of bacteria based on 16s rRNA gene sequencing and is analyzed by a phylogenetic tree.

16S rRNA gene partial sequence (~1196bp)

TGCCGAGGAGTCTGCCTGGTACATGGGGGATAACGTTTCGAAAGGAACGCTAAT
ACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCA
GATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCCCACCAAGGCGA
CGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCC
TGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAA
GTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAA
TAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGC
GTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGAT
GTGAAAGCCCCCGGGCTCAACCTGGGAATTGCATCCAAAACTACTGAGCTAGAGT
ACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGA
AAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTG
CGAAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCG
ATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCTGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTC
GGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGG
GTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCG
GTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTA
GTCCGGATCCCAGT

Phylogenetic tree

Phylogenetic tree generated from the 16S rRNA gene sequence data using neighbor-joining tree building algorithm. The colored name indicates the sequence generated from the present study. The bacterial strain I was identified as Pseudomonas otididis.

SDS-PAGE analysis

The crude enzyme was extracted from Pseudomonas otitidis. The optimized substrate, pH, temperature, and incubation time were maintained in the broth and the crude enzyme was partially purified by dialysis and separated by SDS-PAGE. The molecular weight of the separated enzyme was determined as 55 KD.

6.0 Discussion

The use of pectinase in food industries has been prevalent for many decades and several microbial sources exist for the efficient production of this enzyme. The bacteria isolated from dump yards were screened for pectinase production on a Polygalacturonic acid agar medium. Among the Nine bacterial strains, five strains were isolated from vermicompost and four isolates from fruit dump yards soil and named A – I. During screening on specific media, it is found that only 3 strains showed pectinase activity. Based on the diameter of the zone observed, only one potential isolate strain I isolated from the fruit dump yard soil was further processed for the optimization study. Based on 16s rRNA sequencing, the bacterial strain I was identified as Pseudomonas otitidis.

The Pectinase production of the selected isolate was optimized for culture conditions like incubation time, pH, and temperature using Pectin (polygalacturonic acid) containing minimal media and natural substrates containing pectin. Table 1 and Chart 1 show the effect of pectinase production on different incubation time and the maximum amount of pectinase production was observed after 24 hours (52 µg/ml) and the minimal amount of pectinase production was observed at 72 hours (30 µg/ml) of incubation. Table 2 and Chart 2 show the effect of various pH on pectinase production after 24 hours of incubation at 37°C. The maximum pectinase production was observed at pH 6.0 (125 µg/ml) and the minimum amount of pectinase production (10 µg/ml) was recorded at pH 9. Table 3 and Chart 3 show the effect of various temperatures on pectinase production. The maximum pectinase production was obtained at 35°C (80 µg/ml).

Soares et al., 1999 reported that the best pH value for pectinase production was 6.0. Commercial pectinase works well at a pH of 4.5 – 5.5 which coincides with the current study. Atala et al., 2015 reported that the highest production of pectinase by Pseudomonas species was obtained at 30°C and this result disagrees with the current study, and the optimum temperature was determined as 35°C

Table 4 and Chart 4 show the effect of various natural substrates like orange peel powder, sweet potato extract, cassava extract, and rice bran extract on pectinase

production. The maximum enzyme production (320µg/ml) was measured for orange peel powder, followed by cassava extract (200µg/ml). The pectinase activity of Pseudomonas otitidis with optimized cultural parameters was determined as 1.568 U/ml. In the present study, the pectinase production by Pseudomonas otitidis is reported for the first time. Raju et al., 2013 reported three bacteria pectinase-producing isolates – Bacillus licheniformis, Bacillus cereus and Staphylococcus aureus from dump yards of vegetables. Reetha et al., 2014 reported that Pseudomonas fluorescens were able to produce a high amount of pectinase when compared to Bacillus subtilis. Pectinase production occupies about 105 of the overall manufacturing of enzyme preparations. These enzymes are widely used in the production of juices, fruit drinks, and wines.

The enzyme was extracted and partially purified by ammonium sulfate precipitation and dialysis. The extracted enzyme was separated by SDS – PAGE and the molecular weight was determined as 55KD. Jayanthi et al., 2005 stated that the microbial pectinases possess the molecular weight ranges between 35 and 79KDa.

7.0 Conclusion

The efficient Pectinase-producing bacteria Pseudomonas otitidis was isolated from fruit dump yard and identified by 16s rRNA partial gene sequencing. The optimum cultural parameters for the isolate were identified as pH 6.0, temperature, 35°C, and incubation time, 24 hours. The optimized natural substrate for Pectinase production was found as orange peel powder and the molecular weight of the enzyme was determined as 55 KD. The present study made a successful primary attempt to enrich and isolate the potential bacterial strain, Pseudomonas otitidis from the dump yard soil producing an industrially important enzyme, pectinase.

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