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Studies on Pectinase Production by *Bacillus Subtilis* using Agro-Industrial Wastes.

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ABSTRACT

Pectin is a heterogeneous structural polysaccharide present in primary cell wall and middle lamella of fruits and vegetables. Pectinases are the enzymes, used for pectolysis which show wide range of industrial applications. *Bacillus subtilis* is a gram +ve bacteria, isolated from soil samples. Microbial, biochemical and molecular characterization was done to identify the present bacteria. Agro-industrial wastes like sugarcane bagasse, wheat bran, rice bran, wheat straw, rice straw, sorghum stems, saw dust, corn cobs, sun flower heads, coconut coir pith, banana waste, tea waste, sugar beet pulp, apple pomade, orange peel, soya bean pulp powder, lemon peel etc. can be used for the pectinase production as carbon source. Presently, dry orange and banana peel powders are employed to study the influence of different concentrations (0.5 to 2.5%) of these natural wastes as substrates on the rate of enzyme production. When *Bacillus subtilis* was seeded into flasks containing both the substrates with optimized pH (pH 7) and the temperature (37^0C) constant, pectinase activity was increased from 0.5% to1.5%. Further increase in substrate concentration from 2% resulted in considerable decrease in enzyme activity. Maximum activity of the pectinase enzyme was obtained with 1.5% of Orange peel powder and 1.5% of Banana peel powder (2.5 and 2.8 IU/ml/min) respectively. Enzyme activity was determined by DNS method. Results showed that the pectinase activity of *Bacillus subtilis* was higher with banana peel powder than orange peel powder when used as natural carbon source. Thus, *Bacillus subtilis* is one of the most versatile of bacteria which is more useful to society and the environment with an eye to plethora of biotechnological applications. Scientists can use pectinases still more effectively as pectinases are one of the important upcoming enzymes in the commercial sector nowadays. So, It is clear that the fundamentally positive characteristics and productive fermentation strains *like Bacillus subtilis* can be made to involve in making many new and improved products with which the emerging genomic and proteomic era moves forward. In this way, agro-industrial solid wastes can be recycled in higher extent to produce pectinase enzymes.

Keywords*: Pectin, Pectinolysis, Pectinases, Agro-industrial wastes etc.*

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INTRODUCTION

Pectin is a heterogeneous structural polysaccharide present in primary cell wall and middle lamella of fruits and vegetables (Voragen *et al*., 2003; Prathyusha and Suneetha, 2011) that shows wide range of industrial applications (Doran *et al*., 2000; Hutnan *et al*., 2000; Liu *et al*., 2006; Chambin *et al*., 2006; Schols *et al*., 2009; Morris *et al*., 2010).

Role of Microbes in Pectinase Production

Enzymes are the Bio-active compounds that regulate many chemical changes in living tissues (Prathyusha and Suneetha, 2011). Pectinases are a group of at least seven different enzymatic activities that contribute to the breakdown of pectin. The main source of the microorganisms that produce pectinolytic enzymes are yeast, bacteria and large varieties of fungi, insects, nematodes and protozoas (Patil and Dayanand, 2006; Yadav *et al*., 2009; Jayani *et al*., 2010). Thus by breaking down pectin polymer for nutritional purposes, microbial pectolytic enzymes play an important role in nature (Yadav *et al*., 2009). These enzymes are inducible, produced only when needed and they contribute to the natural carbon cycle (Hoondal *et al*., 2000).

Microbial pectinases account for 10-25% of the global food and industrial enzyme sales (Jayani *et al*., 2005; Murad and Azzaz, 2011) and their market is increasing day by day. Many acidic and alkaline pectinases are found in microbes. Acidic pectinases are mainly produced by fungi and are widely used in the production and clarification of fruit juices, in maceration and solubilization of fruit pulps. *Bacillus* sps. generally produce alkaline pectinases and are used in several areas (Hoondal *et al*., 2000).

