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Bacillus pectinlyase, A Cock Tail Bacterial Enzyme from Kangayanellore Coconut Waste

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ABSTRACT

The objective of this study was to screen pectin lyase producing *Bacillus* from coconut waste. Soil enzymes are the biocatalysts which mediate all the synthetic and degradative reactions in living organisms. They increase the rate of slow and imperceptible reactions without undergoing any net change and are remarkable because of their extraordinary specificity and catalytic power. However, in many cases the enzyme activities can still act on the same composition, as the composition can have a complex chemical structure having various types of chemical bonds, requiring different enzyme activities for breakdown. Such pectinase composition often contains one or more of the following activities: polygalacturonase, pectin lyase and pectin methyl esterase. Pectinase preparations are often used in fruit juice processing. Pectin lyase that hydrolyze the pectic substances, present mostly in plants. Although they can be derived from several sources, such as plants, animals and micro-organisms, the enzymes from microbial sources generally meet industrial demands.

Key Words: Pectinase, Pectin lyase, Fruit juice, Catalytic Power, Enzyme Activity.

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INTRODUCTION

Proteins are the highly complex structures with conjugations formed with metals, carbohydrates or lipids. In a similar way all enzymes are proteins, but all proteins are not enzymes. The enzymes are called biocatalyst since they increase the rate of biochemical reactions without affecting the kinetics of the biochemical reactions. Enzymes in various forms and ingredients have been used to prepare food since before the recorded history, but the production and usage of purified enzymes has gained importance in the recent past.

Nature is a large laboratory for the development of novel and innovative biocatalysts for industrial processes. Microorganisms in particular have been investigated as source for new enzymes and other bioactive molecules [5]. Pectinases, the cock tail enzymes are important industrial enzymes which are used for cloud point stabilization in juices, to increase pulp extraction from fruits and vegetables, in cocoa bean fermentation and for soluble tea preparations [25, 27, 3]. More recently, they have been used in the textile industries for the degumming of fiber crops, in wastewater treatment and in the paper industries [12]. Actually, there are processes using a specific type of pectinase, such as the preparation of citrus and orange juices, where endopolygalacturonases are preferred to maintain the turbidity and opaque aspect of juices [14]. Pectic substances, present in the primary cell wall and middle lamella of higher plants, contribute to the firmness and structure of plant tissues [22]. Different pectinolytic enzymes are involved in the breakdown of pectin and are widely distributed in higher plants and microorganisms [9, 15]. They are important for plants as they help in cell wall extension and fruit softening. They have a role in maintaining ecological balance by causing decomposition of plant material.

PECTINOLYTIC BACTERIA

Bacillus, Clostridium, Citrobacter, Enterobacteriacea and pseudomonas were found to be the potent pectinolytic bacteria. Since bacteria have the ability to produce enzymes [1, 8]. Pectinolytic bacteria have the ability to process industrially cheap and possibility of strain improvement gives high yield. There is a huge scope for genetic manipulations and strain improvement in microorganisms that makes process commercialized [9, 17, 21].

Table V: Classification of Pectinase from Enzyme Nomenclature

Name	Reaction type
Pectinesterase	Hyhrolysis of carboxylic ester
Polygalacturonase	Hyhrolysis of O-glycosyl bond
Galacturan 1,4-α-galacturonidase	Hyhrolysis of O-glycosyl bond
Exopoly- α-galacturonidase	Hyhrolysis of O-glycosyl bond
Endo-pectatelyase	Elimination (C-O bond cleavage)
Exo-pectatelyase	Elimination (C-O bond cleavage)
Endo-pectinlyase	Elimination (C-O bond cleavage)

MATERIALS AND METHOD

Collection of soil samples

Soil samples were collected from coconut waste enriched soil in Kangayanellore, Tamilnadu, India. Soil samples are taken with help of sterile gloves, in sterile plastic box the samples were brought to Microbiology laboratory and kept in deep freeze for screening processing.

Isolation of Microorganisms

The soil samples were carried out by adopting baiting technique with the peels of apple, orange and papaya were cut into small pieces aseptically this is used as baits which acts as substrate. Screening for pectinolytic strains from samples was done by following modified protocol of Good fellow and Cross 1984, as used by Suneetha *et al.* 2011(26)(27).

