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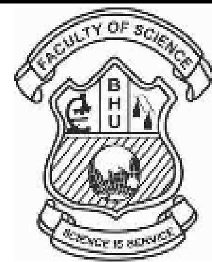

Green Enzymes from Fruit Waste *Aspergillus Sp*-Derived Pectinase

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Abstract: **Pectinase is one of the essential enzymes used in industry and biotechnology, along with cellulase and amylase. This study aimed to isolate pectinase-enzyme-producing organisms and characterise them. Fungal cultures with pectinase activity were isolated from different samples of rotten fruits like oranges, bananas, pomegranates, and soil samples. The fungal culture S2A, which was taken from the orange, had the most polygalacturonase (PG) enzyme activity out of the five samples. Its activity peaked on the fourth day at 0.179 U/mL of incubation under stationary conditions. According to microscopic examination and morphological characteristics, the culture was Aspergillus sp. The optimum pH for the activity of the enzyme was 6.0. The polygalactorunase enzyme from S2A culture was not thermostable at 70 °C. The pectinase enzyme was concentrated using the solvent precipitation method at 3.55 U/mL. Pectinase enzymes from isolated cultures have shown positive results in clearing apple juice through pectin degradation, which can be used in the fruit industry.**

***Keywords: Aspergillus*, pectinase, and Polygalacturonase (PG) production.**

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I. INTRODUCTION

Pectin is a structural heteropolysaccharide present in the primary cell walls of terrestrial plants. Henri Bracannot was the first to isolate and describe pectin in 1825. There are four main types of pectic substances: pectic acids, pectinic acids, protopectin, and pectin. Pectic acid mainly consists of colloidal polygalacturonic acid and mostly lacks methyl ester groups. Polygalacturonic acid and pectic acid are other names for demethylated pectin. (Bharadwaj et al., 2017; Maskey et al., 2018).

It has the highest concentration in the middle lamella, which plays a crucial role as a strengthening substrate between adjacent cells. Pectinases are a group of seven different enzymes. They are known as pectinesterase (EC3.1.1.11), polygalcturonase (EC3.2.1.15), galacturan 1, 4-α-galactouronidase (EC3.2.1.67), exopoly-α-galactouronosidase (EC3.2.1.82), endopectate lyase (EC4.2.2.2), exopectate lyase (EC4.2.2.9), and endopectin lyase (EC4.2.2.10) (Thakur et al., 2021).

Pectinase enzymes are capable of breaking down pectin during the ripening of fruits. (Bharadwaj et al., 2017; Mehmood et al., 2019).

Pectinase is well-known worldwide for producing helpful enzymes and for the enzyme-catalysed breakdown of polygalacturonic acid into monogalacotouronic acid by breakage glycosidic linkage. (Bhardwaj et al., 2017; Mehmood et al., 2019).

Pectinase enzymes are widely used in different industries, such as in the food processing industry, for the production and clarification of fruit juices to develop the stability of fruit and vegetable juices and to produce high-density fruit juice

concentrates for haze elimination from wines, in the paper and pulp industry for the acidifying of cotton and plant fibres, discoloration of paper, wastewater treatment, extraction of vegetable oil, tea and coffee fermentation, and in alcoholic beverage production. (Dhillon et al., 2004; Kc et al., 2020).

Producing polygalacturonase from microbial sources has advantages such as resistance to climatic or seasonal changes and the ability to implement genetic manipulation to enhance productivity. (Dhillon et al., 2004). Some of the factors that affect the choice of the microbial source for making the polygalactouronic enzyme are its genetic make-up, how stable the enzyme is at different temperatures and pH levels, and how well the culture works in food applications. (Khairnar et al., 2009).

Peels of fruits, rotten fruits and vegetables, soil, and dump yards are common sources for the isolation of pectinase-producing cultures. Various genera of bacteria, yeasts, and moulds have been utilised in the production of polygalacturonase such as Bacillus spp., Clostridium spp., Pseudomonas spp., Aspergillus spp., Monillalaxa, Fusarium spp., Verticillium spp., Penicillium spp., Sclerotinia libertiana, Coniothyrium diplodiella, Thermomyces lanuginosus, Polyporussquamosus, etc. (Dhillon et al., 2004).