Pectinases are constitutive or inducible enzymes that can be produced by both submerged and solid state fermentation. SSF incorporates microbial growth and product formation on or within particles of a solid substrate under aerobic conditions, in the absence or near absence of free water, and does not generally require aseptic conditions for enzyme production.

Substrates and various parameters used for the production of Pectinases

Substrates that are employed in the production of enzyme should be solid, as solid substrate can give good encourage to the growing cells. Substrates should provide all needed nutrients to the microorganisms for their growth. Other factors like particle size, moisture levels (Leda *et al*., 2000; Martin *et al*., 2004), concentration of nutrients, pH, temperature, are also to be taken for consideration. Generally agro-industrial wastes are employed for the pectinase production. Various substrates that are being used are sugarcane bagasse, wheat bran, rice bran, wheat straw, rice straw, sorghum stems, saw dust, corn cobs, sun flower heads, coconut coir pith, banana waste, tea waste, sugar beet pulp, apple pomade, orange peel, soya bean pulp powder, lemon peel etc. The effect of carbon and nitrogen sources on the productivity of pectinases also can be studied (Maria *et al*., 2000; Catarina and Almeida *et al*., 2003; Patil and Dayanand, 2006).

Because of the potential and wide applications of pectinases, there is a need to highlight recent developments on several aspects related to their production. Microbial production of pectic transeliminases was reviewed (Gummadi and Kumar, 2005). However, aspects regarding the most common microorganisms and processes for hydrolytic depolymerising pectinase (PGase) production have not been considered until now. The aim of this review is to present an overview of the pectinase activity obtained by *Bacillus subtilis* as well as the strategies used to obtain higher activities. In this study, we report the nutritional and environmental conditions required for the production of Pectinase by *Bacillus subtilis* using Orange peel powder and Banana peel powder.

The pectinolytic enzymes from microorganisms have generally focused on induction enzyme production under various conditions, fermentation process, various substrate purification and characterization and use of this enzyme for different industrial processes. The enzyme system used by microbes for metabolizing and for complete breakdown of pectin are most important tools for elaborating the economical, ecofriendly and green chemical technology for using pectin polysaccharide in nature.

As there are several biotechnological applications of pectinases, the study was presently undertaken which is aimed at screening and isolating pectinolytic bacteria from the soil samples. Identification of isolates was based on staining, biochemical and molecular characterization. Further, efforts were also made to optimize the cultural and environmental conditions for maximizing the yield of an enzyme.
The objectives of present study are:

- To isolate bacteria from soil samples that produce pectinase enzyme using a selective medium after enrichment.
- To identify the bacterial isolates based on biochemical and molecular characterization by sequencing the 16S rRNA coding gene.
- To optimize various physico-chemical factors such as temperature and pH that influence growth and production of the pectinase.
- To study the influence of different concentrations of natural substrates on the rate of enzyme production.

MATERIALS AND METHODS

Isolation of Bacterial Strains

50 soil samples were collected from different areas of agricultural lands at Duggirala and Burripalem in Guntur district, A. P. and were brought to the laboratory for enrichment with 1g of pectin.

Selective Media

A selective medium i.e. Vincent's mineral salts broth (Vincent, 1970) was employed here which allows only the desired bacteria to utilize pectin as the sole carbon source and inhibits the growth of the bacteria that cannot use pectin as the carbon source.
Pure cultures were maintained on Vincent's agar medium.

Identification of the Bacteria

The isolated bacteria were identified using different staining (Simple, Gram), biochemical and molecular techniques.

Biochemical Tests

The strain isolated from soils was identified by conventional biochemical tests in accordance with Bergey's Manual of Systematic Bacteriology (Taiwo and Oso, 2004). The isolate was subjected to different tests like Indole production test, Methyl red and Voges proskauer tests, Gelatin Hydrolysis, Starch Hydrolysis, Oxidase Production, Catalase Activity, Citrate utilization test, Nitrate reduction test, Caesin Hydrolysis etc.

Identification of the Isolated Bacteria by Sequencing the Amplified 16S rRNA Gene

This was done using the PCR (Mullis, 1990) and the amplified product has been subjected to sequencing and the sequence obtained has been compared with the sequence obtained from the Nucleotide Database of NCBI.

Scanning Electron Microscopy of *Bacillus subtilis*

SEM studies were carried out by taking 24 hrs old cultures of *Bacillus subtilis.*

Stock seed preparation

About 5 ml of sterile distilled water was added on to the well grown culture and vortexed for few minutes to make uniform bacterial suspension. One ml of this suspension was used to incubate 50 ml of sterile seed medium present in 250 ml Erlenmeyer flask. These inoculated flasks were kept on a shaker and maintained at 37°C for 24 to 36 hours and this seed was used for all the other experiments.

Production of Pectinase

For production of pectinase, 2.0 ml of the seed was taken and inoculated in to 250 ml Erlenmeyer flasks containing 50 ml sterile productive medium (Vincent Broth). The inoculated flasks were incubated at 37°C for 96 hours using aerated and agitated condition on a rotary shaker with 250 rpm for 96 hours. At the end of the fermentation cycle, 5.0 ml of the fermented broth was aseptically removed and centrifuged at 2000 rpm for 10 minutes. The clear supernatant containing the enzyme was used in the enzyme assay. For all the experiments, triplicates were maintained for consistent observations.

Enzyme Assay

Enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method (Miller, 1959). For this, to 0.2 ml of 1% pectin solution, 2.0 ml of sodium citrate buffer of pH 7.0 and 1.0 ml of enzyme extract were added. The reaction mixture was incubated at 37°C±1°C for 25 min. After 25 min, 1.0 ml of this reaction mixture was withdrawn and added to test-tubes containing 0.5 ml of 1M sodium carbonate solution. To each test-tube, 3.0 ml of DNS reagent was added and the test-tubes were shaken to mix the contents. The test-tubes were heated to boiling on the boiling water-bath for 10–15 min. The tubes were cooled and 4 ml of the reaction mixture was measured at 550 nm. The enzyme and substrate blanks were run parallel to one another and the standard curve was prepared for reducing sugars with glucose. One enzyme unit of endopolygalacturonase is the number of µM of reducing sugars measured in terms of glucose, produced as a result of the action of 1.0 ml of enzyme extract in 1 minute at 37°C ± 1°C.

The OD of test was subtracted from the OD unknown

= OD of Test – OD of Unknown = to the OD of the Colour intensity of the liberated product.

From graph, the concentration of the reducing sugar liberated by the action of the enzyme is determined and the Enzyme activity is expressed.

Enzyme Activity = µ moles of the product liberated per mole of enzyme per ml per Minute of Glucose liberated.

$$\text{IU/ml/min enzyme} = \frac{(\mu\text{mol of glucose equivalent released})\ (11)}{(1)\quad (10)\ \ (2)}$$

11 = Total volume (in milliliters) of assay
10 = Time of assay (in minutes) as per the Unit Definition
1 = Volume of enzyme (in milliliter) used
2 = Volume (in milliliters) used in Colorimetric Determination

Optimization of Physico-chemical parameters for Pectinase Production by *B. subtilis*

Pectinase enzyme production in any species such as bacteria can be varied and affected by different physico–chemical parameters (Suneetha and Khan, 2010). These parameters could affect the secretion of the enzyme in different ratios. Therefore, in our present investigation the pectinase enzyme production was optimized at various temperature ranges, pH, and substrate concentration (natural carbon sources).

Optimization of the pH for Pectinase Production by *B. subtilis*

Optimization of the pH of basal medium and production media (Vincent's mineral salts agar medium) was adjusted to 4, 5, 6, 7, 8 and 9 by using 1N HCL or 1N NaOH. All the experiments were carried out in triplicates at 37°C and average values were noted. The optimum pH achieved by this process was followed for subsequent experiments (Patil and Dayanand, 2006).

Optimum temperature for Pectinase Production by *B. subtilis*

The production process was carried out by shake flask method by placing the flasks on a rotary shaker. The production process was carried out at various temperatures such as 25ºC 30ºC, 35ºC, 40ºC, 45ºC, 50ºC and 55ºC, to study the effect of temperature on enzyme production (Soriano Diaz and Pastor, 2005). The optimum range of temperature achieved by this step was followed for all subsequent experiments.

Effect of Different Substrates on Pectinase Production by *B. subtilis*

Since all the plant agro-industrial waste material contains pectin as the cell biomass which can be used as substrates for the production of the pectinase (Aparna and Gupta, 2001; Patil and Dayanand, 2006; Nitinkumar *et al*., 2010). Two different natural substrates such as dried orange peel powder and dried banana peel waste powder (0.5%, 1%, 1.5%, 2% and 2.5%) were used in the present study. The production medium was supplemented with the substrates i.e. dry orange peel powder and banana peel waste powder as the source of carbon and inoculum of *B.subtilis* was added into 250 ml production medium for pectinase production. The study was carried out in triplicates by maintaining temperature, pH at their optimum level and the average values of pectinase production were recorded. The best substrate which gave highest enzyme activity was identified and that substrate was used for all other parameters.

RESULTS AND DISCUSSION

Collected soil sample were enriched with pectin and isolation of Pectinase producing bacteria was done by using Vincent's Selective Medium (Vincent, 1970). The isolates were subjected to simple and Gram's staining (Nasrin *et al*., 2008). The isolate 'A' appeared as Gram positive bacilli (Table 1).

Table 1. Microscopic examination and biochemical reactions of bacterial Population from isolated soil of Duggirala & Burripalem (Guntur Dt.)

Microscopic and Biochemical Examinations of an Isolate	Isolate A
1. Simple staining 2. Gram staining 3. Motility Test	Rod shaped Gram +ve rod Motile
1. Indole production Test 2. Methyl Red test 3. Voges-Proskauer Test 4. Citrate utilization Test 5. Catalase Test 6. Nitrate reduction test 7. Starch Hydrolysis 8. Caesin Hydrolysis 9. Gelatin Test 10.Oxidase Test	Negative Negative Positive Negative Negative Positive Positive Positive Positive Positive

SEM (Scanning Electron Microscopy)

The preliminary study on morphology of the bacterium suggested that the bacterium is a *Bacillus* species. The bacterium was rod shaped and had a positive gram stain reaction. SEM analysis had revealed that the bacterium was rod shaped and size of the bacterium was about 2 - 3 µm (Fig 1).

Biochemical characterization

The isolates were identified by conventional biochemical tests in accordance with Bergey's Manual of Systematic Bacteriology (Taiwo and Oso, 2004). The isolates were considered to be the members of the group *Bacillus* based on the staining procedures and the biochemical results (Kasing *et al.,* 2000; Catia *et al.,* 2008). The isolate 'A' was negative for Indole production, Methyl Red test, Citrate utilization and Catalase test and the organism was positive to Caesin Hydrolysis, Gelatin, Nitrate reduction, Starch hydrolysis, Voges-Proskauer and Oxidase Tests.

Basing on the microbial and biochemical characterization (Virginia *et al*., 2004; Wen-Jing *et al.*, 2005), the test organism showed that it was capable to produce pectinase and the Culture "A" can be inferred as *Bacillus subtilis* (Table 1). However, it cannot be concluded at this stand point itself and for the identification of the bacteria exactly upto the species level, it is evident to follow molecular based techniques and hence an attempt was carried out further for the characterization of the test bacteria based on the DNA coding for 16s rRNA sequences.

