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***Research Article***

# Production and purification of pectinase by newly isolated *Bacillus megaterium* PBIL-2 using agro residue

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## Abstract

Pectinases are enzyme that hydrolyze the pectin, and are commercially important in clarification of fruit juices and wines. In the present study, pectinase enzyme was produced by *Bacillus megaterium* PBIL-2 isolated from fruit and vegetable market waste of Koyambedu market, Chennai, India, and identified by 16S rRNA sequencing. The results showed that *Bacillu*s species were predominant in all fruit and vegetable waste. Under optimized conditions, maximum pectinase production with 62 U/mL was obtained in the presence of orange peel at 35°C and pH 4 after 18 h of incubation. Partial purification of pectinase was done with an increased purification fold of 1.56. Thermo stability of purified pectinase activity was found to be maximum at 40°C and pH 5. Based on the results, it is concluded that *Bacillus megaterium* PBIL-2 could be responsible for the breakdown of pectin by production of pectinase. Thus, they may be potentially useful for production of pectinase using agro residue.

**Keywords:** Pectinase; Fermentation; *Bacillus megaterium* PBIL-2; Purification.

## Introduction

Pectinases are enzyme that hydrolyze the pectin substance, which is major component of middle lamella of plant cell wall and is broadly distributed in higher plants and microorganism [1]. These enzymes are classified into three main groups that catalyze depolymerization, demethylation and de-esterification reactions [2]. Polygalacturonase (EC.3.2.1) that catalyzes the breakdown of α 1-4 glycosidic bonds between two adjacent galacturonic acid residues present in the pectin is a depolymerizing enzyme. Pectin lyase (EC.4.2.2) that acts the elimination reaction between two methylated residues is a demethylating enzyme and Pectin esterase (EC.3.1.1) catalyzes the splitting of methoxyl groups releasing methanol is a de-esterifying enzyme [3].

Agricultural and food wastes are the major sources of pollution. Biological degradation helps to produce various valuable compounds using microorganisms such as proteins, polysaccharides, oligosaccharides, vitamins, hormones and enzymes for industrial exploitations [4]. Several microbes are capable of using these substances as carbon and energy sources for the biosynthesis of immense range of pectinolytic enzymes in different environmental places. The commercial value of microbial derived pectinase have amplified predominantly for escalating the filtration efficiency and clarification of fruit juices and wines, processing of vegetables, textile industries, plant tissue maceration, wastewater treatment, degumming of plant webbers [5].

Pectinases are synthesized by different sources such as bacteria, fungi, yeasts, insects, nematodes, plants and protozoan. Most of the Bacterial isolates such as *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp., and *Staphylococcus* sp., were reported as good pectinase producers [6-8]. In this present study, bacterial strains were isolated from fruit and vegetable wastes and screened for pectinase production.

## Materials and methods

### *Sample collection*

Fresh, dried and decomposed of fruit and vegetable waste samples were collected in sterile polythene covers from different places around Koyambedu fruit and vegetable market, Chennai,

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Tamil Nadu, India. These samples were stored at 4 °C for further purposes.

### *Isolation of microorganism*

For isolation of bacteria, suspensions of samples were prepared in sterile saline solution, which is plated on modified pectin agar medium. A loop of the homogenate was then streaked onto pectinase screening plates composed of (g/L) (pH 7.0) pectin 5, ammonium sulfate 2, yeast extract 1, $KH_2PO_4$ 0.3, $K_2HPO_4$ 0.2, $MgSO_4$ 0.01 agar 20 and incubated at 37ºC for 24 to 72h. All morphological contrasting colonies were purified by repeated streaking. Pure cultures were sub-cultured onto slants media [2].

### *Screening for pectinolytic activity*

Pure cultures were inoculated by making puncture in the medium and incubated for 48 h [9, 10]. After which iodine –potassium iodide solution (1 g Iodine, 5 g of potassium iodide and 330 mL distilled water) was added to determine the pectin hydrolysis zones. The isolates that exhibited highest pectin clear zone (PCZ) were selected.

### *Identification of bacteria*

The bacterial isolates were identified by 16S rRNA sequencing analysis using with 8F and 1541R primers. Total genomic DNA contents were extracted from bacterial cells cultured onto nutrient agar and 16S rRNA sequence were performed using ABI 3730xl sequencer (Applied Biosystems).

### *Total protein determination*

Total protein contents present in crude and purified were evaluated using Lowry's method by taking bovine serum albumin as the standard and were expressed as µg of protein present per ml [11].

