

USE OF *CITRUS LIMETTA* PEELS FOR PECTINASE PRODUCTION BY MARINE *BACILLUS SUBTILIS*

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Abstract

The aim of this study was to evaluate the efficiency of locally available pectin rich *Citrus limetta* peels as a substrate for pectinase production by marine *Bacillus subtilis* in submerged fermentation (SmF). The marine *Bacillus subtilis* strain used in this study was isolated from marine sediment sample collected from Chinchani beach, India. *Citrus limetta* peels was selected as one of the main constituent in medium formulation for extracellular pectinase production as they are pectin rich and easily available. Different physical and biotechnological parameters such as incubation time, temperature, pH, substrate concentration and nitrogen source, were optimized. The enzyme is maximally produced in late exponential phase of the growth of the culture during submerged fermentation after 28 hours in medium containing *Citrus limetta* peel powder (3%). The pectinase activity was found to be maximum when pH of medium containing *Citrus limetta* peels was 5.0 at 40°C.

Key words: marine, *Bacillus subtilis*, pectinase, *Citrus limetta*

Introduction

In nature, microorganisms have been endowed with enormous potentials. They produce a vast array of enzymes, which have been exploited commercially over the years. With the recent advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties.

Marine microorganisms have recently emerged as a rich source for the production of industrial enzymes (Chandrasekaran *et al.*, 1997). Marine bacterial enzymes have several advantages for

industrial utilisation. Most marine bacterial enzymes are considerably thermotolerant, remaining stable at room temperature over long periods. In addition the optimum activity of marine bacterial enzymes usually occurs at high salinity, making these enzymes utilisable in many harsh industrial processes, where the concentrated salt solutions used would otherwise inhibit many enzymatic transformations (Mohapatra *et al.*, 2003).

Pectinases are heterogenous group of related enzymes that catalyze breakdown of pectic substances. Microbial pectinases have tremendous

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potential to offer mankind. Pectinases are widely used in textile industry in retting and degumming of plant bast fibres (Saleem *et al.*, 2007) scouring of cotton (Kalantzi *et al.*, 2010), in pectic wastewater treatment (Tanabe *et al.*, 1987), papermaking (Reid *et al.*, 2000) extraction and clarification of fruit juices (Pinelo, 2010; Joshi, 2011), oil extraction (Najafian *et al.*, 2009), coffee (Murthy *et al.*, 2012) and tea fermentations (Jayani, 2005; Thakur, 2012).

Pectinase find novel applications in production of pectic oligosaccharides as functional food components/ as prebiotic component (Combo, 2012; Sabajanes, 2012) and extraction of DNA from plants (Rogstad *et al.*, 2001). Emerging new applications underline the importance of screening pectinase producing microorganisms with novel properties, greater enzyme activity and also large scale production of these enzymes. (Singh and Mandal, 2012) Higher cost of production is perhaps the major constraint in the industrial application of the enzyme. Also for the process to be commercially viable it should be cost effective. In this concern, use of fruit waste or agricultural residues for pectinase production had been explored by many researchers. (Bayoumi, 2008; Ali, 2010; Trujillo, 2011; Geetha, 2012) Fruit wastes are perishable materials and their disposal often is a problem in processing industries. Production of enzymes using these waste residues by using technology of fermentation is one of the many ways of exploiting them profitably (Venkatesh *et al.*, 2009).

Pectic substances are widely distributed in fruits and vegetables. Citrus fruits are rich source of pectin such that substantial portion of commercial pectin is derived from citrus peel. The inner white spongy portion of citrus peel, albedo is rich in pectin. On dry weight basis *Citrus limetta* peels are reported to contain 30.28% calcium pectate which represents an important natural substrate for pectinases (Sastri, 1950).

The present study reports nutritional and environmental conditions required for the production of pectinase by marine *Bacillus subtilis* in submerged fermentation using *Citrus limetta* peel powder as substrate.

Materials and reagents

Citrus limetta (Mosambi) peels were obtained from a local market and were dried in hot air oven at 40°C, ground using a mill and then autoclaved at 121 °C for 15 minutes. The chemicals viz. pectin, yeast extract, and agar were of bacteriological grade and supplied by HiMedia, Mumbai. Polygalacturonic acid (PGA) was purchased from Sigma chemicals (St. Louis, MO, USA). All the other chemicals were procured from S. D. Fine chemicals ltd., Mumbai.