Production of pectic enzyme on solid-state (SSF) and submerged (SmF) fermentation

Strains presenting large clearing zones were used for enzyme production assays on liquid and solid medium. The liquid medium containing 0.14% (NH4)2SO4, 1% citrus pectin 0.6% K2HPO4, 0.20% KH2PO4 and 0.01% MgSO4 7H2O, pH 6.0 was inoculated with a suspension containing 143.cells/ ml. Cultures were grown in 250ml Erlenmeyer flasks with 100 ml of medium in a rotary shaker (100rpm) at 30ºC. The SSF was done using a 250 ml Erlenmeyer flask containing 5g of wheat bran and 10 ml of 1% (NH4)2SO4 and 0.02% MgSO4 (67% of moisture). 106 cells per gram of wheat bran were added to each flask and maintained at 30ºC. After 72h, fermented material was mixed with 40 ml distilled water and filtered at vacuum and centrifuged.

Pectin lyase assay

The enzyme solution was then analyzed by continuous spectrophotometric rate determination method [2] at 235 nm under 40°C and pH 5. When the crude enzyme solution added to pectin solution in the above said conditions the enzyme starts to break the glycosidic bonds of pectin by b elimination. Due to this action the solution will become turbid. The increase in A was recorded for 235nm 5 minutes. A minute using the maximum 235nm linear rate for both the test and blank was obtained One unit of enzymatic activity (U) was defined as the amount of enzyme which released 1 µmol of unsaturated uronide per minute, based on the molar extinction coefficient (5500) of the unsaturated products. The enzyme production was expressed in units per ml of initial dry solid substrate (U/ml).

Units/ml enzyme = (A235nm/min Test - r A235nm/ min Blank) (2.5) (df) / (1.0) (0.6)

2.5 = Total volume of assay (in milliliters)
1.0 = Change in A 235nm per minute at 40 C as per the Unit Definition

0.5 = Volume (in milliliter) of enzyme
df = Dilution factor

Optimization of pH

In microbial process pH of the medium plays a vital role. The effect of pH was studied by using different pH values from 5 to 9 for pectin lyase production.

Optimization of Temperature

Under pH 6.5 the optimization, production of Pectin lyase was studied using the LB medium. The cultures were incubated for 24-72 hrs in different temperature such as 30°c to 45°c.Observation was made every 12 hours.

Optimization of Agitation

Based on the agitation process the growth of the bacteria varies in rapid. Optimization of agitation was done by keeping the pH and temperature constant value. The agitation process is done from 50 rpm to 200 rpm.

Pectin Estimation

Pectin estimation was done by gravimetric methods and the pectin estimation was done in carrot, orange, sweet lime, papaya and potatoes, in this method the percentage of calcium pectate is calculated. For this the reagents required are Hcl, NaOH, $AgNO_3$ and phenolphthalein

Phenol estimation

A series of five test-tubes were taken, labeled and 0.1, 0.2, 0.6, 0.8 and 1 ml of culture filtrate was added to respective test-tubes. 0.2ml of 0.2 N folin's reagent was added to each. Volume of solution was made up to 4ml using distilled water. These test-tubes were incubated for 3 minutes at 37°C.In this experiment standard solution was taken as galic acid solution. 1ml of 20% sodium carbonate solution was added to each and they were incubated for 2 hours at room temperature. Absorbance readings were taken for each by setting UV spectrophotometer at 765 nm and standard graph was plotted [21, 25, 26].

Total amino group estimation

A series of seven test tubes taken from 1-5 were taken as standard with different concentration of leusine from 1ml to 5ml and the 6, 7 were the test samples. Volumes of the standard and test sample were made up to 5ml using phosphate buffer and 0.5mg of pectin substrate were added to all the test tubes. The test tubes were incubated at 50°c for 30min in water bath. 1ml of TCA (Trichloroacetic acid) added to standards and test sample. Then the all

the test tubes were filtered with whatman filter no-1 from that 0.5ml was taken and added with 1ml of ninhydrin. With phosphate buffer as a blank the standard and test sample were analyzed in spectroscopy at 520 nm.