### *Pectinase activity assay*

The quantitative assay of pectinase was carried out by using pectin as substrate [12, 13]. The reaction mixture containing 0.5 mL of the crude enzyme and 0.5 mL of 1% pectin in 0.1 M acetate buffer with pH 6.0 was incubated at 40°C for 10 min. The reaction mixture was then added with 1 ml of DNS reagent and the mixture was boiled for 5 min at 90°C. One milliliter of Rochelle's salt was added to stop the reaction. The absorbance was read at 595 nm. Standard glucose solution was used to generate standard graph. One unit of Pectinase activity was defined as the amount of enzyme which liberated 1 µmol glucose per min.

### *Production of pectinase*

The selected bacterial isolate was grown in 1 L Erlenmeyer flasks containing 250 mL modified pectin broth, wherein pectin was replaced with respective substrates and inoculated with *Bacillus megaterium* PBIL-2 strain. Various fruit peel wastes were selected like orange peel, onion peel, cabbage waste and apple pomace as substrate. The unsoiled raw materials were dried at 50-60°C and ground to fine particles, packed in airtight hygienic containers and stored at room temperature [6]. All these processed substrates were used at 1% (w/v) level in fermentation media directly instead of using of pectin, and enzyme production was carried out by incubating the flasks at 37±2°C for 24 h. The required physical parameters like temperature, pH and agitation were kept at 37±2°C, 7.0 pH and 120 rpm, respectively. At the end of fermentation, samples were assayed for the enzyme activity.

### *Optimization of pectinase production*

Various fermentation parameters were established for the enhanced production of pectinase. The process parameters optimized were pH (3-10), temperature (25-50°C at 5°C interval), fermentation time (12-36 h), inoculum age (12-46 h) and inoculum size (1-10 %) [14].

### *Purification and characterization of pectinase*

Crude extract of pectinase was precipitated [15] by addition of saturated ammonium sulphate 20-80% (w/v), and the precipitate was re-suspended in 10 mL of acetate buffer (50 mM) pH 7, and dialysed against 100 mL of acetate buffer (50 mM) pH 7 for 24 h. Gel filtration chromatography was employed for concentrated sample and elution of the proteins was done using acetate buffer (50 mM, pH 4). Characterization of produced pectinase [16, 17] was done by measuring pectinase activity in various pH conditions (4-10) and temperatures (30-60°C) respectively.

## Results and discussion

### *Sample collection*

There are about nearly 100 fruit shops in Koyambedu market in Chennai district, T.N., India leading to generate high quantity of waste

 

that rise to give the problem of environmental pollution. Twenty mixed fruit samples were collected from different places around the market as a source for pectinolytic microorganisms.

### *Isolation and identification of bacterial strains*

Bacterial strains used in this present study were isolated from collected mixed fruit wastes. Totally eight bacterial strains were identified and they were given by names such as PJIB-1, PBIL-2, PBIL-3, PBIl-4, PBIL-5, PBIL-6, PBIL-7, and PBIL-8.

### *Screening for pectinolytic activity*

All the isolates were considered as good producers of pectinase with their pectin clear zone value of 28 mm. Based on the high PCZ value, only one strain that is PBIL-2 has been selected for the production of pectinase.

### *Species identification of PBIL-2 strain*

The 16S rRNA gene sequence of the selected isolate PBIL-2 showed 97% similarity to Bacillus sequences in NCBI data base. The bacterial strain PBIL-2 clustered with *Bacillus megatirium* and *Bacillus aryabhattai* strains in the phylogenetic tree constructed using NCBI data base. Thus, 16S r-RNA sequence of isolated bacterial strain PBIL-2 has shown (Fig. 1) more homology with reported *Bacillus megaterium*.