Researchers have focused on new microbial isolates that produce the pectinase enzyme with desirable properties and low production costs. (Bibi et al., 2018).

Pectin is degraded by applying these enzymes to fruit pulp. Hence, the fruit juice can be handled easily due to its reduced viscosity. (Bibi et al., 2018). Therefore, the main aim of this study was to isolate pectinase-producing organisms from agro-waste and implement it for juice clarification.

II. MATERIALS AND METHODS

A. Sample collection and Isolation of Pectinase enzyme-producing organisms:

The samples were collected from a nearby fruit and vegetable waste dump in Market Yard (Pune, Maharashtra, India, latitude: 18.4877° N and longitude: 73.8684° E). Rotten fruit and soil samples were spread-plated, and these samples were inoculated on minimal media that contained pectin as a sole carbon source. (g\L; $NaNO_3$ 0.2 g, $MgSO_4$ 0.05 g, $FeSO_4$ 0.01 g, K_2HPO_4 1 g, pectin 10 g, agar 30 g) (Bibi et al., 2018).

The cultures were isolated from rotten fruit peels and soil samples. The peels were suspended in sterile saline, and this saline with peels was kept in a shaker for an hour to allow the release of adhered spores and cells into the saline. Purified cultures were spot-inoculated on pectin agar plates to check pectin hydrolysis.

B. Screening of culture for enzyme production:

0.1 mL of the saline was spread and plated on a minimal medium containing 1% pectin as a sole carbon source; the plates were incubated for 48 h at 37°C and 25°C. The hydrolysis zone was checked by adding a 1% iodide solution. (Bibi et al., 2018).

C. Identification and characterization of microorganisms:

The lactophenol cotton blue stain method was used to identify the fungal isolate that showed acid production. A bright field microscope with a 40X magnification was used to observe the morphology, characteristics of the hyphae, the presence or absence of asexual spores, and the arrangement of the conidia. (Kc et al., 2020).

D. Determination of polygalacturonase activity:

To determine the polygalacturonase PG activity, the 3, 5-dinitrosalicylic acid DNSA method (Miller, 1959) was used. For this, in a test tube, 0.5 mL of the crude enzyme was added to 0.5 mL of the substrate. A 1% pectin (6% esterified) solution was prepared in 0.1 M acetate buffer, pH 5.0. The test tube was then heated to 35°C and incubated at room temperature (25–26°C) for 20 minutes. 1 mL of the 3, 5-dinitrosalicylic acid DNSA reagent was added to each test tube, and the test tube was shaken to mix the contents. The test tubes were heated to boiling in the boiling water bath for 10–15 minutes. The tubes were then chilled, and distilled water was added to the contents before measuring absorbance at 540 nm (Dhillon et al., 2004). The amount of enzyme required to catalyse the liberation of 1 micromole of galacturonic acid from pectin per minute at room temperature (25–26°C) is defined as one unit of enzyme activity. (Patil & Dayanand, 2006).

E. Time course for enzyme production:

To study the production of enzymes, the most potent fungal isolate was allowed to grow in pectin broth and incubated for 6, 12, 24, 48, 72, 96, 120, 144, 168, and 192 hours, respectively. The culture supernatant was obtained, and the enzyme assay was carried out at pH 5.0 at room temperature (25–26°C). (Dhillon et al., 2004).

F. Optimization of pH for enzyme production:

The study of the effect of pH on polygalacturonase PG production was determined by measuring the polygalacturonase PG activity at different pHs (5, 6, 7, and 8) by using 0.1 M acetate buffer. The enzyme assay was performed at 5.0 pH at room temperature (25–26 °C). (Deshmukh et al., 2012).

G. Optimization of Temperature for enzyme production:

The spore suspension was inoculated into a flask containing 100 mL of sterile minimal medium containing 1 g% pectin and incubated at room temperature for 48 hours. Centrifugation at 10,000 rpm for 10 minutes produced aliquots. The supernatant was used as a crude enzyme and was kept at 70 °C in a water bath for different time intervals (0 min, 15, 45, 90, 150, and 165 min).

H. Precipitation of enzyme by solvent precipitation method:

1 mL of spore suspension was inoculated in a sterile minimal medium containing 1 g% pectin at optimised conditions. The flask was incubated at room temperature under stationary conditions for 48 hours. After incubation, the culture supernatant was obtained by centrifugation and was used as an enzyme sample for solvent precipitation. An equal volume of chilled acetone was used for precipitation. The dilution of the precipitated enzyme used for determining enzyme activity was 1:50. Enzyme activity was determined by the 3,5-dinitrosalicylic acid DNSA method. (Scopes, 1982).

I. Fruit Juice Clearance:

For juice clarification 5 g of crushed apple with 20 mL of acetate buffer was added to one test tube, and 10 mL of crude enzyme pectinase was added to another test tube. 5 g of crushed apple with 20 mL of 0.1M acetate buffer, pH 5.0, was added to the test tube, along with 10 mL of distilled water. This mixture was incubated for 48 hours at room temperature (27°C) (Dange & Harke, 2018).

III. RESULTS

A. Isolation and screening of pectinase producing bacterial organism:

Five organisms were isolated from the soil, orange and banana peels, and primarily screened based on pectinolytic activity using the spread plate method. Cultures S2A and S2B were from orange internal tissue; culture S4 was from a banana soil sample; and culture S7 was from a banana sample (Table 1). The culture with the largest hydrolysis zone in the potassium iodide assay was selected as the positive culture. The culture (S2A) was found to have pectinolytic activity. (Figure 1).

Table 1. Results of screening for pectinase enzyme-producing cultures

Culture from Sample	Zone of diameter (cm)
Orange peel S1	0.3
Orange internal tissue S2A	1
Orange internal tissue S2B	1.3
Banana Soil Sample S4	2.1
Banana Sample S7	1.7

Fig.1. Cultures Number S2A on pectin agar plate

B. Identification and characterization of organism:

The growing cultures were taken, and lactophenol cotton blue staining was carried out and observed under the microscope at 40X to determine morphological characters. According to microscopic examination, the culture S2A belonged to *Aspergillus sp.*

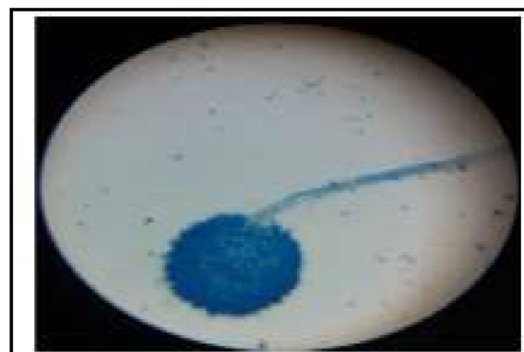
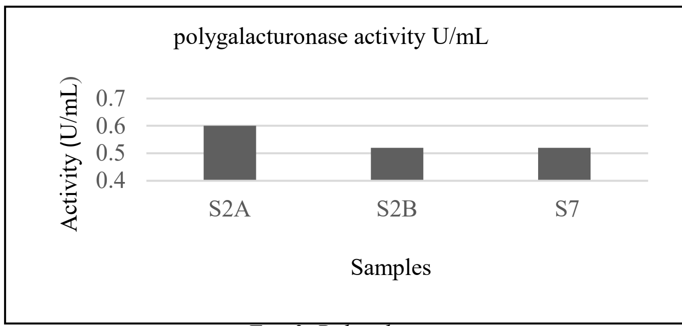


Fig 2. Lactophenol cotton blue stain

C. Determination of polygalacturonase activity:

The polygalacturonase enzyme breaks down pectin into galacturonic acid subunits, which are reducing sugars that may be determined by the 3,5-dinitrosalicylic acid (DNSA) method. The culture (S2A) had the highest pectinolytic activity (0.6 U/mL).



ss *Fig. 3. Polygalacturonase activity*

D. Time course for enzyme production:

The maximum enzyme activity of culture S2A was seen on the fourth day; enzyme activity was observed at 0.179 U/mL. The enzyme activity showed a decline as the incubation period rose (96 h).

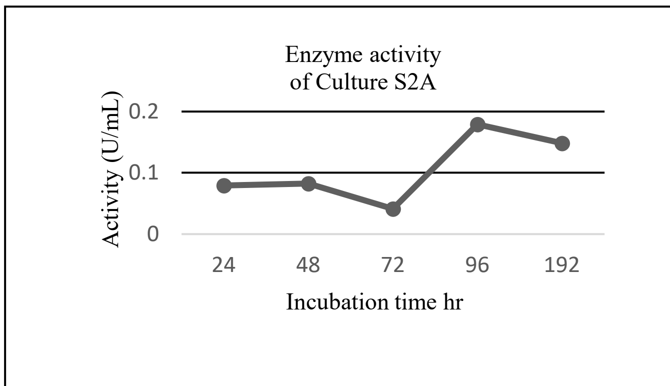


Fig. 4. Enzyme activity of Culture S2A

E. Optimization of pH for enzyme production:

PH greatly influences enzyme function. In this study, pectinase showed strong activity between pH 5.0 and 8.0, with the most optimal pH being 6.0 on the third day of incubation. As shown in Figure 5.

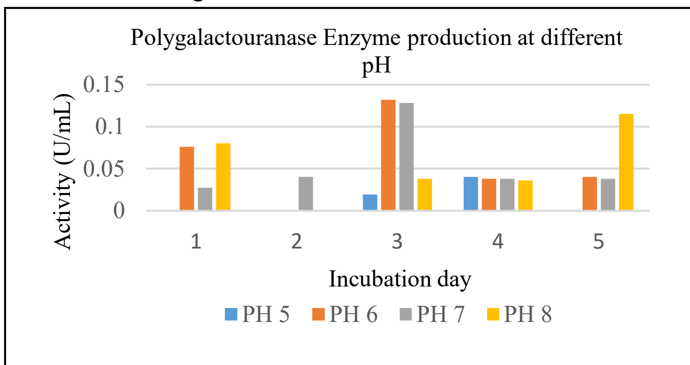


Fig. 5. Polygalactouranase Enzyme production at different PH

F. Determination of enzyme stability at 70 °C:

Enzyme activity is significantly influenced by the enzyme's stability at higher temperatures. In this study, the enzyme activity is decreasing because of temperature. As shown in Figure 6,

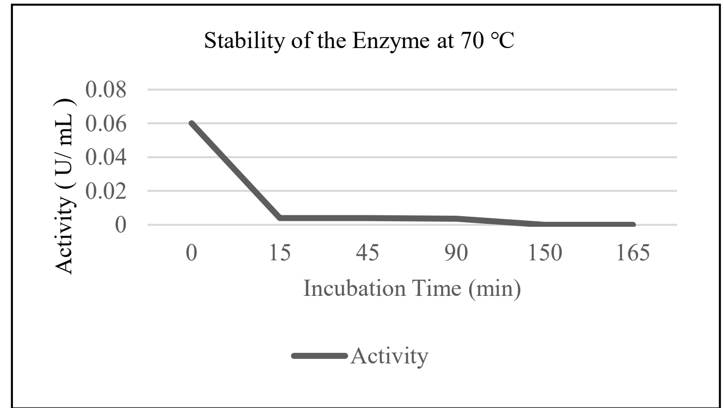


Fig. 6. Stability of the Enzyme at 70 °C

G. Concentration of Enzyme by solvent precipitation:

The pectinase enzyme was concentrated by the solvent precipitation method. The precipitated sample was diluted 1:50 times and was used to determine enzyme activity. 3.55μmole/ml/min (U/mL).

H. Clarification of Apple Juice by Pectinase.

This study aimed to assess the effect of pectinase on the clarity of juice through the use of apple fruits. The experiment utilised partially purified crude pectinase, as indicated by the results in Figure 7. After 48 h, the pectinase helped in the clearance of juice by degrading pectin and showed less turbidity.