Anti-Oxidant Activity of Pectin Lyase by reducing Power Assay Method

Anti-Oxidant activity was measured by reducing power assay method. Three different test tubes were taken respectively blank, test and standard to all the test tubes 2.5 ml phosphate buffer and potassium ferri cyanide then the test tubes were incubated at 50°c for 30 min.10% of trichloro acetic acid (TCA) was added to the mixture after adding all the 3 mixture were centrifuge at 3000rpm for 15 min. Then the supernatant was then for the analysis. 5 ml of distilled water is added to all the 3 supernatant then 0.5 ml of ferric chloride is added. Test sample was added to test and ascorbic acid is added to standard and the absorbance was measured at 700nm.
The percentage scavenging activity was calculated by using the formula:

Scavenging activity (%) = (A control-A sample)*100/A control

Where,
A control is the absorbance of the solution without extract and
Asample is the absorbance of the solution with extract

Estimation of Crude Fibre

Crude fibre consists largely of cellulose and lignin (97%) plus some mineral matter. It represents only 60–80% of the cellulose and 4–6% of the lignin. For this type of estimation the culture filtrate was boiled with 200 ml of sodium hydroxide solution for 30 min. After that it was filtered through muslin cloth and washed with 25 ml of boiling 1.25% H2SO4, three 50 ml portions of water and 25 ml alcohol. Removing the residue and transferring to ashing dish (pre-weighed dish W_1) and the residue has to be dried for 2 h at 130 ± 2°C. After that it was cooled the dish in a desiccators and weigh (W_2).After that W_2 was kept at 600°C in muffle furnace and was cooled in a desiccators and reweighed (W_3)

RESULT AND DISCUSSION

Screening and detection of pectinolytic activity possessing Microbes

Baiting technique was used to analyze isolated pectinolytic bacterial strain (fig.2, 3, 4). Initially we took 90 soil samples to analyze the bacterial strain and these samples were collected from different places situated in Kangayanellore where soil is enriched with coconut industrial waste (fig1). Coconut industrial waste contains pectin substance in excess, and potential pectinolytic species were isolated by using luria bertini agar medium and production media. Different isolates of pectinolytic species were observed during the process of isolation from a number of samples, and these strains were identified by observation of growing pattern

on or around the baits of pectin substrate [15, 24, 23] So the 2 isolates were observed to seen the complete degradation of pectin substrate at different level on the plates of selective media. These isolates were selected as potential pectinolytic strain from the initial screening. After that we took the colonies of these organisms and by repeating the baiting procedure we obtained the pure cultures from plates of selective media [3, 10, 18, 27].