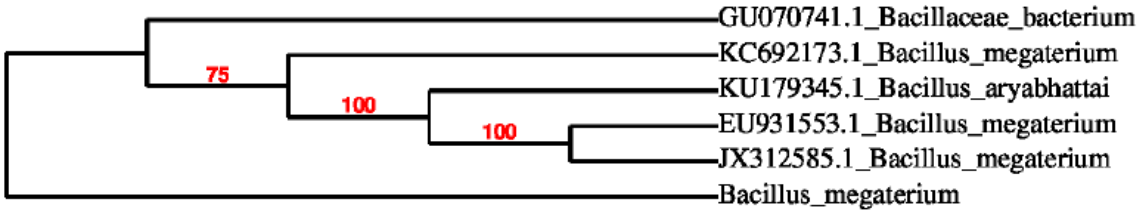


Fig. 1. Phylogenetic tree constructed for bacterial isolate PBIL-2

### *Effect of substrates on production of pectinase*

At industrial scale, the development of enzyme mainly depends on the cost of enzyme production. In order to meet industrial needs, high yielding pectinase producing microorganisms are appropriate that can cultivate on agro-industrial residues to produce the enzyme cost effectively [18]. We have developed a cost-effective medium for production of pectinase using agro wastes as substrates. Microbial synthesis of primary metabolites is greatly influenced by their growth, which in turn is evaluated by the availability of nutrients in the substrate. Improvement of nutritional value of production medium by its supplementation with a suitable substrate will improve the growth of the bacterial strain, resulting in enhanced enzyme production [19]. On adding various substrates to the production medium, pectinase production by *B. megaterium* PBIL-2 was observed to be slightly lower in the presence of onion peel, cabbage waste and apple pomace (Fig. 2). In contract, enhanced pectinase production was observed on supplementation of production medium with processed orange peel powder. Previously it was reported that citrus pectin was found to increase production of pectinase in *G. stearothermophilus* Ah22 [20] and *B. pumilus* P9 [21].

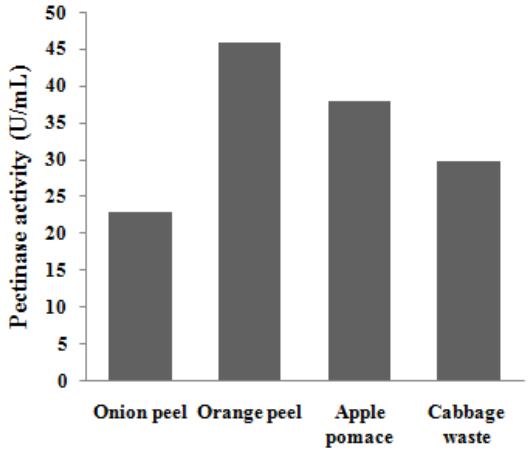


Fig. 2. Effect of substrates on production of pectinase

### *Optimized production parameters*

*Effect of medium pH*

The effect of pH of the production medium was evaluated in the range of 3-10 on the production of pectinase enzyme. It was noticed that pectinase production by *B. megaterium* PBIL-2 increased with increase in pH up to 6.0, followed by continuous decline with further increase in pH up to 10.0. Hence it was concluded that optimum pH for production of

 

pectinase by *Bacillus megatirium* PBIL-2 is 6.0 (Fig. 3). Due to less growth of the bacterial strain at pH value above the optimum value, enzyme production might be decreased. In contract, Simran et al. [6] reported that pH 4.0 was optimum for production of pectinase by *B. subtilis* SAV- 21.

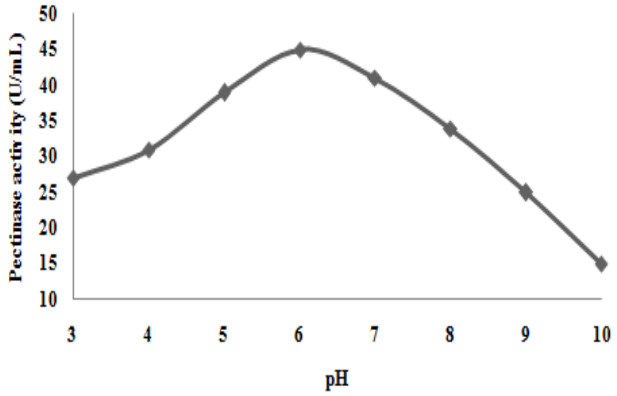


Fig. 3. Optimized pH for pectinase production

*Effect of temperature*

Microbial growth and metabolic activity of enzymes is greatly affected by temperature. The effect of temperature was estimated in the range of 25-50°C on the production of pectinase enzyme. The highest production (Fig. 4) of pectinase by *B. megaterium* PBIL-2 was found to be 35°C. Hence it was concluded that optimum temperature for production of pectinase by *B. megaterium* PBIL-2 is 35°C. Simran et al. [6] reported that 35°C was optimum temperature for production of pectinase by *B. subtilis* SAV-21 using agro residues.

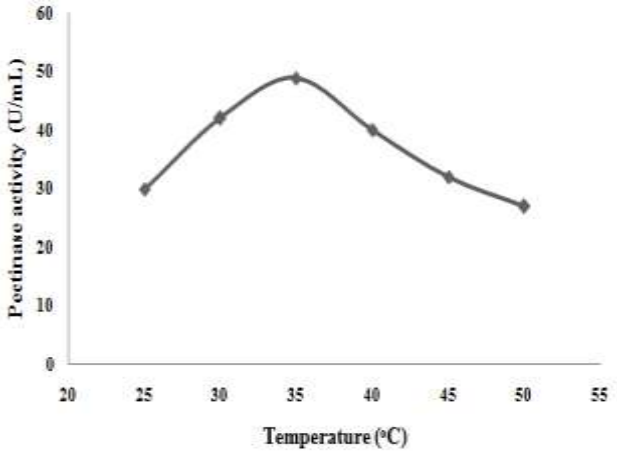


Fig. 4. Optimized temperature for pectinase production

*Effect of fermentation time*

The effect of varying fermentation time was tested for 12-36 h on the production of pectinase enzyme. The data indicated (Fig. 5) that maximum production of pectinase by *B. megaterium* PBIL-2 was occurred after a period of 18 h. Further increase in incubation period decreased pectinase production due to depletion of the nutrients.

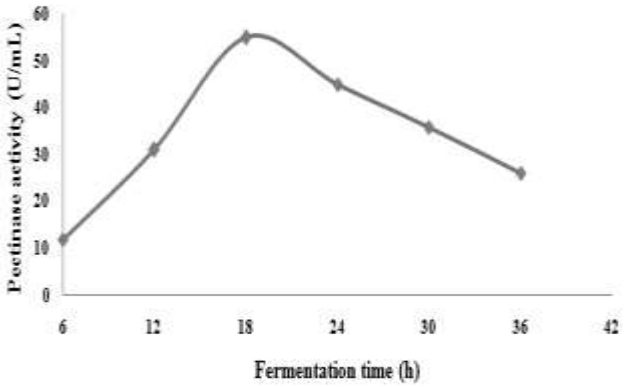


Fig. 5. Optimized fermentation time for pectinase production

*Effect of inoculum age and size*

All other production parameters like pH, temperature and fermentation time were kept constant at their respective maximum value. Then, the effect of inoculum age and size were estimated on the production of pectinase enzyme in the range of 12- 46 h and 1-10 % (v/w) respectively. It was noted that maximum production of pectinase was found at 24 h of inoculum age (Fig. 6) and 3% of inoculum size (Fig. 7). Similar to present study, Amanjot et al [14] reported that 24 h old inoculum was suitable for pectinase production using *B. pumilus* AJK. Growth of the microorganism can be influenced by inoculum size and hence the competence of enzyme production depends on inoculum size. Large size of inoculum results in lower enzyme yield as it limits the metabolic activity due to the limitation of nutrients, whereas a small size inoculum consequences in lesser growth by lowering the number of microbial cells in the production medium [22].

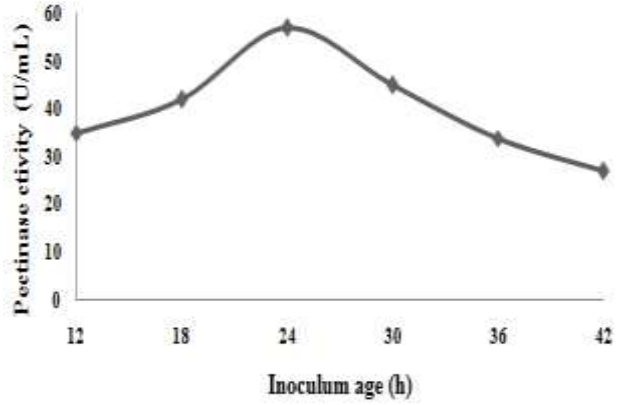


Fig. 6. Optimized inoculum age for pectinase production

 

