Studies on Pectinase Production by *Enterobacter* sp. using Mango Fruit Processing Industrial Waste as Whole and Sole Carbon Source

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Abstract:
Mango Fruit Processing Industrial Waste (MIW) is a pectin rich carbon source utilized as substrate for the production of pectinase from *Enterobacter* sp. in submerged fermentation process. Eight bacterial strains were isolated and screened (Pectin Clear Zone technique) for their ability to produce pectinase. Among them, *Enterobacter* sp. has given highest PCZ value of 34 mm. In secondary screening, pectinase production by *Enterobacter* sp. has been studied under the suitable fermentation conditions such as temperature- 38 °C, pH- 6.0, inoculum-size- 0.6 ml/100 ml, incubation- 96 hrs, substrate concentration- 0.6 g/100 ml, carbon source-fructose (1 %), Riboflavin (1 %). The effect of different amino acids, vitamins also studied. Under these suitable conditions the highest pectinase activity of 82.647 U/ml observed. These results suggesting that, the production of pectinase in large scale using MIW is a low cost method with high value product i.e., pectinase. The utilization of this waste for pectinase production will also control the environmental pollution.

Key words: Mango Fruit Processing Industrial Waste (MIW), *Enterobacter* sp. Solid State Fermentation (SSF), Submerged Fermentation (SmF), Pectinase.

Introduction
Mango (*Mangifera indica* L.) is an important fruit crop of India. India stands at top position in the mango production in the world, with its 12749.8 million tons of production per year (1, 2). Many number of mango fruit processing industries have established in Andhra Pradesh as it is contributing major amount of mangoes to an average production of India. A huge amount of waste have been generating, while processing the mango fruit and causing severe environmental pollution due to its microbial contamination. In order to control this, the mango fruits processing industrial waste was utilized for the production of commercially valuable products. The microbial transformation of agro industrial wastes had produced various valuable products like bio-gas, ethanol, enzymes, volatile flavoring compounds, fatty acids and microbial biomass (3). Similarly, dried mango industrial waste also used for production of pectinase, as it contains an appreciable amount of pectin and carbohydrates, proteins, the fat content, however, is low (4). Pectin acts as the inducer for the production of pectinolytic enzymes by both submerged and solid state fermentations. But submerged fermentation requires high volumes of water, continuous agitation and generates lot of effluents (5). Earlier many bacteria like Bacillus, Aeromonas, and Lacto bacillus etc., used for the production of pectinase. It is well known that as compared to intracellular enzymes, the extra cellular enzymes are easier to be extracted. Pectin itself can be extracted from mango industrial waste (mainly from peels) as a commercially important by-product. Earlier some other wastes like apple pomace, mango waste, orange waste, other fruit and vegetable industrial wastes and different agro-industrial wastes were used for the production of enzymes such as
pectinase, cellulase, α-amylase, esterase and peroxidase (6, 7). Pectinases are group of enzymes that attack pectin and de-polymerise it by hydrolysis and transelimination as well as by de-esterification reactions, which hydrolyses the ester bond between carboxyl and methyl groups of pectin (8). These enzymes act on pectin, a class of complex polysaccharides found in the cell wall of higher plants and cementing material for the cellulose network (9). The present investigation was undertaken to produce pectinase by the isolated bacterial strain Enterobacter sp. for the conversion of mango fruit processing industrial waste into humus via microbial transformation in submerged fermentation process.

Materials and Methods
Sample Collection: The processed waste produced from mango fruit processing industries (wet, dry and soil) were collected in sterile polythene covers from different MIW yards around Chittoor district, A.P., India and stored at 4 °C for further use. This waste (dried and powdered) used as carbon source/Nutrition for isolates.

Isolation and Identification of Bacteria: The bacterial strains were isolated by serial dilution of 1.0 g MIW. The pure cultures of isolates were made by Streak-plate method on nutrient agar media slants. The isolates were identified by gram’s staining, morphological and biochemical characterization of colonies on agar slants described in K R Aneja lab manual (10).

Primary Screening (Screening test-I): Modified Czapec dox’s broth used as production medium and screening assay has done on nutrient agar medium supplemented with 4 % Pectin.

Pectinase Production Medium (PPM): This medium consists of part (A) and part (B). Part (A) contained (g/l): NaNO₃ - 2.0; KH₂PO₄ - 1.0; KCl - 0.5; MgSO₄.7H₂O - 0.5; Yeast extract - 1.0. These contents were dissolved in 40 ml distilled water. The pH was adjusted to pH 7.0 by NaOH (5 %, w/v). Part (B) contained (g/l): Pectin, 5.0, dissolved in 10 ml of distilled water. The two parts (A) and (B) were mixed and sterilized. Then inoculated with bacterial isolates and incubated at 37 °C for 96 hrs, then assayed for pectinase activity.