Fig.1: Collection and Enrichment of soil samples

Fig.2 Baiting Technique

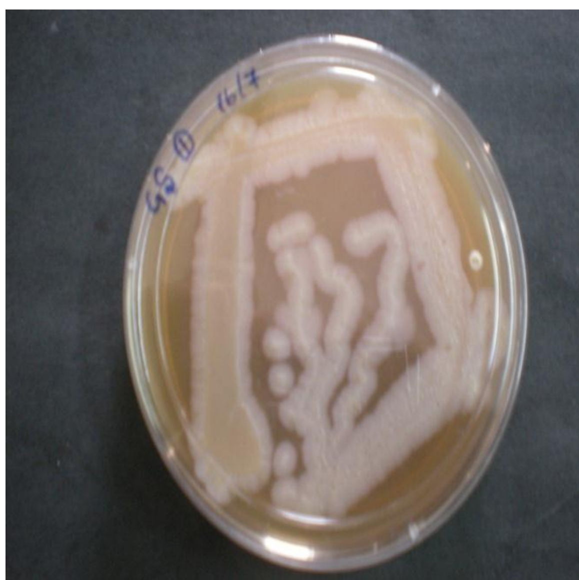

Fig.3 Screened bacterial Strain VITSG-1

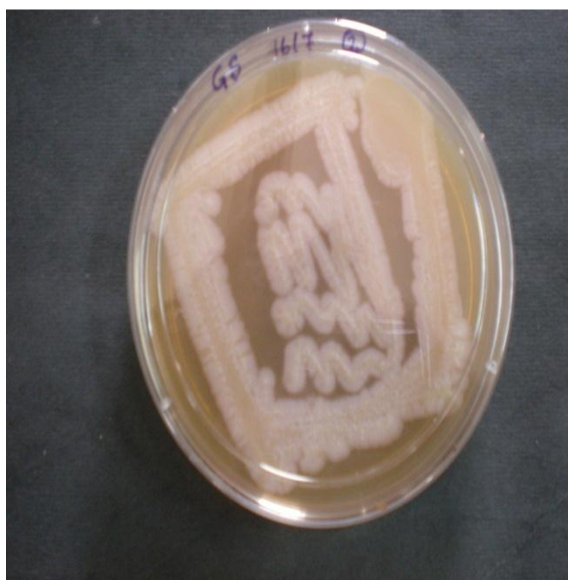

Fig.4 Screened bacterial Strain VITSG-2

Effect of temperature

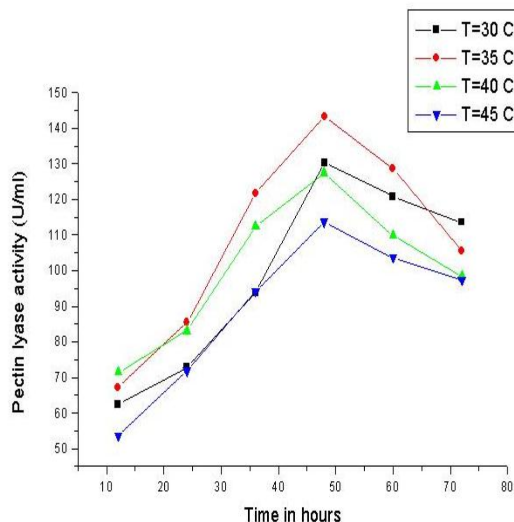

Fig 5

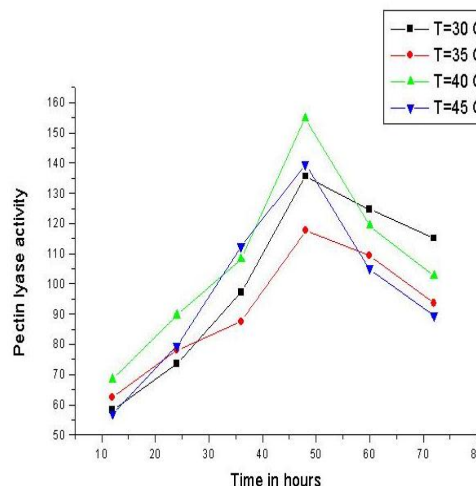

Fig 6

Fig.5 and Fig.6 denotes pectin lyase activity at different for strain-1 and strain-2 respectively

From the observation the optimum temperature for VITSG-1 was found to be 35°C (Fig.5) were the maximum activity of pectin lyase 143.55U/ml and for VITSG-2 the optimum temperature is 40°C (Fig.6) pectin lyase activity was 154.31 U/ml.

Effect of pH

From the results the optimum pH was found to be 6 (Fig.7) for VITSG 1with the maximum activity of pectin lyase of 249.72 U/ml and for VITSG 2 the maximum activity of pectin lyase is 230.88 U/ml and the optimum pH is 6(Fig8.).

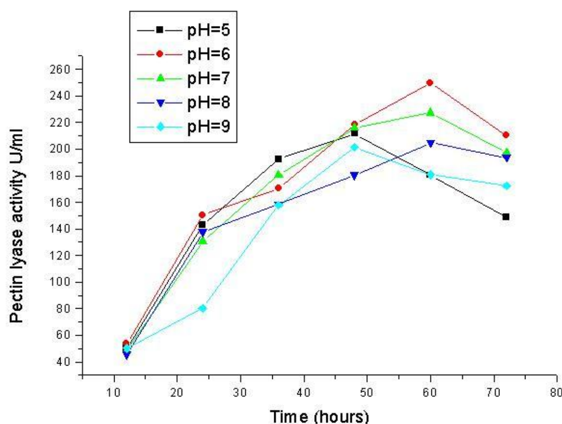

Fig 7

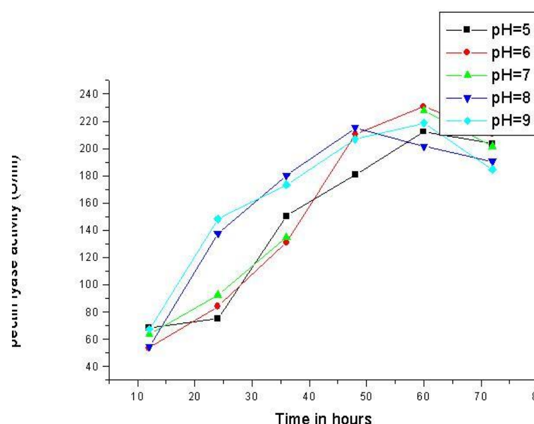

Fig 8

Fig.7 and Fig.8 denotes Pectin lyase activity for VITSG -1 and VITSG -2 at different pH