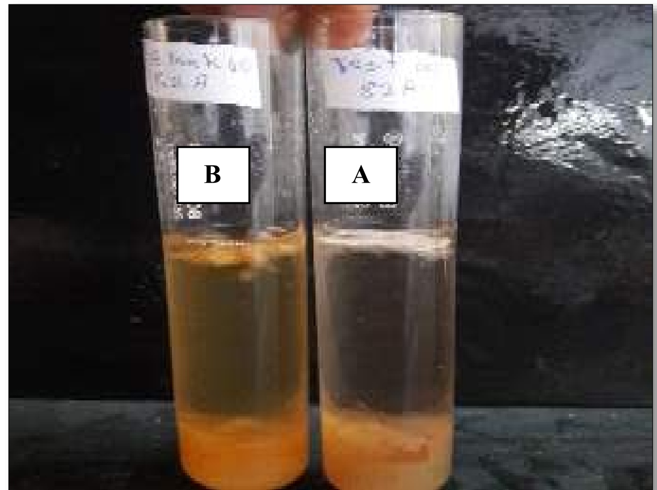


Fig.7. the effect of pectinase on fruit juice clarification: B-(control), A- Test (After clarification)

IV. DISCUSSION

The pectinase enzyme has different applications in the biotechnology sector; it is used to clarify fruit juice by breaking down the pectin. Manufacturing enzymes is one of the most developing fields, with over 1.5 billion enzymes produced globally. Most industrial enzymes are microbially derived. The previous literature indicates that rotten fruits are a source of the pectinase enzyme. (Bayoumi and colleagues, 2008; Bhardwaj et al., 2017).

Previous studies have highlighted the significant role of *Aspergillus sp*. in the industrial manufacturing of the pectinase enzyme. (Bibi et al., 2018; Kc et al., 2020). Our results showed that *Aspergillus sp*. could be significantly exploited as a potential source for the production of the pectinase enzyme. Compared to other results showing that *Aspergillus sp*. could also be used for enzyme production (Bibi et al., 2018),.

The fungal culture was isolated from rotten fruits such as oranges, bananas, and pomegranates, and the soil had pectinase enzyme activity. The fungal culture (S2A) from orange showed the maximum pectinase activity on the fourth day of incubation, and the enzyme activity was 0.179 mole/ml/min at pH 5.0. Our findings are strongly correlated to several studies of rotten fruits that confirm these sources are some of the most natural resources for pectinase. (Kashyap et al., 2001; Dhillon et al., 2004).

Pectinolytic enzymes have been regularly found to have higher enzymatic activity between pH 4.0 and 7.0. But their activity and stability may vary at different pH levels. (Ferreira et al., 2010; Kc et al., 2020). Maximum enzyme activity for S2A culture was about 0.076 mole/ml/min. and was observed at pH 6.0.

Enzyme thermostability refers to its capacity to withstand thermal unfolding without the presence of substrates. It is crucial to comprehend the unique characteristics of each pectinase preparation in order to determine the best circumstances for its utilisation, especially in industrial settings where high temperatures are frequently encountered. The previous literature found that the thermos ability varied depending on the enzymatic extract of the strain. . (Dhillon et al., 2004; Sandri & da Silveira, 2018). The activity of S2A culture decreased by increasing the time of incubation at 70 °C.

Many researchers used the precipitation method as the first step in their purification process. (Ahmed & Sohail, 2020). Therefore, the pectinase enzyme was concentrated by the solvent precipitation method.

Several studies have reported that the increase in enzyme concentration has greatly improved the clarity of the juice. (Sandri & da Silveira, 2018; Haile et al., 2022).

Pectinase produced by S2A showed positive results in the clearance of apple juice by degrading pectin.

Our findings bring up an interesting new line of research focusing on natural sources, among other sources that are cost-effective and eco-friendly. But more research is needed to identify the strain at the genetic level, ensure the stability of the culture, and make sure it can be used commercially in making and processing food on a large scale.

IV. Conclusion

Historically, fruit juice extraction typically produced a cloudy appearance, appealing hue, and thick consistency. The fruits contain a significant amount of pectin.

In the present study, five samples of waste fruits were isolated to produce pectinase enzymes, which highlights the importance of using agro-waste as a pectinase source. This way is environmentally friendly, cost-effective, and proficient. One strain was classified as *Aspergillus sp.* based on morphological characteristics. However, pectinase enzymes produced in this way can be used in the clearance of apple juice by the degradation of pectin. Further study on this enzyme will be necessary to enhance the efficiency of *Aspergillus sp*. for application in the fruit juice industry.

V. Acknowledgment

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