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ISOLATION AND CHARACTERISATION OF PECTINASE ENZYME FROM AGRICULTURE WASTE AND ITS EFFICIENCY IN FRUIT JUICE CLARIFICATION

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ABSTRACT

Pectinases are one of the essential groups of enzyme and has application in various industries including food, textile, paper industry and waste management. Fruit wastes are one of the polluting solid wastes which has been utilized for the production of pectinase enzymes. Fruit wastes of orange, apple, banana, pineapple and grape which are good sources of pectin. A large group of pectinase enzyme that are capable of hydrolyzing the pectin are used in industrial sector to increase the yield and clarity of fruit juices. This study was undertaken with aim of isolating pectinase producing bacteria from rotten orange peel and applied on fruit juice clarification. In which rotten orange were collected from Kochi fruits market and subjected to laboratory activities. Isolation and screening for pectinase producing bacterial strains was done by spread method in pectinase screening agar media. Bacterial strains which produce clear zone around the bacterial colonies are identified as pectinase producers then they were subjected to microscopic and biochemical identification. Based on this process the isolated bacteria were identified as Gram-positive rod. About 0.4 unit of pectinase enzyme activity was quantified based on the DNSA method. In this study the production and optimisation studies revealed that Gram positive Bacillus requires 35^0c, pH 5.5, pectin (carbon source), and peptone (nitrogen source) higher pectinase enzyme production. In the

current study pectinase enzyme produced by *Bacillus cereus* was used in fruit juice clarification and good result was observed.

Key words: Pectinase, DNSA, Agri-waste, Isolation

INTRODUCTION

Over the past decade, the demand for fruit and vegetable juice has risen rapidly and is projected to increase further in the near future due to changing lifestyles, more travel, easy adaptation to western culture, and increased knowledge of health and awareness. Without the use of enzymes, high quality juice production is unlikely. The use of food processing enzymes such as pectin and amylase has increased tremendously in fruit juice industries. Pectinase is the enzyme which is in use since ancient time and its commercial applications were first observed in 1930 for the preparation of wines and fruit juices. Generally, food and fruit processing industries use a variety of enzymes to enhance the quality of products as well as reducing the overall production cost. Enzymes are biocatalysts that catalyse and accelerate various biological reactions. Fruit Juice industries mainly involve juice clarification that is carried out by depectinization enzymes i.e. pectinase enzyme **[1]**.

Microorganisms are essential elements in our environment. In the manufacturing sector, enzymes derived from microbes are used because of their environmentally friendly nature. In both technical and industrial processes, microbial enzymes replace the use of chemicals, lowering the rate of pollution (Kumar., 2015). One such enzyme is pectinase to be used in different industries that can be produced from different microorganisms such as bacteria and fungi. It is reported that, 50% of accessible enzymes are initiated from fungi and yeast, 35% from bacteria, while the remaining 15% are either of plant or animal origin **[2]**.

Enzymes are naturally occurring catalysts. In order to increase the rate of an enormous and diverse set of chemical reactions needed for life, living organisms produce them. Pectinases are the upcoming industrially important enzyme having major industrial importance and they hold a leading position among the commercially produced industrial enzymes. Microorganisms including yeast, bacteria, actinomycetes and a large number of filamentous fungi are commonly recognized as the best natural sources for the production of pectinase enzyme. The chief source of acidic pectinases is fungi but alkaline pectinases are produced from alkalophilic bacteria, primarily *Bacillus* spp **[3]**.

Pectinases constitute approximately 10% of the total enzyme production in the world market and 25% of global sale in the food industry. The consumption of fruits in our daily life is increasing day by day. Fruits are major source of pectin and the high viscosity of fruit juices is due to mechanical crushing of fruits which are rich in pectin. To extract juice by mechanical method is difficult. Pectinase along with some other enzymes replaces the mechanical extraction process and clarify the juice obtained from pectin containing fruit **[4]**.

The juices from a wide variety of fruits such as apple, pear, plum, mango, banana, grape, orange and papaya can be extracted to produce natural beverages.The most important characteristic affecting the extraction of juice is the fruit cell wall, which is a complex structure of interwoven polymers composed of bundles of crystalline cellulose micro fibres embedded in an aqueous gel of hemicellulose and pectin **[5]**.

