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Insights of Pectinase from Fungi

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ABSTRACT

The plant's cell walls contain pectin, a gelatinous heteropolysaccharide that is integrated into the cell and other parts of the cell wall that allow cells to co-exist and are essential for plant growth. Enzymes called pectinase serves as an important agent in the breakdown of the components of pectin substances. Pectinase is used to degrade the pectin present in vegetables and thus helps to reinforce fruit juice extraction from fruits such as apples, tomatoes, and oranges. The current study aims to isolate, screen, and optimize the pectolytic enzyme from fungi of environmental samples. Twenty of the 25 isolates showed a zone of hydrolysis, indicating an organism's ability to produce pectinase enzyme. By performing screening of pectinase, 80% of positive isolates were obtained, and these isolates were optimized to achieve high pectinase production by carrying out various parameters such as pH, temperature, and carbon source, and it was found that pH 5, 30 °C and 2% of carbon source were found to be optimum for the production of pectolytic enzyme. The molecular weight of the isolated enzyme was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and was determined to be approximately 66 kDa.

***Key words**: Pectin, Heteropolysaccharide, Pectolytic enzyme, Optimization, Gel electrophoresis.*

Introduction

Pectinase are the enzymes that invade the pectin materials by using the process of depolymerisation, trans-elimination and with the aid of hydrolyzing the ester bond inside the middle of methyl and carboxyl groups of pectin (Geetha *et al*., 2012). *Pectinase* is also known as pectin lyase, pectin methylesterase and polygalcturonase, commonly called pectic enzymes. The enzyme has been found to be seen in various plant cell walls and microbial cells. Human and animal cells are not able to synthesize the pectinase enzyme on its own. Microorganisms utilize economically cheaper substrates as a source, for production of both extra and intracellular enzymes. Microorganism such as bacteria, yeast and fungi are synthesizing pectolytic enzyme and it is found to be important for the plant-microbe symbiotic relationship, in the process of carbon cycle and in decaying plant resources (Anisa *et al.,* 2013; Pedrolli *et al.,* 2009). Fungi are the major source of pectinase and can be employed commercially.

According to the FDA, pectolytic enzyme obtained from the source of *Aspergillus niger*, *Aspergillus oryzae*, and *Penicillium expansum* is considered to be a safe source for industrial production of pectolytic enzymes. The significance of the pectinase enzyme has been seen in several regions such as agricultural, pharmaceuticals, food and bioremediation methods (Raju and Divakar, 2013). Pectinase producing enzymes are required for the degradation of substances having pectin and these enzymes have a lot of industrial importance.

Clarification of the fruit juice is one of the essen-

(*Associate Professor)

tial applications of the pectolytic enzyme. Fruit juices have colloids which basically are composed of polysaccharides. Pectin and starch are some of the polysaccharides. These polysaccharides can cause fouling and can interfere with filtration. Hence, the viscosity of the juices due to these colloids can be drastically reduced employing pre-treatment strategies with enzymes such as pectinase. This increases the clarity of the juice. Besides pectinases play a vital role in solid waste management in several industries.

The purpose of the study was to isolate the fungi from the environmental source and screening of the isolates for the enzyme production followed by the optimization of the enzyme to reach the maximum enzyme activity and molecular weight determination by SDS-PAGE. Evaluation of the pectinase enzyme has been studied by performing clarification of the fruit juice. Future these enzymes can be applied to the industrial applications that have been mentioned above.

Materials and Methods

Sample collection

Fruit peels degraded soil, and spoiled fruits were collected from the fruit market, Chennai. Fruit samples include *Musa acuminutesata, Musa balbisiana*, *Malus domestica*, *Psidium guajava*, *Citrus X sinensis*, *Citrus limetta*.