Sample for bacteria isolation

Marine sediment samples were collected from Chinchani beach, Tarapore, India. Samples were collected from five different areas within range of 1 km around coast and were carried to laboratory and stored at 4°C until using.

Bacteria isolation and screening

For enrichment of pectinase producing bacteria, collected samples were inoculated in Pectin Yeast extract (PYE) broth. The broth contained 0.25% pectin, 1.0 % yeast extract, 50 ml distilled water (D/W) and 50 ml filtered sea water. The pH was adjusted to 7.0 and the broth was autoclaved at 121°C, for 15 minutes. Then, 1g of soil sample was inoculated in aseptic conditions in the above broth and incubated for 24 h.

A volume of 0.5 ml from the enrichment broth was serially diluted to 10⁻⁷ dilution and was spread plated on nutrient agar prepared using 50 ml sea water and 50 ml D/W. Colonies obtained were further subjected to secondary screening by using Pectin Yeast Extract (PYE) Agar, pH 7.0 and incubated at 40°C, for 24 h. Slant agar medium in petri plate was overlaid with 0.25% congo red solution for 10 minutes.

Congo red solution was then poured off and slant agar medium in petri plate and then was washed with several exchanges of 0.1% NaCl solution in order to remove any superficially adhered congo red dye. Diameter (D) of the clear hydrolytic zone around the colonies and the diameter (d) of the colonies were measured. The values of ratio D:d was calculated and on basis of this value, the pectinolytic strains were selected (Anand *et al.*, 2010).

The isolate with enzymatic activity was primarily identified by Gram staining and morphological studies. The taxonomic identification was carried out at National Centre for cell sciences (NCCS), University of Pune Campus, using 16S rRNA method.

Pectinase production and fermentative conditions optimisation

Pectinase production using selected strain of *Bacillus subtilis* was carried out by submerged cultivation.

A liquid medium containing 1.0% yeast extract and 1.0% *Citrus limetta* peel powder with pH adjusted to 7.0 was sterilized by autoclaving at 121°C, 15 psi for 15 min. 1 % inoculums of 24 h old *Bacillus subtilis* culture (10^8 CFU/ml) was then added to 50 ml of the medium (in conical flask of 100 ml volume).

The flasks were incubated at 40°C for different time intervals on a rotary shaker at 150 rpm. The absorbance of the culture was measured by spectrophotometer at wavelength of 600 nm. The culture was then centrifuged at 5000 rpm, 20 min and the cell free supernatant was assayed for pectinase activity (Kashyap *et al.*, 2000)

In order to achieve maximum pectinase activity by the isolate; medium parameters such as, incubation period, pH of the medium, incubation temperature, pectin concentration, yeast extract concentration were optimized.

Experimental methods for studying all the parameters were carried out using sterile PYE broth. Medium optimization studies were carried out by studying one parameter at a time while keeping other parameters constant. Biomass production and pectinase biosynthesis was determined at intervals of 1 h. It is to be noted that all experiments were done in triplicates.

Pectinase activity assay

Polygalacturonase activity was assayed by the colorimetric method (Miller, 1959).

A volume of 250 μ l of cell free supernatant was incubated with 500 μ l of substrate (PGA, 1.0% w/v, pH 8.0) at temperature of 40°C for 30 minutes under static conditions.

After adding 1 ml of 3, 5- dinitrosalicylic acid (DNSA) reagent, the mixture was boiled for 5 min. Mixture was finally diluted to 10 ml with deionised water (8 ml). The absorbance of the colour developed was measured at wavelength of 540 nm.

One unit of enzyme was defined as the amount of enzyme which catalyses the formation of 1 μ mol of galacturonic acid during one minute.

Pectate lyase activity assay

The pectate lyase activity was assayed by the spectrophotometric method (Macmillan and Phaff, 1966).

Briefly, a stock solution of substrate is prepared by mixing 150 ml sodium polygalacturonate, 90 ml 0.1M Tris buffer and 30 ml 0.005 M CaCl₂. To 2.7 ml of substrate solution a volume of 0.3 ml diluted enzyme was added and the increase in absorbance was measured at 235 nm and 25°C.

One unit of pectate lyase activity is the amount of enzyme which will produce 1 μ M of unsaturated product per minute at pH 8.0. The molar extinction coefficient for pectate lyase is 4600 M⁻¹ cm⁻¹.

Statistical analysis

All determinations were obtained from triplicate measurements and results were expressed as mean \pm standard deviation. Student's T test was used to analyse data and statistical significance was declared at $p < 0.05$.