As a result, application of pectinases and amylases (collectively called macerating enzymes) has been observed useful in the extraction and clarification of fruit and vegetable juices **[6]**.

Pectinases degrade pectin and other high molecular weight components in the cell wall, resulting in increased juice yield and decreased viscosity, thus giving a crystalline appearance to the final product with reduction in filtration time up to 50%. In the process of extracting juice, pectinases can be used to obtain a higher yield of sugar and soluble solids, resulting in a higher juice yield **[7]**. Hence the study was taken with the following objectives: To

- Isolate and purify the pectinase producing bacteria from decayed fruits
- Microscopy and biochemical characterisation of pectinase
- Efficiency of pectinase in juice clarification

METHODOLOGY

A. Sample collection

The pectinase activity was found to be higher in the orange peel extract. Hence orange peels were chosen to extract the pectinase enzyme. Partially decayed oranges were collected using sterile polythene bags form the local market to isolate the pectinase producing bacterial strain

B. Isolation of pectinase producing bacteria

For isolation, fruit peels were ground and made into slurry by using mortar and pestle. 10 ml of this sample made into serial dilutions from 10^{-2} to 10^{-7}. A total of 0.1 ml from dilutions (10^{-4}) were spread plated on pectinase specific media (PSAM) with pectin as a sole source of carbon for bacterial growth. These plates were

incubated for 24 to 72 hours at 37^0C along with control plate.

C. Screening for pectinase producers

Pectinase Screening Agar Medium (PSAM) is used for the selective growth of those microbes which release pectin. After the preparation and sterilization of the media, the bacterial colonies with the zone from the isolation plate was taken and made spot culture on PSAM. The plates were stored at 37^0C 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. Then colonies were subculture on nutrient agar.

D. Identification and characterization of bacterial strains

The isolated bacterial cultures were characterized based on colony morphology, Gram staining, biochemical test. The isolated bacteria were further subjected to biochemical characterization. The following biochemical tests were carried out.

a. Indole Test

Prepare tryptophan broth tubes, then aseptically inoculate one tube with test organism and one uninoculated tube was kept as control. Incubate all tubes at 37 c for 24 hours. After incubation 1 ml of kovac's reagent was added to each test tubes and agitated gently. Observe the tubes for the development of cherry red colour reagent layer and result were noted. The test is used to determine the ability of the organism to convert amino acid tryptophan to indole by the action of the enzyme tryptophanase.

b. Methyl Red Test

Test is used to determine the ability of an organism to oxidise glucose with the production and stabilization of high concentrations of acid end products. Sterile MR VP broth tubes were prepared. Aseptically inoculate one tube with test organism and one uninoculated tube was kept as control. Incubate all tubes at 37°C for 24 hours. After incubation added 5 drops of methyl red to each test tubes and agitated gently. Observe the tubes for the development of cherry red colour.

c. Voges Proskauer Test

MR VP broth tubes were prepared and sterilized. Then aseptically inoculate one tube with test organism and one uninoculated tube was kept as control. Incubate all tubes at 37 c for 24 hours. After incubation add 10 drops of Barrit's reagent A and shake the culture. Immediately add 10 drops of Barrit's reagent B and shake. Tubes were carefully observed for 15 minutes. Red colour indicates positive result. This test is used to determine the ability of organisms to produce neutral end product acetyl methyl carbinol from glucose metabolism.

d. Citrate Utilization Test

Test is used to detect the ability of an organism to utilize citrate as the sole source of carbon and energy. Simmon citrate agar slants were prepared and asceptically inoculate one slant with test organism and other slant were kept as control. All the tubes were incubated for 24 hours at 37^0C. After incubation tubes were observed for blue colour.

E. Pectinase enzyme production

The enzyme production media, Yeast Extract Pectate broth (YEP) has to be prepared and autoclaved at 121^0C for 15 minutes. The selected isolate has to be inoculated into fermentation media and incubated in shaker incubator at 37^0C for 3-4 days. After the incubation the culture should be centrifuged at 12,000 rpm for 15minutes to get cell free culture filtrate. This is used as enzyme source to determine pectinase activity.

