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Original Article

Purification and Production Optimization of Bacterial Pectinases Using Fruit Waste

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ABSTRACT

Objectives: Pectinase plays a crucial role in the commercial food processing industry by aiding in the breakdown of pectin and facilitating various processing stages like liquefaction, clarifying, and juice extraction. To meet the increasing demand for these essential enzymes for industry, a thorough investigation of pectinases from different organisms will be beneficial in optimising growth conditions and reducing the cost of production. **Materials and Methods:** The present study focussed on pectinase production from Bacillus species, optimization of the carbon, nitrogen and growth factors in achieving cost-effective production of pectinase. **Results:** Higher concentrations of pectinases were produced with 5% pectin concentration, 5% inoculum size and fermentation broth with extra carbon source i.e., 1% dextrose. **Conclusion:** We were able to increase the extracellular pectinase yield to 4.8 IU/mL fermentation broth by using *Bacillus licheniformis*.

Keywords: *Bacillus licheniformis*, Pectinases, Fruit waste, Enzymatic assay, Column Chromatography.

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INTRODUCTION

Pectinases are the enzyme that breaks down pectic compounds present in agricultural and culinary products.[1] Renewable resources, such as agricultural and forest leftovers, are becoming increasingly important to meet global energy demands. The main components of these residues are pectin, lignin and cellulose.[2] These substances may serve as carbon and energy sources for microorganisms, which can produce a wide variety of enzymes.[3] All terrestrial plants' main cell walls contain a significant amount of pectin. Colloidal polysaccharides with a galacturonic acid backbone joined by α (1-4) links are known as pectin polymers.[4] Pectin induces the synthesis of pectinolytic enzymes in microbial environments.[5] Several studies have been conducted on pectinase synthesis using several genera of filamentous fungi like Penicillium, Rhizopus and Aspergillus. SSF accounts for the majority of pectinase synthesis. Pectinase breaks down ester bonds between carboxyl and methyl groups and opens glycosidic connections to produce monoglacturonic acid from the pectin polymer polygalacturonic acid.[6] The extensive range of uses in the food business necessitates a huge pectinase market. Pectinases are commonly employed to break down pectin on plant primary cell walls in food production sectors because they speed up liquefaction, clarifying, and juice extraction.[7,8] It is currently utilised in the beverage and oil extraction sectors to handle pectin-rich industrial waste, in addition to its extensive use in the winemaking and fruit juice industries.[9-11] Filamentous fungi like *Trichoderma viridiae, Aspergillus niger, Phanerochaete chrysosporium* and several bacterial genera like Bacillus and Serratia were identified as potent pectinase-producing organisms.[12-15] Submerged fermentation is the most beneficial technique for producing pectinase on a wide scale because of its downstream processes, simplicity of scaling, and optimisation using different bio-processing technologies, like fed-batch and continuous culture methods. Low-cost carbon sources such as rice bran, molasses, and fruit waste from industries are among the alternative substrates for pectinase synthesis.[16-20] Peptinases are among the most significant industrial enzymes. Research is being done to produce pectinases with better activity and characteristics as well as to use less expensive substrates for better process economics. Several studies have been conducted on pectinase synthesis by fungal species of Penicillium and Aspergillus.[21] SSF is the method used for the majority of pectinase manufacturing. Acidic pectinases have a broader range of uses in the food industry compared to alkaline pectinases.[22] Since pectinases are essential to the natural decomposition process, they can be found abundant in decaying fruit and vegetable waste. Pectinase is used in the fruit processing industry for a variety of purposes, one of which is fruit juice clarity. *Bacillus licheniformis* produces

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alkaline pectinase, which can be utilised to remediate wastewater from the food and vegetable processing sectors textile industry and food processing industries.[23] Pectinases are enzymes that aid in the depolymerization and de-esterification processes needed to break down pectic substances. Microbial pectinases are more advantageous since they are involved in the Phyto pathologic process, plant-microbe symbiosis, and the decomposition of dead plant material.[24-27] Pectinases are categorised based on how they operate on the galacturonan component of pectin molecules.[28]

In this study, we report on the synthesis, refinement, and enhancement of pectinases from Bacillus sp. using submerged fermentation with various fruit peel wastes as substrate.

MATERIALS AND METHODS

Extraction of Pectin from Fruit peel

Various fruits such as mango, banana, orange, pomegranate, grapes, and papaya were bought from local vendors in Guntur. Fruit peel waste was then dried overnight in the shade followed by oven drying (80ºC for 30 min) and ground using a mortar and pestle. The powder is then filtered using a fine muslin cloth. The resultant fine powder was kept until it was used at 4ºC.

Microorganism and inoculum preparation

100 mL of LB broth were inoculated with 1% *Bacillus lichenformis* culture, and incubated at 37ºC for 4 days. After that, the Bacillus culture was moved to the production medium.