Pectinase Activity Assay: Nutrient agar medium with 4 % Pectin, pH 7.0 was used as assay medium. Plates of the same size poured with equal amounts of sterilized assay medium. Upon solidification three wells poured with 0.1 ml of culture filtrate. These plates incubated at 37 °C for 2-4 days and then plates flooded with Hexadecyl Trimethyl Ammonium Bromide (HTAB) solution, clearing zones of the medium investigated and taken as the criteria for determining the pectinase productivity.

Secondary Screening (Screening test-II): In secondary screening, pectinase produced in submerged fermentation and its activity assayed by DNS method. Mango fruit processing industrial waste powder Basal medium was used as production medium (MIWP-BM).

MIWP-Basal Medium: The basal medium (BM) was prepared according to Vincent method. It contained the following (g/l): NaNO₃ - 2.0; K₂HPO₄ - 0.5; KCl - 0.5 and yeast extract 1 %. These were dissolved in citrate phosphate buffer at pH 7.0 and supplemented with MIW powder (4 % w/v) separately. Then pH of this medium was adjusted to 7.0 and sterilized at 121°C. This medium was inoculated with 0.5 ml of overnight culture broth of Enterobacter sp. incubated at 37 °C for 6 days. Then the pectinase activity was assayed for every 24 hrs.

Pectinase Assay: The pectinase activity assayed by DNS method (11). 0.5 ml of culture filtrate was used as enzyme source and 0.5 ml of 1% pectin was used as substrate. One unit (1U) of enzyme activity is equal to the 1 µMol of reducing sugars released, measured in terms of D-galacturonic acid, produced as a result of enzyme-substrate reaction.

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Factors influencing the pectinase production:

Effect of Temperature: The Enterobacter sp. grown in MIWP-Basal Medium at different temperatures viz. 18 °C, 28 °C, 38 °C, and 48 °C, for about 6 days for the production of pectinase. Measurement of pectinase productivity was performed for every 24 hrs at 540 nm by spectrophotometer.

Effect of pH: The pH of the production medium of different culture flasks was adjusted to 5.0, 6.0, 7.0, 8.0, by using 0.1 N NaOH and 0.1 N HCl. Then flasks were inoculated with Enterobacter sp. and incubated at 38 °C. Then the Pectinase activity was assayed.

Effect of Substrate concentration: Different concentrations of substrate (g/100 ml flask, (w/v)) 0.2, 0.4, 0.6, and 0.8, added in production medium, pH adjusted to 6.0. Then flasks were inoculated with Enterobacter sp. and incubated at 38 °C. Then the Pectinase activity was assayed.

Effect of Inoculum-size: The overnight broth culture of Enterobacter sp. was used as inoculum. The inoculum sizes (ml/100 ml) 0.2, 0.4, 0.6, and 0.8 inoculated in to production medium containing 0.6 g of substrate at pH 6.0. Then the inoculated flasks were incubated at 38 °C and Pectinase activity was assayed.

Effect of different Incubation periods: Under the suitable culture conditions such as pH-6.0, substrate concentration (0.6 g) production medium was inoculated with 0.6 ml of Enterobacter sp. culture broth and incubated at 38 °C. Then the pectinase activity was measured every day at 2 hrs, 4 hrs, 8 hrs, and 16 hrs of time intervals.

Effect of Carbon source: Different external carbon sources were introduced into the production medium at an equimolecular amount located at 1 % (w/v) sucrose. Parallel experiment was made with no sugar as a control. The carbon sources, dextrose, fructose, and lactose and mannose and pectin were introduced at the level of 1 % (w/v). Under the above mentioned (in 2.4.5) cultural conditions the culture flasks were incubated for 52 hrs and Pectinase activity was assayed.

Effect of Nitrogen source: Production medium was supplemented with different nitrogen sources at an equimolecular amount of nitrogen that present in sodium nitrate (0.2 %, w/v) in basal medium. The applied nitrogen sources ammonium oxalate, potassium nitrate, peptone, urea introduced as organic nitrogen source at the level of 1 % and the control was devoid from any nitrogen source. All the experiments were carried out pectinase activity was assayed.