F. Pectinase enzyme assay

a. DNSA Method

Pectinase enzyme assay was based on the determination of reducing sugars produced as a result of hydrolysis of pectin by Dinitrosalicylic Acid reagent (DNS) method **(Miller, 1959)**. For enzyme assay, 1.5 ml of freshly grown culture was taken and centrifuged at 10,000 rpm for 15 minutes. The supernatant (100µl) from the culture broth was served as the source of the enzyme. In addition, substrate was prepared by mixing 0.5% (w/v) citrus pectin in 0.1M of pH 7.5 phosphate buffer.

From the prepared substrate, 900µl was added to three clean test tubes; one for enzyme, one for enzyme blank and one for reagent blank. Then 100µl of crude enzyme was added to test tube labeled as enzyme and 100µl of distilled water was added to test tube labeled as reagent blank while test tubes labeled as enzyme blank remained as it was. Then the test tubes were incubated at 50^0 c for 10minutes in water bath. After incubation 2000µl of DNS was added to the all test tubes to stop the reaction. Meanwhile, into test tube labeled as enzyme blank 100µl of crude enzyme was added after DNS. Then all the test tubes were placed in a boiling water bath at 92^0C for 10minutes. Finally, the tubes were cooled and Optical Density (OD) was measured using spectrophotometer at 540 nm. Enzyme activity was measured against enzyme blank and reagent blank. The enzyme unit defined as the amount of enzyme that catalyses µmol of galacturonic acid per minute (µmol min^{-1}) under the assay conditions.

G. Optimisation of pectinase enzyme

a. Effect of pH

Enzyme activity was tested at different pH level from 3.5 to 6.5.

b. Effect of Temperature

The enzyme activity is determined by incubating enzyme at different

temperature 30°C, 35°C, 40°C and 45°C under optimum pH.

c. **Effect of Substrate concentration**
Substrate concentration for the production of pectinase enzyme was studied under varying the concentration of substrate from 2 to 8(gm/ml.)

d. **Effect of Carbon source**
Carbon sources such as glucose, starch and pectin were supplemented as individual components to the production medium to check their effect on pectinase production.

e. **Effect of Nitrogen source**
The effect of nitrogen sources on pectinase production were studied by supplementing various nitrogen sources, namely peptone, ammonium chloride and casein.

H. Purification of the pectinase enzyme

The ammonium sulphate precipitates of various percentage of saturation (10-80%) were desalted by dialysis. Pre-treatment of dialysis bag (Himedia, Molecular Weight Cut off (MWCO) of 12kDa) play an essential role in dialysis. Dialysis membrane was taken and treated with distilled water at 65ºC for 10 minutes to remove glycol and then soaked in 10mM disodium Ethylene Diamine Tetraacetic Acid (EDTA). It was then treated with 10mM Sodium bicarbonate to remove Sulphur molecule and finally washed with distilled water. The membrane was clipped at one end and one third of the bag was filled with ammonium sulphate precipitates and the other end was also clipped using dialysis tubing clip. The samples were then dialyzed against phosphate buffer saline (pH 7.4) by changing buffer in every four hours for overnight. Then the dialysis bag was taken and the samples were carefully drawn and collected for further analysis.

I. Application of the pectinase enzyme in fruit juice clarification

Fruit juices are turbid and cloudy after their extraction from pulp. This unclarified juice can be clarified using pectinase enzyme under its optimized conditions. To observe the effect of crude pectinase enzyme on fruit juice clarification, six beakers were labeled as test and control. 10 ml of orange, pineapple, grape juice and 1ml of crude pectinase enzyme was taken into the beaker labelled as test while the other beaker containing juices was kept as control without adding enzyme. The contents of the tubes were agitated to mix the enzymes throughout the juice. The experiment was kept for 3-4 hrs and the results were recorded.