Pectinase Production Medium

Pectinase was produced using a simple liquid fermentation medium with 1-5% (w/v) pectin powder from fruit peels, 1% yeast extract and with or without 1% dextrose as an extra carbon source. Production media to carry out submerged fermentation was made in 250 mL conical flasks and autoclaved for 20 min at 15 psi. Flaks were then inoculated with 1-5% of Bacillus broth and incubated under continuous agitation for 5 days at 37ºC. After centrifuging the broth for 10 min at 8000 rpm, the supernatant obtained was used as a source of unprocessed enzymes Table 1.

Purification of Pectinases

Seventy per cent saturation of ammonium sulphate was used to precipitate the crude enzyme. The solution after precipitation was centrifuged for 10 min at 8000 rpm. 1 mL of potassium phosphate buffer (10 mM) was used to resuspend the pellet. Dialysis was carried out using 10 mM potassium phosphate buffer for 3 hr, with a buffer change every hour to eliminate salts. Dialysed samples obtained are collected and stored at 4ºC till further analysis. Purification of the enzyme was done on a Sephadex G-100 gel filtration column (400 mmx20 mm) with a column matrix volume of 40 mL. Equilibration was done using 10 mM potassium phosphate buffer and the flow rate was set to 1 m/min during fractionation. Plotting a chromatogram was done after eluted fractions were analysed at 280 nm. Pectinase active fractions obtained from the gel filtration column were pooled and concentrated.

Pectinase activity assay

Pectinase splits polygalacturonic acid into mono-d-galacturonic acid by reducing glycosidic linkages. The DNS (3,5-Dinitrosalicylic acid) method was employed to estimate the concentration of galacturonic acid emitted to test pectinase activity. 0.5% pectin dissolved in 10 mL 10 Mm potassium phosphate buffer was used as a substrate. 500 µL of purified pectinase enzyme and 500 µL of pectin substrate were taken in test tubes and incubated for 30 min at 50ºC. After adding 3 mL of DNS reagent, the mixture was heated in a boiling water bath and incubated for 10 min. The UV-visible spectrophotometer was then used to measure absorbance at 540 nm. Galacturonic acid produced during the enzymatic reaction was used to quantify pectinase activity. Mono-d-galacturonic acid (1 mg/mL) was utilized in plotting the standard curve. A standard graph of mono-d-galacturonic acid was plotted with concentrations 0-1000 µg/mL and their corresponding absorbance values at 540 nm. The amount of

Table 1: Pectinase optimization studies.

% Inoculum	Flask-1	Flask-2	Flask-3	Flask-4
1% Inoculum	1% Pectin Yeast Extract (1%) Dextrose (1%).	3% Pectin Yeast Extract (1%) Dextrose (1%).	5% Pectin Yeast Extract (1%) Dextrose (1%).	1% Pectin Yeast Extract (1%) No Dextrose.
	Flask-5	Flask-6	Flask-7	Flask-8
3% Inoculum	1% Pectin Yeast Extract (1%) Dextrose (1%).	3% Pectin Yeast Extract (1%) Dextrose (1%).	5% Pectin Yeast Extract (1%) Dextrose (1%).	1% Pectin Yeast Extract (1%) No Dextrose.
	Flask-9	Flask-10	Flask-11	Flask-12
5% Inoculum	1% Pectin Yeast Extract (1%) Dextrose (1%).	3% Pectin Yeast Extract (1%) Dextrose (1%).	5% Pectin Yeast Extract (1%) Dextrose (1%).	1% Pectin Yeast Extract (1%) No Dextrose.

pectinases producing one micromole of galacturonic acid during enzyme reaction in one minute was defined as one unit (U).[29]

Statistical analysis

The data was presented as Mean±SD. Every outcome was determined to be statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Pectinase production

A 1%-5% *Bacillus lichenformis* inoculum size was utilised to maximise pectinase output. Similarly, various initial substrate concentrations were maintained with pectin (1%, 3% and 5%), dextrose (0-1%) and 1% yeast extract to optimize pectinase production. For five days, submerged fermentation was performed in a 250 mL flask with the variables mentioned above. Centrifuging the broth for 10 min at 8000 rpm isolated the pectinase that had been released into the medium. Ammonium sulphate precipitation was used to recover the pectinase enzymes in the supernatant.

Purification of pectinase using chromatography

Crude enzyme obtained from ammonium sulphate precipitation was fractionated using CM Sephadex G 100 gel filtration chromatography. The chromatogram produced by gel filtration chromatography is displayed in Figure 1. Pectinase activity was examined in all of the chromatogram's peak fractions. The red-highlighted protein peak indicated a strong level of pectinase activity.

Effect of inoculum size on pectinase production

Pectinase was produced via submerged fermentation with three different *Bacillus lichenformis* inoculum sizes (1%, 3%, and 5%). In comparison to the other two versions, we found that the 5% inoculum size produced the highest amount of pectinases Figure 2.