Effect of Amino acids: The production medium was added at an equimolecular amount of nitrogen located in the best inorganic nitrogen source for the pectinase productivity. This experiment was controlled by performing of parallel one containing the original nitrogen source i.e., sodium nitrate and was devoid of any amino acid. The supplemented amino acids: alanine, glycine, phenyl alanine, and methionine. All the experiments were carried out and pectinase activity was assayed.

Effect of Vitamins: Different vitamins are ascorbic acid, riboflavin, vitamin-B6 and vitamin-E added separately to flasks containing the pectinase production medium, while the control applied free from any vitamin. All the experiments were carried out and assayed pectinase activity was assayed.

Results and Discussion

Sample collection: Thirty mango fruit processing industrial waste samples collected from ten different mango fruit processing industries around the Chittoor district. They were used as a source for the isolation of pectinase producing bacterial strains. Earlier the wastes like Orange peel, Citrus peel and Potato peel were also used as a source for isolating pectinolytic microorganisms (12).

Isolation and identification of bacteria: Eight bacterial strains were isolated and pure cultured on nutrient agar slants. Based on the results of
morphological and biochemical characteristics (Table-1 and 2), the isolated bacterial strains were identified as *Enterobacter* sp., *Serratia* sp., *Enterobacterium* sp., *Providencia* sp., *Raoutlella* sp., *Pectobacterium* sp., *Entero-bacteriaceae bacterium* sp., *Morganella* sp. (Table-3). Similarly bacterial isolates such as *Bacillus firmus*-I-4071, *B. firmus*-I-107 and *Bacillus laterosporus*-I-10104 were reported from agro and fruit processing wastes (7, 12 and 13).

**Primary screening (Screening test-I):** All eight bacterial strains considered as good pectinase producers with their PCZ values of pectinase plate assay (Table-4). One of them, *Enterobacter* sp. has given highest pectinase productivity with its PCZ value of 34 mm (Fig. 1). The PCZ value of *Enterobacter* sp. strain was very much similar with that of 3 bacterial isolates; 4071, 107 and 10104 with pectin clear zones of 32, 34 and 34 mm respectively (by using *Solanum tuberosum* peels as substrate) (14).

**Secondary screening (Screening test-II):** Since 1940s, pectinases have been used in several conventional industrial processes, such as textile, plant fiber processing, tea, coffee, oil extraction, treatment of industrial wastewater, purification of viruses, in making of paper, for increasing filtration efficiency and clarification of fruit juices, in wood preservation and used in maceration, liquefaction and extraction of vegetable tissues (15). For this reason, *Enterobacter* sp. have examined for its ability to utilize mango fruit processing industrial waste as substrate.

![Fig. 1: Pectin Clear zones of Enterobacter sp.](image1)

**Fig. 1 and 3.** Effect of Temperature (°C) and pH on the pectinase production by *Enterobacter* sp.

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Fig. 4 and 5. Effect of Substrate concentration (g/100ml) and Inoculum size (ml) on the pectinase production by Enterobacter sp.

Figure 6 and 7: Effect of Incubation periods (Hours) and Carbon Source (1%) on the pectinase production by Enterobacter sp. Dex- Dextrose, Fru- Fructose, Lac- Lactose, Man- Mannose, Con- Control

3.4.4. Effect of Nitrogen Source (1%) and Amino acids (1%):

Fig. 8 and 9: Effect of Nitrogen Source 1% and Amino acids 1% on the pectinase production by Enterobacter sp. Ala-Alanine, Gly-Glysine, Phe-Phenyl alanine, Met-Methionine

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of the fermentation medium play an important role in pectinase production from *Enterobacter* sp. (Fig. 2). The highest pectinase production was observed at 38°C, upon increasing or decreasing the temperature the pectinase production was decreased. Similarly pH of the medium, incubation time, substrate concentration and inoculum size will also affect the pectinase production. It was observed that from Fig. 2 to Fig. 6, shows that the optimal cultural conditions increased the pectinase activity 40.859 U/ml to 67.24 U/ml. Similarly the high pectinase production was observed at 72 hrs of incubation at 35°C with the initial pH of 6.5 using *Bacillus* sp. MFW7 (16). The presence of external carbon source (Fructose) and added vitamins (Riboflavin) in the production medium increased the pectinase productivity to 82.647 U/ml while the presence of external nitrogen source and added amino acids in production medium decreased the pectinase productivity to 71.123 U/ml [(Peptone) (Fig. 7 to Fig. 10). In presence of 0.5% of pectin (carbon source) the FW2 isolate showed highest activity of 22 U/ml (17), similarly Lactose in combination with peptone supported maximum pectinase production by *Bacillus* sp. MFW7 (18). The pectinase activity of *Enterobacter* sp. 82.647 U/ml was observed under the standardized cultural conditions is very much higher than the activity of reference bacterial strain OS-IV (29.1 U/ml) isolated from soil of a plum tree orchard and agro-industrial waste (19, 20). This shows that the chemical composition of the medium will also play an important role in pectinase production from microbes. The economic and ecological function of pectinase enzymes in industries is gaining much attention with the need of highly productive strains of microorganisms to reduce production cost. Production of pectinase by *Enterobacter* sp. using MIW as substrate in submerged fermentation is a very low cost method. In this process 25 g of purified pectinase can be extracted using 1000 g of MIW. The cost for whole fermentation and purification processes of pectinase is half of the market value of 25 g pectinase ($295.0) (21). This shows that the utilization of freely available MIW for pectinase production by *Enterobacter* sp. is more suitable for industrial large scale production.