RESULTS AND DISCUSSION

A. Isolation and Identification of Pectinase Producing Bacteria

a. Isolation of Pectinase Producing Bacteria

The growth of bacterial colonies on the plate was observed in the serial dilution of the extracted sample on PSMA which indicated the presence of pectinase activity. An isolated colony from 10^{-4} dilution plate was spot cultured onto the same pectinase screening agar medium for screening purpose. The enzyme producing bacteria was identified in spot culture technique. On adding Congo red clear zone was formed around the colonies due to pectin hydrolysis. Then pure culturing was performed on Nutrient agar.

b. Identification of Pectinase Producing Bacteria

i. Colony Morphology

Colony morphology is used to describe the characteristics of an individual colony of bacteria growing on agar in a petri dish. The following Table -1 describes the colony morphology.

Table 1: Colony Morphology

Size	**Medium**
Shape	**Irregular**
Colour	**Creamy white**
Opacity	**Opaque**
Elevation	**Flat**

The bacteria found on the agar plate was irregular, medium sized, creamy white, flat opaque colony.

ii. Gram Staining

The bacteria were Gram stained and the slide was observed under microscope. Purple color rod shaped cells were observed under microscope and it indicates that the organism is a Gram positive *Bacilli*.

iii. Biochemical Tests

1. Indole Test

Kovac's reagent is added after incubation, the isolated strain did not show cherry red colour ring at the top of the medium. It indicated that the strain was indole negative. It occurs because the test organism is not capable for producing tryptophanase

2. Methyl Red Test

After 24 hours of incubation, test reagent methyl red is added then there was no colour change was observed which indicated the negative result.

3. Voges Proskauer Test

Colour change was observed after adding Barrit's reagent A and B into the inoculated bacterial broth as compared with control. The appearance of red colour indicated the positive results that they had the ability to produce neutral end product acetyl methyl carbinol.

4. Citrate Utilization Test

Colour change from green to Prussian blue was noted in the medium which indicated the positive result that the organism had the ability to utilise citrate.

B. Pectinase Enzyme Production

The enzyme pectinase was produced by using isolated bacterial species which is carried out with pectinase

production medium by submerged fermentation after incubation of 3-4 days. After submerged fermentation the culture was centrifuged, the crude enzyme was recovered and used to determine pectinase activity and further optimization process. Pectinase enzyme assay was done by DNSA method and it was found to be 0.4µmol/min.

C. Optimisation Of Pectinase Enzyme

a. Effect of pH

The effect of pH on pectinase production in YEP broth was studied in different pH level ranged from 3.5 to 6.5. It was observed that pH around 5.5 is optimum for enhanced pectinase production.

b. Effect of Temperature

To study the effect of temperature on pectinase production, the YEP broth was inoculated and incubated at 30°C, 35°C, 40°C and 45°C and it was observed that maximum pectinase production was seen at 35^0c.

c. Effect of Substrate Concentration

Effect of substrate concentration on the production of pectinase was studied under varying the concentration of substrate (g/ml) at 2, 4, 6, and 8 and the maximum production was observed to be at 6g/ml in production media.

d. Effect of Carbon Source

Production media was supplemented with 1% of different carbon sources such as glucose, starch, and pectin to study their effect. Pectin was found to be the right carbon source for bacterial strain for higher production of pectinase.

e. Effect of Nitrogen Sources

The effect of nitrogen sources on pectinase production were studied by supplementing various nitrogen sources, namely peptone, yeast extract, ammonium chloride and casein. The highest production was achieved when peptone was used as the nitrogen source.

D. Application of Pectinase Enzyme

Fresh orange, pine apple and blue grapes were selected and juice was extracted using juicer. The pectinase was added to orange, pine apple and grape juice. 10 ml of each unclarified juice was taken in different beakers. About one ml of the crude pectinase enzyme was added to all the beaker. It was observed that fruit juice got clarified in 3 to 4 hours. It indicated that the bacterial pectinase was effective in the clarification of fruit juices.

CONCLUSION

The present investigation reports a cheap and easy method for obtaining pectin degrading bacteria from fruit waste. The pectinase enzyme producing bacterial strain was isolated from rotten orange peels. The isolated bacterial strain is identified as Bacillus cereus. In the present study it was

observed that the viscosity, bitterness and clarity of the enzyme treated juice was decreased because of the degradation of pulp in it. This study has shows the potential of utilizing agricultural waste provide cost effective and eco-friendly methods for pectinase production and its efficiency in fruit juice clarification. It can be used as cost effective method in fruit industries.

Conflict of Interest: Nil

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