Effect of Agitation

Based on the observation taken under constant pH and temperature with varying Agitation speed the optimum rpm for VITSG 1 was 200 rpm (Fig.9) with maximum activity of pectin lyase is 240.47U/ml and for the VITSG 2 the optimum rpm was 200(Fig.10).

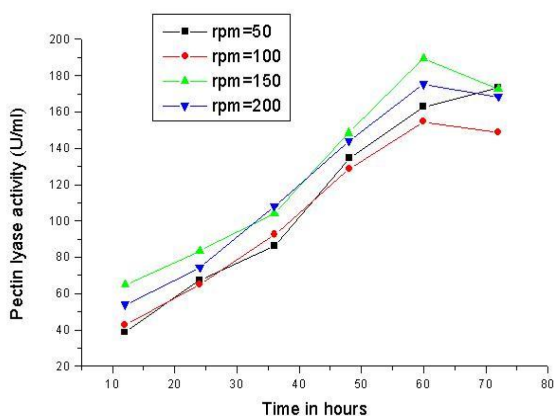

Fig 9

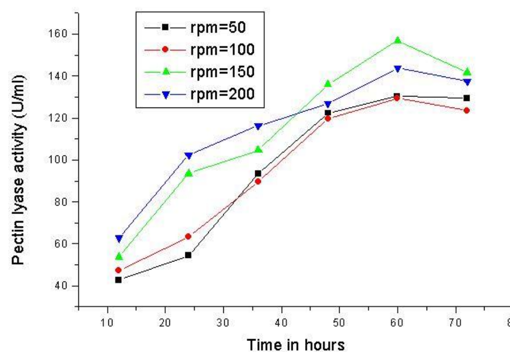

Fig 10

Fig.9 and Fig.10 denotes pectin lyase activity at different rpm for VITSG -1and VITSG -2

Pectin estimation

The pectin estimation was done for different fruits and vegetables by the gravimetric method and the following results which have been given in table-II.

Table I Screening of Pectinase Producing bacterial

Strains	Zone of inhibitions (pectinolytic activity)
2	>50%
46	>25%
32	<10%

Table II Estimation of Pectin content in different fruits and vegetables

Fruit and vegetables	Pectin concentration(%)
Carrots	6.9-18.6
Orange pulp	12.4-24.0
Potatoes	1.3-2.6
Papaya	0.8- 2 3.0
Sweet lime	3.5-5.1

Pectinlyase activity estimation

The pectin lyase estimation was done in production media as well as in pellet obtained by purification with $(NH_4)_2SO_4$ precipitation method by continuous spectrophotometric rate determination method. Pectinlyase activity and specific activity was calculated given in table-III.

Table III Effect of media on pectin lyase producing bacterial

Media	VITSG-1	VITSG-2
Production media	52	45

Estimation of Biodegradable product in the Culture filtrate

Various types of bio products from the culture filtrate was estimated and calculated which has been summarized in table-IV**.**

Table-IV Amount of released bio-product from the culture filtrates along with the production of pectin lyase.

Name of Bio-products	VITSG-1	VITSG-2
Phenol	0.53 mg/ml	0.68 mg/ml
Crude fiber	1.93 µg/ml	2.14 µg/ml
Antioxidant analysis	2.906 µg/ml	2.923 µg/ml
Alcohol	0.62 mg/ml	0.78 mg/ml
Amino group	0.095mg/ml	0.315mg/ml

CONCLUSION

The Pectin Lyase Producing bacterial strain from Kangayanellore coconut soil sample was screened and degradable products estimated which emphasis on the significance of Microbial pectinlyase. The bacterial strains obtained from the coconut wastes soil are very potent strain producing pectinlyase that is very useful for degradation of pectin. The pectinlyase activity was calculated. Scope of this research is to analysis the structural component of pectin lyases with mass spectroscopic analysis and the use of agaro industrial waste to use as a substrate for the screened microbes for better production of the pectin lyases.

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