Molecular Characterization based on 16s RNA Sequence

The DNA extracted from the isolate was checked for purity by spectroscopy and by agarose gel electrophoresis confirmed to be pure (Fig 2) and the same containing the gene (DNA) coding for 16s rRNA was amplified by Polymerase chain reaction, yielded a DNA band of 800 base pairs (Fig 2). Sequencing of the 16S rRNA gene of *B. subtilis* was also done (Sergei Bavykin *et al*., 2004; Catia and Orlando, 2008). The gene sequence was submitted to NCBI and the **GenBank accession number** assigned was **JQ946083**.

Effect of Physical Factors on Pectinase Activity of *B. subtilis*

Enzymes are biocatalysts, i.e. substances which accelerate certain chemical reactions without actually being consumed themselves. They are found in every cell of all living beings from simple single cellular organisms to highly complex multicellular organisms including human beings. The rate of enzyme activity depends on various factors such as temperature, pH, substrate concentration, and source of nitrogen etc (Neeta Raj *et al*., 2011).

Effect of pH on pectinase activity of *Bacillus subtilis*

The effect of pH on the pectinase activity was studied by incubating the culture in production medium at different pH ranges from 4 to 9 using different buffered compositions at 37°C on a temperature regulated rotatory shaker at 200 rpm (Chawanit *et al.,* 2007). One unit of enzyme activity was defined as the amount of enzyme that released 1 µ mol of glucose per ml per minute (Fig 4).

The pectinase activity was found to be maximum at pH 7. The amount of the enzyme activity was expressed in IU/ml/min**.** It was observed that the enzymatic activity increased from 24hrs at pH 7 (0.9 IU/ml/min) and reached maximum after 96hrs (3.2 IU/ml/min) (Jayani *et al*., 2010; Nitinkumar *et al*., 2010; Ranveer *et al*., 2010). Further increase in pH to 8 and 9 resulted in considerable decrease in enzyme activity (Baladhandayutham and Thangavelu, 2010; Praveen Kumar *et al.,* 2011; Elagovan Namasivayam *et al.,* 2011; Neeta Raj *et al.,* 2011 and Biswapriya *et al.,* 2011).

Effect of different temperature on pectinase activity of *B. subtilis*

Like pH, temperature is one of the most important parameter essential for the success of a fermentation reaction (Arun *et al.,* 2007). The effect of temperature on the pectinase activity of *B. subtilis* was determined at various temperatures ranging from 25°C to 50°C at pH 7 (Urmila *et al.,* 2005; Ranveer *et al.,* 2010; Neeta Raj *et al*., 2011) (Fig 5).

The pectinase enzyme activity of *B. subtilis at* 0.5% substrate concentration of pectin at varying temperature showed that the activity increased from 25°C, 30°C, 35°C & 37°C with pH 7 after 96hrs of incubation (1, 1.6, 2.4, 3.4 IU/ml/min) (Janani *et al*., 2010; Nitinkumar *et al*., 2010; Elagovan Namasivayam *et al.,* 2011). Further increase in temperature (40, 45 & 50°C) resulted in a considerable decrease in enzyme activity (2.2, 2, 1.2 IU/ml) (Phutela *et al*., 2005; Ranveer *et al.,* 2010; Hayrunnisa *et al.,* 2010).

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Effect of the Substrate conc. (Orange and Banana peel powder) on pectinase activity of *B. subtilis*

The pure culture of *B. subtilis* was inoculated into the conical flasks containing production medium at different concentrations (0.5%, 1%, 1.5%, 2%, 2.5%) of the natural substrate i.e., Orange peel powder and Banana peel powder (Prathyusha and Suneetha, 2011) with optimized pH and the temperature constant (Fig 6 & 7). The pectinase activity increased from 0.5% to 1.5%. Further increase in substrate concentration from 2% resulted in considerable decrease in enzyme activity (Neeta Raj *et al*., 2011). Maximum activity of the pectinase enzyme was obtained with 1.5% of Orange peel powder and 1.5% of Banana peel powder (2.5 and 2.8 IU/ml/min) respectively. There was a steady increase in the pectinase activity of *B. subtilis* from 24hrs of incubation and reached maximum at 96hrs but the pectinase activity decreased from 120hrs. Maximum pectinase activity was recorded with 1.5% of Orange peel powder and Banana peel powder after 96hrs of incubation at pH 7 and temperature 37°C (Palaniyappan *et al*., 2009; Arpita Dey *et al.,* 2011).

The pectinase activity of *B. subtilis* showed maximum activity at 1.5% concentration of Orange peel powder and dry Banana peel powder at temperature 37°C and pH 7. *B. subtilis* showed high pectinase activity in the presence of Banana peel powder than in Orange peel powder at pH 7 and temperature 37°C. The enzymatic activity increased from 24hrs at conc of 1.5% of Orange peel powder and Banana peel powder (1 IU/ml/min for Orange peel powder and 1.5 IU/ml/min for Banana peel powder) and reached maximum after 96hrs at 37°C and at pH 7 (2.5 and 2.8 IU/ml/min) respectively and again there was a decrease in pectinase activity from 120hrs at 37°C and at pH 7 (1.8 and 2.2 IU/ml/min).

Fig 1. SEM of *Bacillus subtilis*

Fig 2. Isolated genomic DNA of *B. subtilis*

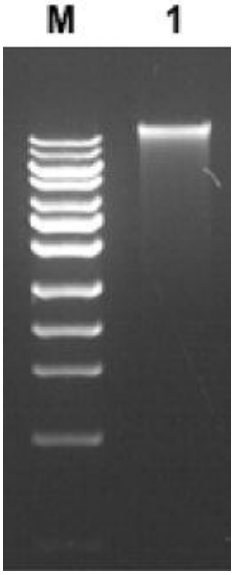
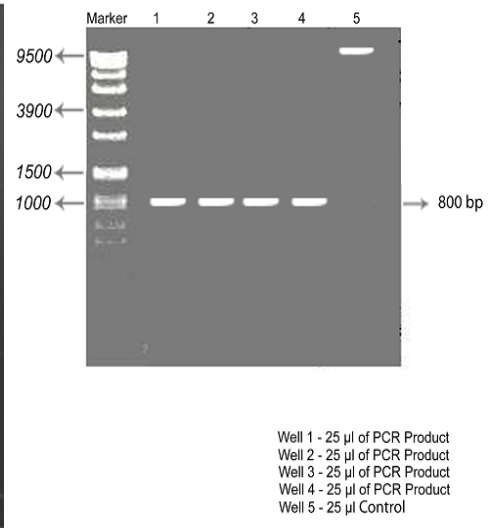


Fig 3. Amplified 16S rRNA of *B. subtilis*



M – DNA Marker
Well 1 – Genomic DNA of *B.subtilis*

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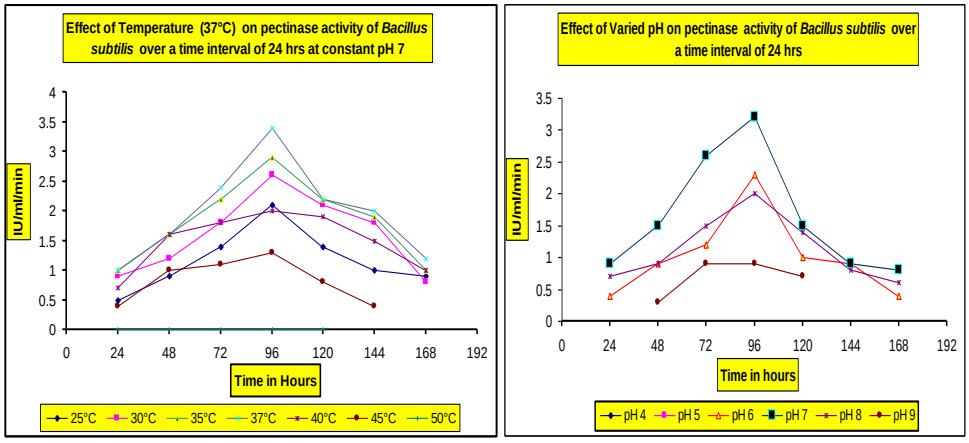