![Fig. 10. Effect of vitamins (1%) on the pectinase production by Enterobacter sp. Aa- Ascarbic acid, Rib-Riboflavin, V- B6-Vitamin- B6, V-E- Vitamin- E](image)

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Table 1. Morphological characters of isolated bacterial pure cultures:

<table>
<thead>
<tr>
<th>Character Isolate</th>
<th>Colony Color</th>
<th>Temperature (ºC)</th>
<th>Growth Form</th>
<th>Margin</th>
<th>Elevation Density</th>
<th>Gram's Staining</th>
<th>Identified as</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSTB1</td>
<td>White</td>
<td>35ºC Agar(+)</td>
<td>Rhizoid</td>
<td>Filamentous</td>
<td>Flat</td>
<td>Translucent</td>
<td>Rod+ Bacilli (+)</td>
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<td>PSTB2</td>
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<td>35ºC Agar(+)</td>
<td>Circular</td>
<td>Raised</td>
<td>Flat</td>
<td>Translucent</td>
<td>Round + Ccci (+)</td>
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<tr>
<td>PSTB3</td>
<td>White</td>
<td>35ºC Agar(+)</td>
<td>Irregular</td>
<td>Filamentous</td>
<td>Flat</td>
<td>Translucent</td>
<td>Rod chains- Strept bacilli (-)</td>
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<tr>
<td>PSTB4</td>
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<td>35ºC Agar(+)</td>
<td>Irregular</td>
<td>Lobate</td>
<td>Flat</td>
<td>Translucent</td>
<td>Round bunch + Staphylococcus (-)</td>
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<td>PSTB5</td>
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<td>35ºC Agar(+)</td>
<td>Irregular</td>
<td>Lobate</td>
<td>Raised</td>
<td>Translucent</td>
<td>Round bunch - Staphylococcus (-)</td>
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<td>Translucent</td>
<td>Round chains+ Diplo coccus (+)</td>
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<td>Lobate</td>
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<td>Round - Ccci (-)</td>
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<tr>
<td>PSTB8</td>
<td>Pink</td>
<td>35ºC Agar(+)</td>
<td>Irregular</td>
<td>Filamentous</td>
<td>Flat</td>
<td>Translucent</td>
<td>Round bunch + Staphylo coccus (+)</td>
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</table>

Note: '+' = Positive, '-' = Negative

Table 2. Bio-Chemical characteristics of isolated Bacterial Strains:

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<th>Isolate Test name</th>
<th>PSTB1</th>
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<th>PSTB3</th>
<th>PSTB4</th>
<th>PSTB5</th>
<th>PSTB6</th>
<th>PSTB7</th>
<th>PSTB8</th>
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<td>Casein Hydrolysis</td>
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<td>Lactose Fermentation</td>
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<td>Dextrose Fermentation</td>
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<td>Sucrose Fermentation</td>
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Conclusion:

In the present study, the production of pectinase from Enterobacter sp. has been investigated under submerged fermentation. The high productivity of pectinase 82.647 U/ml, under suitable fermentation conditions suggested that Enterobacter sp. is a good pectinase producer than the earlier reported bacterial isolates. In addition to these properties, some additional properties like external carbon source (Fructose) and added vitamins (Riboflavin), low substrate concentration, less incubation time for pectinase production indicating the potential of Enterobacter sp. to be used at commercial level in fruit processing industries. This is the first ever report of pectinase production using mango processing industrial waste used as whole and sole carbon Source for the production of pectinase while the pectinase itself can find a number of applications in the mango processing industry.

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References:


