



## PRODUCTION, PURIFICATION AND ASSAY OF PECTINASE ENZYME FROM *ASPERGILLUS NIGER*

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### Abstract

Production of microbial enzymes at the industrial scale and their commercialization has gained a lot of focus and importance. Use of Microbes as bioreactors was also started due to these developments. Some of the industrially important enzymes from microbial origin include Lipases, Amylases, proteases, Xylases and Pectinases. The current work involved the use of microbes isolated from the decaying fruit peel for the extraction of the Pectinase enzyme. The organisms were isolated from the fruit peel and were cultured onto SDA agar Plate as the isolation is of Fungi. The organisms were screened for the production of the Pectinase enzyme using Pectin agar media. Microscopic identification and Morphology study revealed that the Fungi isolated was *Aspergillus niger*. Pectinase Production media was later used for the Lab scale production of Pectinase enzyme by inoculating *A. niger* and incubating for 10 days. The enzyme thus produced was purified by several steps including Salt precipitation and Dialysis. The enzyme thus produced was assayed and compared with the normal fruit juice extraction production. Quantitative estimation of protein was done using Lowry's method at 660nm to know the concentration of protein. The results thus obtained can conclude that the use of *Aspergillus niger* in the industrial production of Pectinase is highly beneficial. The amount of juice extracted in the control samples was less compared to the samples extracted using Pectinase enzyme. The enzyme pectinase was then preserved using microencapsulation technique by Sodium Alginate and stored at 4°C.

### Keywords:

Extracellular Enzyme, Pectinase, *Aspergillus niger*, Fruit wastes, purification, Enzyme assay, Immobilization.

### Introduction

The Pectinases are the enzymes that are capable of hydrolyzing the Pectin polysaccharide into smaller fragments. All the peels of the fruits are made of pectin layer which can be easily digested by the microbial pectinases so as to extract the juices of the

fruits. Therefore, pectinase enzymes are commonly used in processes involving the degradation of plant materials, such as speeding up the extraction of fruit juice.

At present, almost all the pectinolytic enzymes used for industrial applications are produced by the fungi, namely *Aspergillus* sp., *Rhizopus stolonifer*, *Alternaria mali*, *Fusarium oxysporum*, *Neurospora crassa*, *Penicillium italicum* ACIM F-152, and many others. Orange peels are used as substrate for the growth of *Aspergillus niger*. The high cost used in the importation of industrial extracellular enzymes has led to the high cost of finished industrial products. Besides, orange peels cause waste disposal problems since they are being thrown around indiscriminately. This study is therefore aimed at the production of pectinase from *A. niger*, purifying enzyme and assaying the enzyme activity.

### Materials and methods

#### Isolation of the Fungi from the Fruit Peels:

Few orange peels have been collected from the dump of fruit wastes near a local fruit market in L.B Nagar, Hyderabad; The peels were kept damp for few days in the Zip Lock Bag with minute openings for air passage. Once the decaying of the peels was started the growth of fungi started. The peels were cut into small pieces and ground into fine liquid adding dist water. This was used as the inoculum for the isolation of the organisms from the peel.

#### Pure Culture Preparation:

Sabouraud Dextrose Agar is used specifically for the growth of dermatophytes and other types of fungi. Sabouraud agar typically contains 40 g/L dextrose; 10g/L peptone; 20 g/L agar; pH -5.6. The media was prepared as per the composition and was autoclaved to sterilize. After the sterilization the media was poured into sterile petridishes and was solidified. The previously prepared inoculum was then used to inoculate the plates and were incubated at room temperature for 4 to 5 days for the mycelia to develop.

**Screening for the Pectinase Enzyme Production:**

Pectinase Screening Agar Medium (PSAM) is used for the selective growth of those microbes which release pectin. It was prepared as per the following composition

0.3gms/100ml, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> 0.2gms/100ml-  
KH<sub>2</sub>PO<sub>4</sub> 0.3gms/100ml, K<sub>2</sub>HPO<sub>4</sub> 0.01gms/100ml-  
MgSO<sub>4</sub> 2.5gms/100ml-Agar 1gm/100ml -Pectin  
and P<sup>H</sup>-4.5

After the preparation and sterilization of the media, loopful of microbial culture from the master plate was taken and transferred to petridish with PSAM medium by simple streak plate technique. The plates were Stored at room temperature in inverted position for microbial growth. After incubation the plates were screened for the identification of Zones of hydrolysis indicating the positive result for Pectinase production.

**Colony Morphology of the Culture and Microscopy:**

Different zones of microbial growth are found on the screening plate. Loopful of culture from each zone was taken and teased onto a glass slide, This was stained using Lacto phenol Cotton Blue dye and observed by covering with cover slip under an electron microscope. Morphology of each Mycelia was observed under the 40x magnification. The green color sprangiophore with the Sporangiospores on the sporangium indicates the organism to be *Aspergillus niger*.

**Pectinase enzyme production by liquid state fermentation:**

*Aspergillus niger* is placed in a basal medium used for pectinase production, the medium consist of: 0.3% (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.2% KH<sub>2</sub>PO<sub>4</sub>, 0.01% K<sub>2</sub>HPO<sub>4</sub>, 0.01%-MgSO<sub>4</sub>, 2.5%- Pectin. The type of fermentation used was submerged liquid state fermentation. The culture was incubated for 10-12 days at 25°C.

**Crude Enzyme Extraction by Filtration:**

To separate the liquid enzyme mixture and the mycelia mat along with the spore bodies' filtration technique was employed. Filter broth through Whatman No. 1 filter paper and then centrifuge at 10,000 rpm for 15 minutes. This crude enzyme extract is used for measuring pectinase activity.

**Ammonium sulfate precipitation:**

44 grams of Ammonium sulfate was weighed and used for 100ml of suspension. Pinch-by-pinch Ammonium sulphate was added in to the enzyme suspension for complete saturation maintaining ice

cold condition. Incubate the solution in ice-cold conditions for overnight. This technique is useful to remove large amounts of contaminant proteins and obtain the partially pure protein.

**Dialysis:**

This technique involved activation of the Dialysis membrane using sodium bi carbonate and alternatively boiling it in the hot water. Once the membrane was activated the partially pure extract was added into the dialysis tubing and the tube was sealed to be leak proof. The sealed tubing was suspended in water and was incubated overnight to remove the salt impurities present in the Protein extract.

**Pectin Enzyme Assay:**

Pectinesterase activity is measured by increase in free Carboxyl group by titrating against NaOH in the present of a ph indicator like Phenolphthalein.

For assaying pectinesterase activity 20ml of 1% pectin was dissolved in 0.15M NaCl (p<sup>H</sup> -7) and 4ml of crude enzyme extract is taken in a beaker and incubated for 1 hour. After incubation, the solution is titrated against 0.02 N NaOH to reach P<sup>H</sup> 7 using Phenolphthalein as indicator (colour change from colourless to pink) the heated crude enzyme extract is used as control.

$$\text{Pectin esterase activity} = \frac{V_s - V_b}{V} \times 100/V_t \text{ (Normality of NaOH)}$$

Where, V<sub>s</sub>- Volume of NaOH used to titer sample (ml), V<sub>b</sub>- Volume of NaOH used to titer blank (ml), V - Volume of incubation mixture (ml), t - Reaction time (min). Pectin esterase activity is expressed as milli equivalents of NaOH consumed min<sup>-1</sup>ml<sup>-1</sup> of crude enzyme extract under the assay conditions.

**Estimation of the Protein Concentration by Lowry's Method:**

0.2 ml and 0.4 ml of BSA working standard was taken in 2 test tubes and made up the volume to 1ml using distilled water. Crude enzyme, filtered sample and dialysis sample were taken in different concentrations and made up to 1ml using distilled water. The test tube with 1 ml distilled water serves as blank. 5 ml of Reagent I was added and incubated for 10 minutes at room temperature, after incubation 0.5 ml of reagent II was added and incubated for 30 minutes at Dark conditions.

The absorbance was measure at 660 nm and the standard graph was plotted. The amount of protein present in the given sample was estimated from the standard graph.

### **Application of Pectinases in Fruit Juice Extraction:**

The aim here is to monitor enzyme activity by measuring the amount of apple juice released by pectinase use and compare it with the normal protocol in the absence of the enzyme.

Apples were chopped into cubes that are roughly 5 mm on a side. It is important to chop the apple into very small pieces—added surface area helps the enzyme break down the pectin in the plant cell walls, releasing more juice. The chopped apples were divided equally between the two beakers. Sensitive balance was used to weigh equal amounts of chopped apple (20gms.) into each beaker. 3 ml of the enzyme Pectinase was added to one beaker, and 3 ml of water to the other. The beakers were labeled as "pectinase" and "water" respectively. Chopped apple pieces were stirred in each beaker with a glass-stirring rod. Be sure to wet all of the pieces. The beakers were covered with aluminum foil and were incubated at a 40°C water bath for 25–30 minutes. After removing jars from water bath, a wooden spoon was used to gently stir/squeeze the apple pieces in each. To filter the juice from the filter paper was used in the funnels.

A graph was made using the total volume of juice produced by each treatment and the weight of apples used.

### **Enzyme Immobilization**

0.3g of sodium alginate is dissolved in 10ml of distilled water and vortexed until it dissolves completely, 3ml of purified pectinase enzyme is added to the sodium alginate prepared; 0.5g of calcium chloride is dissolved in 25ml of distilled water. The pectinase-alginate mixture is dropped into 2% calcium chloride solution with a syringe. In the calcium chloride solution, alginate polymerizes to give bead like structure and contains enzyme trapped in it.

### **Results and Discussion:**

#### **Isolation of *Aspergillus niger* from contaminated Peel:**

Light green and black colonies are formed on SDA agar with fruit peel inoculums after 10<sup>th</sup> day of inoculation. The cells were densely populated and filamentous growth was seen after 15 days of inoculation on SDA media. The mycelia thus obtained was used for the staining with Lacto phenol Cotton Blue and observed under 40X magnification. Based on the Colony Morphology the Mycelia were identified as *Aspergillus niger*.

**Fig 1: The colony Morphology of *Aspergillus niger* on SDA Media:**

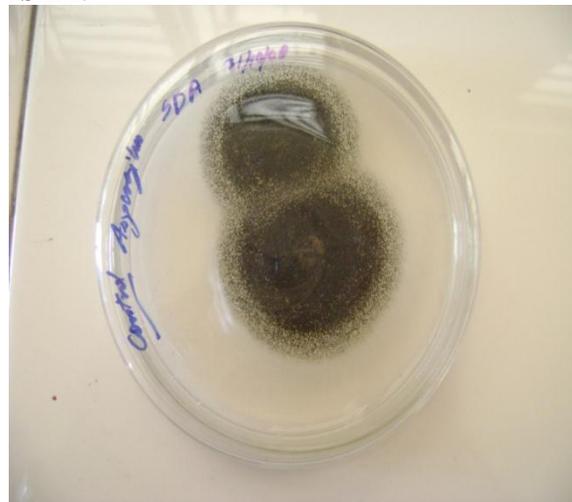


Growth of Pectin hydrolyzing fungi on SDA media with fruit peels inoculums.

#### **Screening for the Pectin Hydrolysis by the Fungi:**

The PSAM medium allowed selective growth of Pectinolytic microbes, different coloured zones were observed on the plate, microscopy on cells from each zone revealed the presence of *Aspergillus niger*.

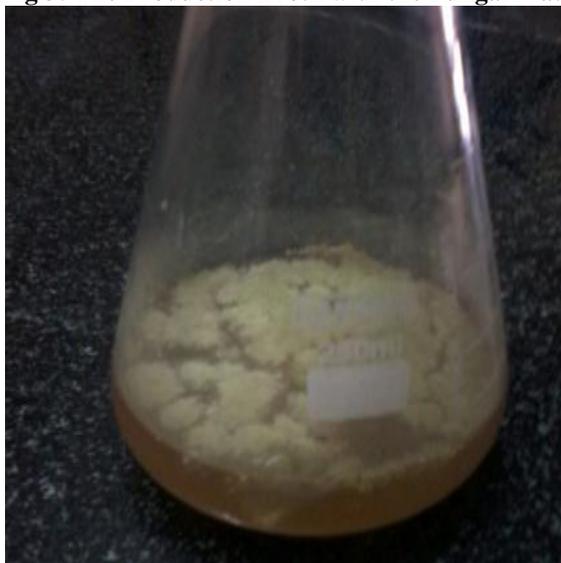
**Fig 2: Growth of Fungi in the Screening Media PSAM:**



#### **Pectinase enzyme production by submerged liquid state fermentation:**

The Pectinase Production broth containing 2% Pectin was prepared and the fungal pure culture was inoculated in the production broth. The broth was incubated for 20 days for the complete production of the enzyme into the extracellular media.

**Fig 3: The Production Broth with the Fungal Mat:**



A light green colored mat is observed on the liquid broth (production medium) after 12 days of inoculation. This is the state of consistent growth and release of secondary metabolites and extracellular enzymes.

**Assay of pectinase activity:**

In order to estimate qualitatively the activity of the enzyme, Enzyme Assay was performed based on titrimetric analysis. NaOH was used for titration and the titer mixture contained Enzyme extract, BSA, water and Phenolphthalein indicator.

Based upon the titer result and Calculations Pectin esterase activity was calculated to be 0.043 μmol/min/mg.

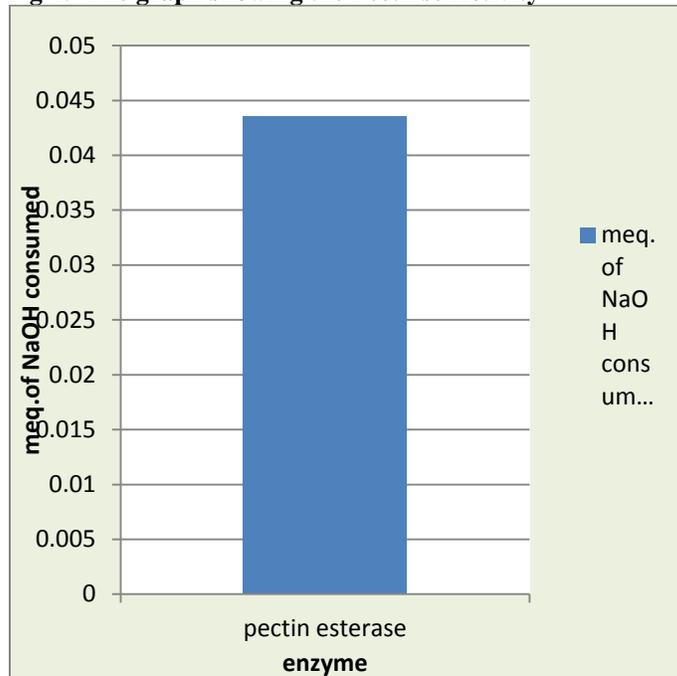
$$\text{Pectin esterase activity} = \frac{V_s - V_b}{(\text{Normality of NaOH}) \times 100/V_t}$$

**Table 1: Showing the Assay of Pectinase Activity by Titrimetry**

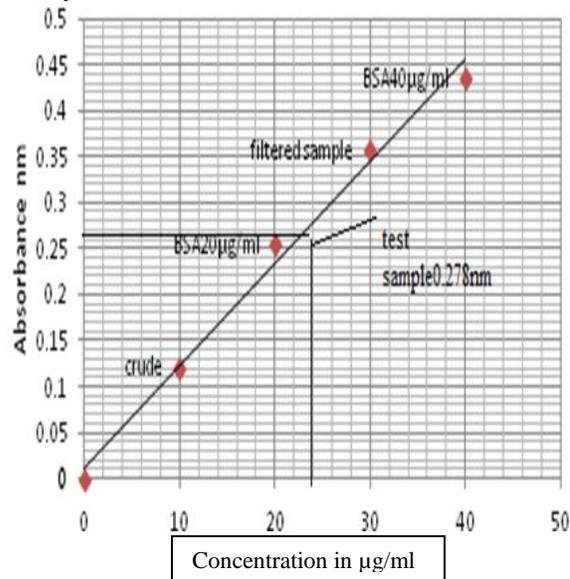
Volume of NaOH used to titrate the sample	45ml
Volume of NaOH used to titrate the blank	7ml
Normality of NaOH	0.02N
Volume of incubation mixture	29ml
Time	60min

Based on the above titre values the Pectinase activity was calculated to be 0.043 μmol/min/mg

**Fig 4: The graph showing the Pectinase Activity**



**Fig 5: Quantitative estimation of protein by Lowry's method:**

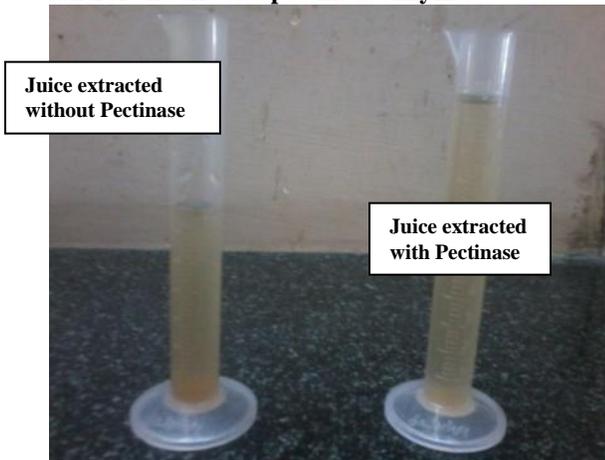


The concentration of test sample was calculated to be 24 μg/ml

**Application in fruit juice extraction:**

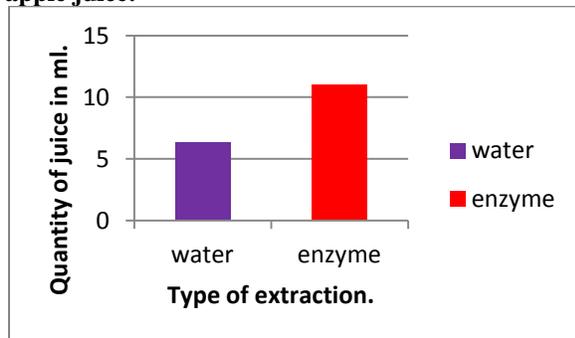
This step was conducted to find the efficacy of pectinase in juice extraction. 6.3 ml of juice was extracted without using pectinase whereas 11 ml of juice has been extracted using pectinase i.e there is a twofold increase in extraction of juice as shown in the Fig6.

**Fig 6: The comparative juice extraction between control and the test pectinase enzyme.**



There is an extensive increase in the volume of the juice extracted with the use of Pectinase enzyme when compared to the control sample.

**Fig 7: Graphical representation of the yield of apple juice:**



**Conclusion:**

Most of the microorganisms are capable of producing various extracellular and intracellular enzymes using various economical substrates. The effect of microbial enzyme activity is higher than the plant and animal derived enzymes. Pectinase is an extracellular enzyme, which is produced from various organisms including bacteria, fungi, and some actinomycetes. In the current work Pectinase enzyme was produced by *Aspergillus niger* which in turn was obtained from the decaying Fruit peels. The Culture was allowed to grow on SDA media containing Pectinase to selectively allow only the Pectinase producers to grow. The mycelia thus obtained after 7 days was stained and observed microscopically. The mycelium was identified as *Aspergillus niger*. The culture was further used for the production of the enzyme using PSAM medium. The culture was incubated for 20 days for the complete production of the extracellular enzyme. The enzyme thus produced was purified by

several techniques like Filtration, Centrifugation, Salt precipitation and Dialysis. The purified product was used for the Enzyme assay which showed that the activity of the enzyme produced was **0.043 $\mu$ mol/min/mg**. Quantitative estimation of the enzyme was performed by Lowry's Method which showed the concentration of the enzyme to be **24 $\mu$ g/ml**. To confirm the applicability of the enzyme, it was used for the extraction of the apple juice in comparison with the normal juice extraction in the absence of the enzyme. The experiment revealed that the juice produced in the presence of enzyme was double the volume of the juice produced using regular protocol.

The work concludes that the enzyme Pectinase can be produced from *Aspergillus niger* and can be used for the industrial extraction of fruit juices to increase the yield.

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