Isolation and Screening of Pectinase

A small part of the rotten fruit was taken and it was homogenized using 3 ml of distilled water in a mortar and pestle. The homogenized suspension was streaked over SDA and the plates were incubated for 3-4 days at room temperature (Preeti *et al., 2015).* For soil samples, spread plate technique was performed to isolate the fungal organisms. Pectinase Screening Agar Media (PSAM) was prepared for confirmation of the production of pectinase producing organisms. PSAM showing the following composition: diammonium hydrogen phosphate 0.3g, dihydrogen potassium phosphate 0.2g, dipotassium hydrogen phosphate 0.3g, magnesium sulphate 0.01g, agar 2.5g and pectin 1g in a 100 ml of distilled water. pH of the medium was adjusted to 4.5. Isolates from the SDA plates were inoculated onto PSAM and the plates were incubated at room temperature for 4-5 days. The plates were screened for the enzyme production by the addition of potassium iodide over the plate and the plates showing zone of hydrolysis have confirms the enzyme production (Kamalambigeswari *et al*., 2018; Sudeep *et al.,* 2020)

Enzyme Production by Submerged Fermentation

The positive isolates from the screening media were selected for the submerged fermentation and the organisms have been cultivated in the Erlenmeyer flask containing the 150 ml PSAM medium. The fungal spores were incubated at room temperature in an orbital shaker at 160 rpm for 4-5 days (Abdullah *et al*., 2018). By carrying out filtration through Whattman No. 1 filter paper, the filtrate was collected and centrifuged at 10,000 rpm for 10-15 minutes at 4 ^{o}C. The crude enzyme was used to determine the enzyme activity.

Enzyme Assay

Pectinase activity was determined using a substrate (pectin). The reaction mixture consists of 1ml of pectin (1%) prepared in sodium acetate buffer (0.1 M; pH 5.5) and 1ml of crude enzyme. This reaction mixture was incubated at 50 ^{o}C in a water bath for 30 minutes. After incubation, 1 ml of DNS reagent was added to all the tubes. The tubes were placed in a boiling water bath for 5 minutes after which 7ml of distilled water was added to each of the tubes. A blank solution was also prepared by adding 1ml of distilled water instead of the crude enzyme. The absorbance was read using a spectrophotometer at a wavelength of 540 nm.

The Galacturonic acid was used as a standard. One unit of pectinase activity was determined as the amount of enzyme that was required to release one micromole of galacturonic acid, using standard assay condition (Sudeep *et al.,* 2020; Shobana *et al*., 2018)

Protein Estimation

Protein estimation was carried out using Lowry's method where Folin Phenol reagent was employed (Sudeep *et al*., 2020; Lowry *et al*., 1951; Shobana *et al*., 2018)

Optimization of Pectinase

To increase maximum enzyme activity, the submerged fermentation method was performed at several parameters such as temperature, pH, and carbon source (Kaur *et al.,* 2016; Ketipally and Ram, 2018; Namasivayam *et al.,* 2011). To obtain the high

enzyme production, pH such as 4, 5, 6 and temperatures such as room temperature, 37 °C, and 40 °C were set to the fermentation medium. To the optimized production medium with pH and temperature, 1%, 2%, and 3% of orange peel powder (carbon source) were added. As discussed earlier, crude enzymes were collected and the enzyme activity was determined by performing DNS reagents.

Molecular Weight Determination by SDS-PAGE

SDS-PAGE analysis was carried out to determine the molecular weight of the pectinase enzyme present in the crude extract.

Clarification of Apple Juice Using Pectinase Enzyme

Apple juice was clarified using the enzyme pectinase and evaluated for the viscosity (Anand *et al*., 2017). One kilogram of apples was washed and dried. They were minced using homogenizer after cutting and the juice was extracted. The juice obtained was transferred to bottles. Following this the entire set up was incubated in a water bath at 85 °C for 10 minutes. Four different enzymatic treatments were employed for clarification of the juice.

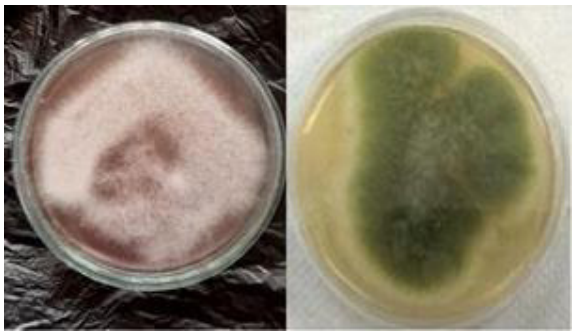
1. Apple juice with no enzyme added served as control
2. Apple juice with a 1% enzyme concentration
3. Apple juice with a 2% enzyme concentration
4. Apple juice with a 4% enzyme concentration

The apple juice treated with the pectinase enzyme was then incubated in a water bath for 1 hour at 40°C. To inactivate enzymatic reactions, the bottle was heated at 90 °C for 5 minutes. Then, the juice was centrifuged at 5000 rpm for 5 minutes to remove impurities. The clarification of the juice was determined by measuring the absorbance at 660 nm using a spectrophotometer, and distilled water was used as a blank. Finally, the pH of the juice was determined to check the acidity levels.

Results and Discussion

Isolation of Fungi

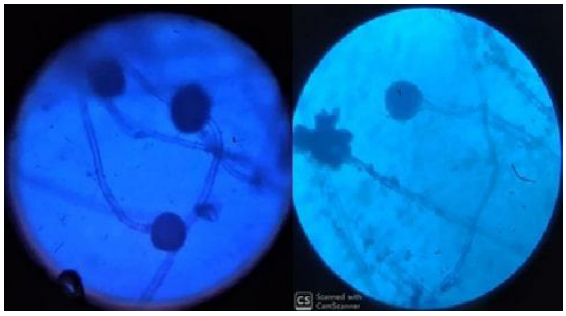
A total of 25 fungal isolates were obtained from spoiled fruits and soil samples. By observing macroscopic and microscopic views, genus level identification was carried out as shown in Figure 1 and 2. Species level characterization was achieved by using VITEK (Preeti *et al*., 2015).



Mucor sp *Aspergillus flavus*

A. fumigatus *Rhizopus sp* *A. niger*

Fig. 1. Isolation of fungi - Macroscopic appearance



Aspergillus niger *Aspergillus flavus*

Fig. 2. Isolation of fungi - Microscopic appearance

Screening of Isolates for Pectinase

Among the 25 isolates, 20 organisms have been shown to be positive for enzyme production. The isolates show a zone of hydrolysis surrounding the colony, which confirms the presence of the pectinase enzyme as shown in Figure 3. By performing the plate assay method, 80% of positive isolates were obtained from spoiled fruits and soil samples.

Submerged fermentation and pectinase assay

Organisms showing a maximum zone of hydrolysis

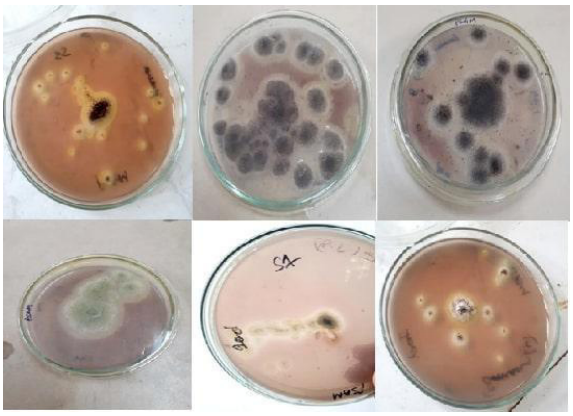


Fig. 3. Zone of clearance around the colonies

Fig. 4. Submerged fermentation for pectinase production

were selected for submerged fermentation for pectinase enzyme production as shown in Figure 4. The crude enzyme was assayed to estimate the amount of enzyme liberated by the organism, and it was found that *Aspergillus flavus* from spoiled *Musa balbisiana, Aspergillus niger* from spoiled *Psidium guajava* and *Rhizopus oryzae* from spoiled *Malus domestica* were found to possess high enzyme activity of 1.87 IU/ml, 0.61 IU/ml and 0.42 IU/ml respectively.

Optimization of Pectinase

Optimization of pH was performed and pH 5 was found to be the optimum, as shown in Figure 5. At this pH, the maximum yield of pectinase enzyme was observed. Similarly, optimization of temperature was performed and it was found that 30 ºC was found to be the optimum temperature as shown in figure 6. Also, Optimization of carbon sources was performed using dried orange peel as a carbon source, and it was found that 2g of orange peel gave a high yield of enzyme as shown in Figure 7. Optimization of carbon, temperature, and pH was performed to see the optimum conditions where the enzyme production was high. It was observed that *Aspergillus flavus* from spoiled banana samples, *Aspergillus niger* from spoiled guava samples, and *Rhizopus oryzae* from spoiled apple samples showed the highest enzyme activity. Hence, they were selected for optimization.

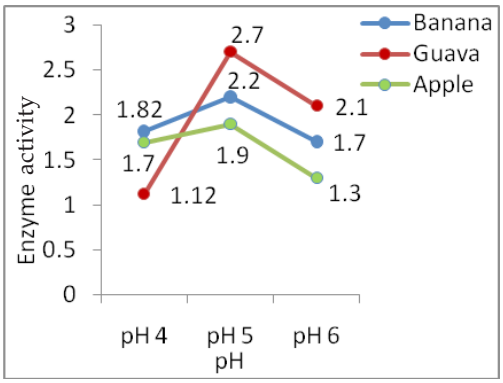


Fig. 5. Effect of pH on enzyme production

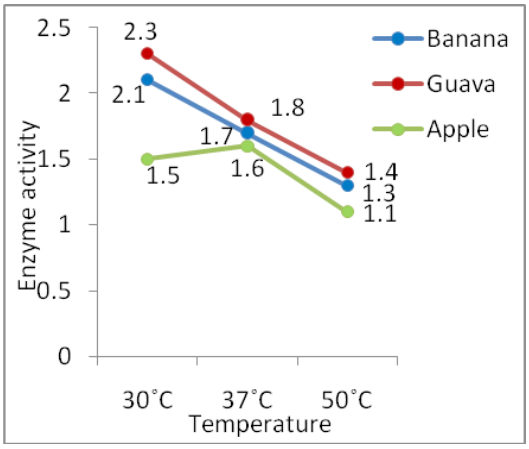


Fig. 6. Effect of temperature on enzyme production.

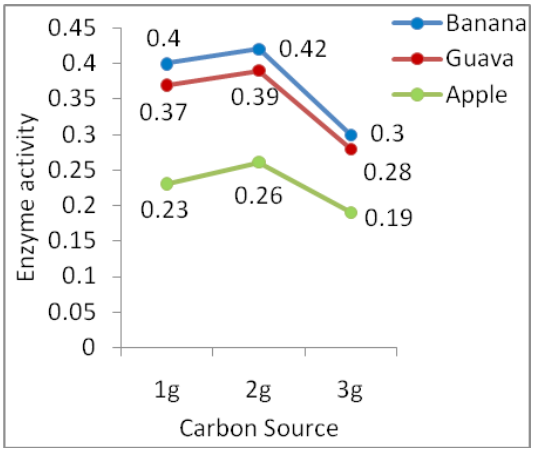


Fig. 7. Effect of carbon source on enzyme production

Protein Estimation

The fungi *Aspergillus flavus* and *Aspergillus niger* isolated from spoiled fruits of banana and guava, were found to possess high protein content, and the amounts were found to be 2080 µg/ml and 1250 µg/ml.

Molecular Weight Determination by SDS-PAGE.

The molecular weight of an enzyme from *Aspergillus niger* was found to be 66 kDa. The presence of the pectinase enzyme was confirmed by observing bands ranging in size from 46 to 79 kDa. Lane 1 is designated for enzymes, while Lane 2 is designated for markers as shown in Figure 8.

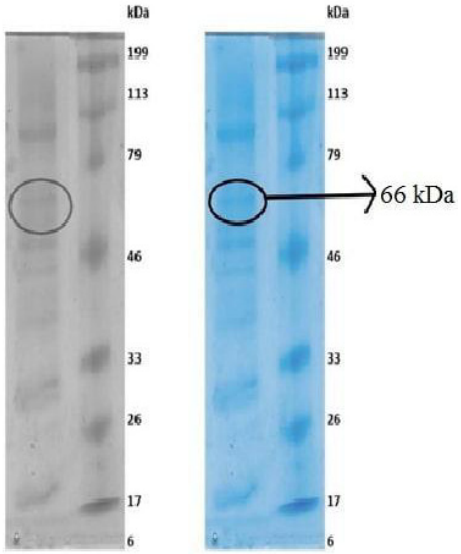


Fig. 8. Enzyme Profile in SDS-PAGE.