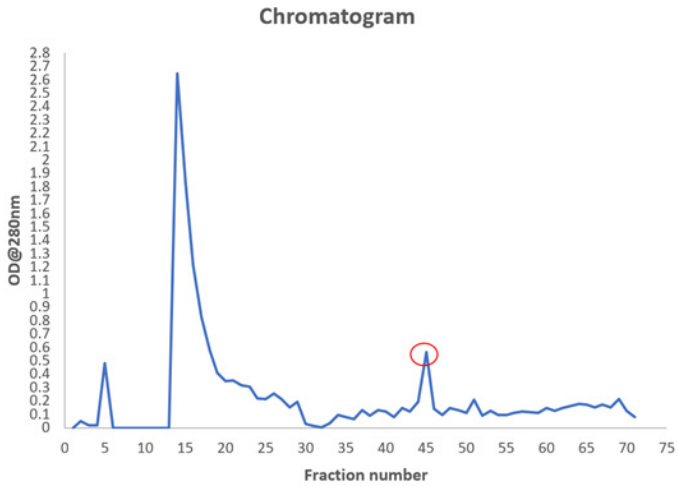

Figure 1: Gel filtration chromatogram.

Effect of inducer on pectinase production

Dried powder of fruit peels was used as a pectin source to induce pectinase production as well as a carbon source for microbial growth. We used 1-5% pectin powder to induce pectinase production. With 5% (W/V) of pectin powder, higher concentrations of pectinases were produced than with the other variations (Figure 3). We observed slightly higher levels of pectinase production when 1% dextrose was added as an additional carbon source to the fermentation media.

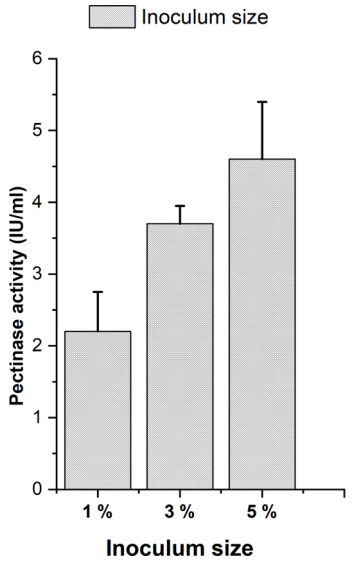

Figure 2: Effect of inoculum size on pectinase production.

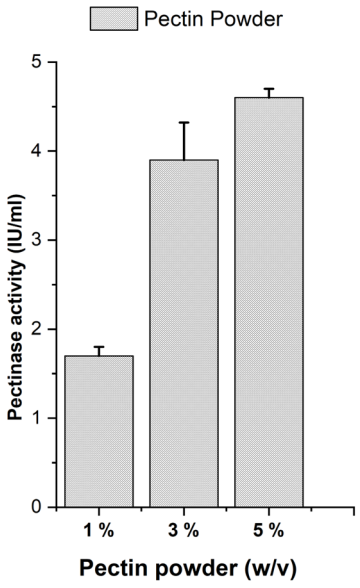

Figure 3: Effect of inducer on pectinase production.

DISCUSSION

Pectinases represent a crucial class of industrial enzymes that belongs to the category of pectin-degrading enzymes used in a variety of sectors, including fruit juice clarification, winemaking, coffee and tea fermentation. These enzymes are highly selective, non-toxic, show long-lasting activity and are environmentally beneficial. Microbial pectinases play a crucial role in nature by aiding in the environment's natural recycling of carbon. Numerous microorganisms have been found to produce pectinases with different molecular mass and catalytic properties. The market demand for pectinases and their application in different industries is constantly increasing. However, the production of pectinases is limited due to higher capital costs associated with conventional substrates used for enzyme production. As a result, cheaper alternative substrates such as agricultural biomasses were prioritised in the production of pectinases. The purification and biosynthesis of pectinases utilising *Bacillus lichenformis* and optimising the carbon, nitrogen and growth factors was the main focus of this investigation. Pectinase production was optimized using different concentrations of yeast extract as a nitrogen source, pectin from various fruit peels as an inducer and carbon source and dextrose as an additional carbon source for submerged fermentation.

Optimization studies were performed with *Bacillus licheniformis* inoculum size ranging from 1-5%, Inducer and primary carbon source concentrations (pectin) (1%, 3% and 5%) and with or without the additional carbon source i.e., dextrose. 1% yeast extract was used as a nitrogen source in all the experimental conditions to optimize pectinase production. In broth made with 1% dextrose, we observed a greater quantity of pectinase. We observed a linear increase in pectinase production by increasing the inoculum size of *Bacillus licheniformis* from 1-5%. The biosynthesis of pectinase was significantly increased when the concentration of pectin (inducer) was increased from 1 to 5%. We observed maximum production of pectinases at 5% inducer concentration. The concentration of pectinases produced by *Bacillus licheniformis* was optimised to 4.7 IU/mL of fermentation broth.

CONCLUSION

Several media variations were used to optimise the biosynthesis of pectinase from *Bacillus licheniformis*. When the pectin concentration was 5%, the inoculum size was 5%, and the fermentation broth contained an extra carbon source i.e., 1% dextrose higher concentrations of pectinases were produced. Based on the results, submerged fermentation with media optimization was found to double the amount of extracellular pectinases produced by *Bacillus licheniformis*.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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