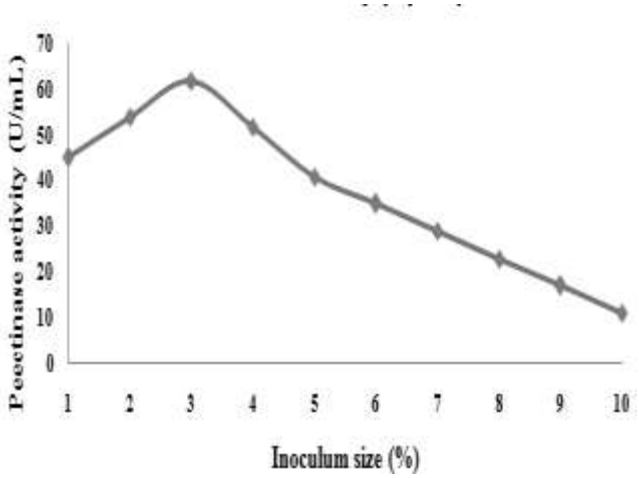


Fig. 7. Optimized inoculum size for pectinase production

### *Purification of pectinase*

Purification of pectinase after ammonium sulphate precipitation (Table 1) showed 0.92 mg/mL protein and 78.26 U/mg specific activity. An increase in enzyme purity was recorded after dialysis with 86.54 U/mg specific activity. Gel filtration chromatography revealed that purified enzyme has 105.71 U/mg specific activity with 1.56 purification fold. Similar work was reported by Ping et al., [23] for the production of pectinase using *Bacillus subtilis* ZGL14.

### *Characterization of pectinase*

*Effect of pH and temperature on activity and stability*

The obtained results confirmed that maximum activity of pectinase produced by *B. megaterium* PBIL-2 was observed at pH 5 (Fig. 8). Produced pectinase enzyme was incubated at various temperatures ranging from 30 to 60 °C. The results confirmed (Fig. 9) that 40 °C was optimum temperature of purified pectinase enzyme.

Table 1. Pectinase purification summary

| Purification steps | Volume (mL) | Enzyme activity (U) | Protein content (mg/mL) | Specific activity (U/mg) | Purification fold |
|---|---|---|---|---|---|
| Crude enzyme | 100 | 62 | 0.92 | 67.39 | 1 |
| Ammonium sulphate precipitation | 25 | 54 | 0.69 | 78.26 | 1.16 |
| Dialysis | 15 | 45 | 0.52 | 86.54 | 1.28 |
| Gel filtration chromatography | 10 | 37 | 0.35 | 105.71 | 1.56 |

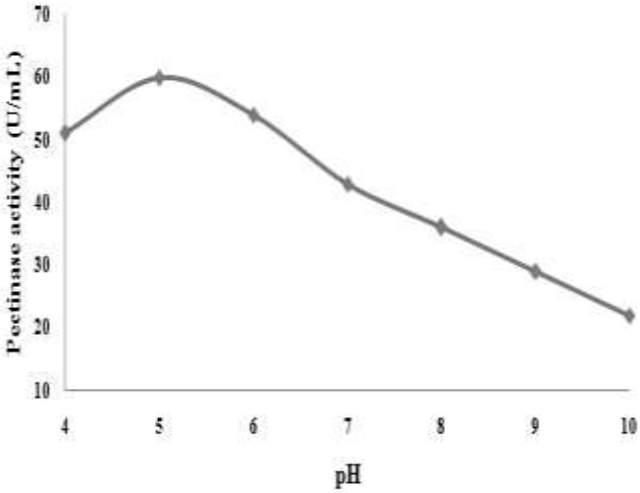


Fig. 8. Effect of pH on pectinase activity

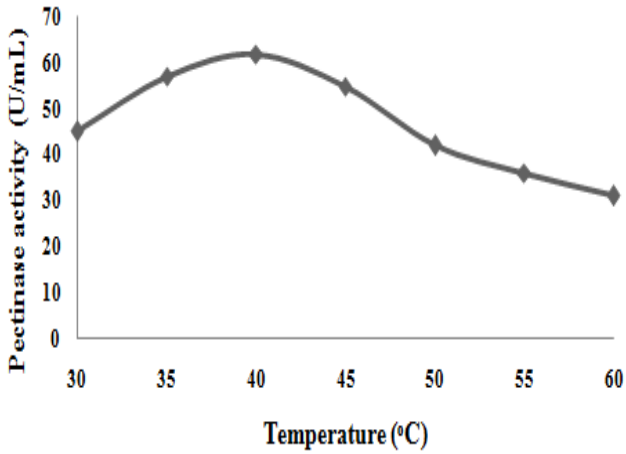


Fig. 9. Effect of temperature on pectinase activity

 

## Conclusions

In the present study, pectinase enzyme was produced by using *Bacillus megaterium* PBIL-2 isolated from fruit and vegetable market waste. The results showed that *Bacillu*s species were predominant in all fruit and vegetable waste. During fermentation, orange peel fine powder has been used as substrate. The maximum specific activity of purified pectinase enzyme was obtained by Gel filtration chromatography (105.71 U/mg). So, these data specified that agro residue was a better source for production of pectinase.

## Conflicts of Interest

Authors declare no conflict of interest.

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