Fig 4&5. Effect of Temperature & P^H

Fig 6. Effect of different concentration of Orange peel powder on pectinase activity of *B. subtilis* at pH 7 and temperature 37°C over a Time Interval of 24 hrs

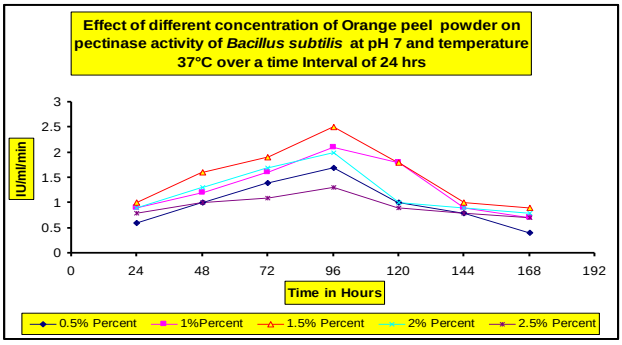
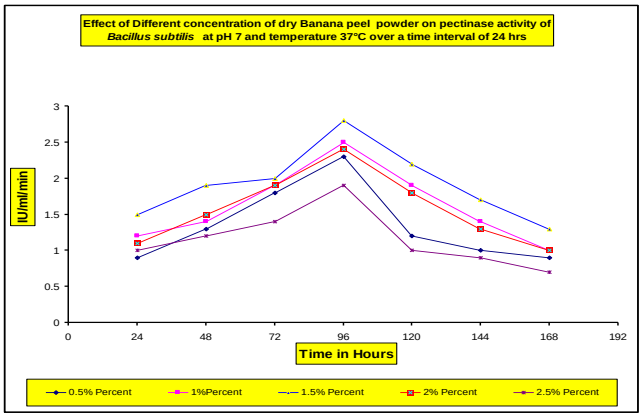


Fig 7. Effect of Different concentration of dry Banana peel powder on pectinase activity of *B. subtilis* at pH 7 and temperature 37°C over a Time Interval of 24 hrs



Based on some of the previous studies using the natural substrates as the source of carbon, it was reported that the agricultural wastes of lignocelluloses are used as a carbohydrate source to produce commercially important products such as ethanol, glucose and single cell protein. Similarly, Ojumu *et al.,* (2003) also reported some lignocelluloses as carbon source for pectinase enzyme production. In the present study, different concentrations (0.5 to 2.5%) of Orange peel powder and Banana peel powder were used as a carbon source to find out their effect on the pectinase activity (Neeta Raj *et al.,* 2011). Thus, the pectinase activity was determined using Orange peel powder and Banana peel powder as the source of carbon (Nitinkumar *et al.,* 2010).

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SUMMARY AND CONCLUSION

Pectinases are a group of hydrolytic enzymes that involve in the breakdown of pectin which show many industrial applications. Pectolysis is an important process in plants as it plays a role in cell elongation, growth and fruit ripening. Pectolytic enzymes are wide spread in nature, produced by different microbes. The present study was aimed at isolation and identification of the pectinase producing bacteria by microscopic, biochemical and molecular characterization and also optimizing the production parameters. The influence of different physico-chemical parameters like pH, temperature, and agro wastes (dry orange peel and banana peel powder) as carbon source on pectinase production was studied. The optimum pH and temperature for the enzyme production was pH 7 and 37°C respectively, while the maximum enzyme production was observed between 72 - 96 hrs. Of the two natural carbon sources tested, the banana peel powder yielded higher enzyme production compared to the orange peel powder. Thus*, Bacillus subtilis* is one of the most versatile of bacteria which is more useful to society.

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