Clarification of Apple Juice Using Pectinase Enzyme

As shown in Figure 9, apple juice was treated with 1%, 2%, and 4% pectinase enzyme. The juice treated with 4% of pectinase enzyme showed the least turbidity. The pH of the juice was decreased after the addition of the pectinase enzyme. The pH value of 4% concentration pectinase was 4. The decrease in pH is due to an increase in the amount of galacturonic acid content. the 4% pectinase treated sample showed an absorbance of 0.094 which was significantly lower than the other enzyme concentrations as shown in Table 1.

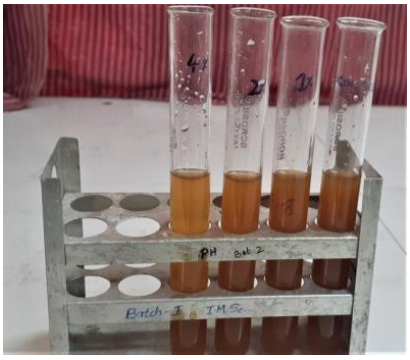


Fig. 9. Clarification of the apple juice

Table 1. Absorbance of enzyme at 660nm of treated apple juice

Treatment	pH	Clarity
Control (Untreated)	4	0.171
Pectinase 1%	4	0.129
Pectinase 2%	4	0.113
Pectinase 4%	4	0.094

The current study has shown that fungal isolates from rotten fruits and fruit peel degraded soil samples produce pectinase enzymes, which have a wide range of industrial applications. Pectinase is an important enzyme which can be used to improve the quality and yield of final products.

This investigation has shown that the diversity of fungal isolates from the fruit peel degraded soil was found to carry an essential source of the pectinase enzyme. Also, it has been observed that soil samples from agricultural waste, spoiled fruit, and vegetables have a potential source for isolating bacterial and fungal pectinases.

The organisms obtained were screened for pectinase production and it has been found that a maximum zone of clearance was observed in *Aspergillus sp*, which possessed high enzyme activity of 1.87 IU/ml. In other studies, organisms such as *Bacillus sp, Bacillus sphaericus, A. niger and P.chrysogenum* exhibited high pectinases by showing large zones of hydrolysis (over 70%) (Jayani *et al.*, 2010; Kalaichelvan, 2012; Okafor *et al*., 2010). This variation in the production of fungal pectinases might be due to differences in the sample collection and strain variations. Using optimization, it was observed that pH 5, temperature 30 °C, and 2g of orange peel were found to be optimum for high enzyme production.

The above results were similar to Kalaichelvan (2012); Kaur *et al*., (2016); Prakash *et al*., (2014). In Jayani *et al*., (2010) studies citrus pectin and xylose were proved as best carbon sources for pectolytic production. In their research work, further increased

or decreased temperature result in less enzyme production. All of these studies have shown that the growth of an organism is reduced at low and high pH levels due to increased acidity and alkalinity.

Also, high temperatures inhibit the growth of the organism, the production of secondary metabolites and the denaturation of the enzymes. The results of this study have suggested using orange peel as a source of carbon due to its wide usage and availability. The enzyme pectinase was characterized by SDS-PAGE and it was found to be 66kDa. Similarly, Kalaichelvan (2012) isolated pectinase enzyme from Bacillus sp, which has a molecular weight of 37kDa and Dey *et al*., (2014) have shown molecular weight of purified pectinase from *Aspergillus awamori* and it was found to be 30kDa. The difference between molecular analyses might be due to strain variation. The use of pectinase enzyme for the clarification of apple juice was investigated, and it was found that a 4% concentration of pectinase enzyme resulted in increased clarification of juice, which is similar to the findings of Berutu *et al*., (2017).

Conclusion

Enzymes from spoiled fruit samples, fruit peels degraded soil, fruit waste, and agricultural waste can act as a potential source for pectinase production. This study has shown that dried fruit peels can be effectively used as a substrate for large scale industrial production of pectinase, which not only reduces the cost but also is environmentally friendly.

To summarize, pectolytic enzymes from fungal isolates with good activity at pH 5, temperature 30°C, and 2 g of carbon source could be beneficial in increasing fruit juice yield. Also, it has been proved that 4% pectin was used for the clarification of the juice more efficiently as compared to other concentrations.

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