Results and discussions

Preliminary agar plate test resulted with the isolation of 12 bacterial strains. Only 4 bacterial strains were detected with pectinase activity after flooding PYE agar plates with 0.25% Congo red solution. These 4 bacterial strains received identification codes like MP1, MP2, MP3 and MP4. The pectinolytic strain showing maximum D/d value i.e. strain MP2 was selected for further experiments (Table 1).

Combination of pectin with yeast extract was found to be an excellent medium for pectinase production (Moran and Starr, 1969; Dave and Vaughn, 1971).

Table 1. Screening *Bacillus subtilis* strains producing extracellular pectinase

<i>Bacillus subtilis</i> strains	Diameter of the hydrolysis zone, mm [D]	Diameter of the colony, mm [d]	Ratio D:d
MP1	14	5	2.8
MP2	20	7	3.4
MP3	9	4	2.25
MP4	14	8	1.75

Morphological characteristics of MP2 strain are presented in Table 2.

Table 2. Morphological characteristics of MP2 strain

Strain	MP2
Test	
Configuration of colony	Circular
Margin	Entire
Elevation	Convex
Pigment	White
Gram Reaction	Positive
Shape	Short rods
Size	~2 μm
Arrangement	Single
Motility	Motile

Gram staining and morphological examinations of the isolate MP2 revealed that it is Gram positive bacteria; rod shaped sporulating microorganisms

with terminal endospores and it shows white colonies by growth on PYE agar.

The taxonomic identification carried out at National Centre for Cell Science (NCCS), Pune by using 16s rRNA method confirmed that pectinolytic strain belongs to *Bacillus subtilis*.

Production of extracellular pectinase was evaluated up to 40 h of submerged cultivation (Figure 1). It was observed that extracellular pectinase production is directly proportional to microorganisms growth. Maximum activity was 24.18 units/ml and it was found after 28 h of incubation in late exponential phase of the growth of the culture after which activity decreased as growth of the *Bacillus subtilis* also showed decreasing pattern. In the case of extracellular pectinase production by *Bacillus subtilis* it was observed that the enzyme is synthesized during the growth phase and rate of enzyme synthesis declines as the culture enters stationary phase (Kurowski *et al.*, 1976).

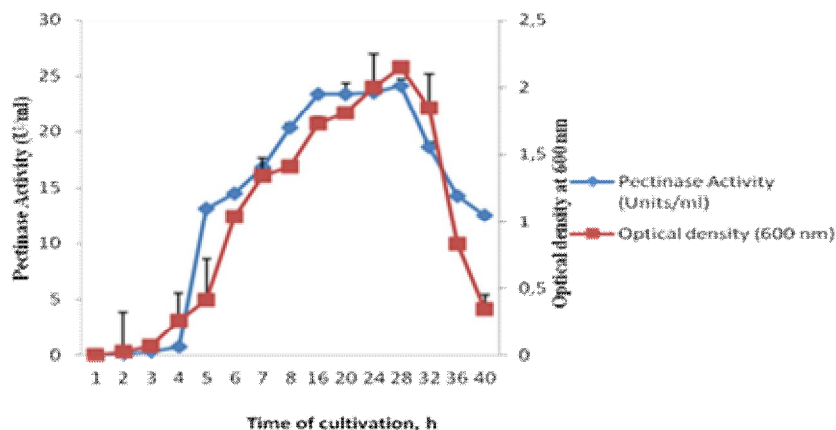


Figure 1. Growth dynamic and extracellular pectinase production by marine *Bacillus subtilis* strain

The pH of growth medium greatly affects extracellular pectinase production. Figure 2 show that pH 5.0 is optimum for maximum yield of the enzyme activity. Similar results of higher pectinase production under acidic condition were reported also by [Vasanthi and Meenakshisundaram, 2012](#).

As can be seen from Figure 2, there is not much difference in extracellular pectinase production at pH 5.0 and pH 11.0. Production of pectinase or polygalacturonase hydrolases is stimulated at acidic ambient pH whereas pectate lyases are

exclusively produced at neutral or alkaline pH ([Romdhane et al., 2012](#)).

Action of both polygalacturonase (pectate hydrolase) and pectate lyase on polygalacturonic acid yields reducing sugars have been estimated by colorimetric method by using 3, 5 dinitrosalicylic acid reagent. The separate assay specific for determination of pectate lyase was performed. As can be seen in Figure 3 the pectate lyase activity was maximum at pH 11.0.

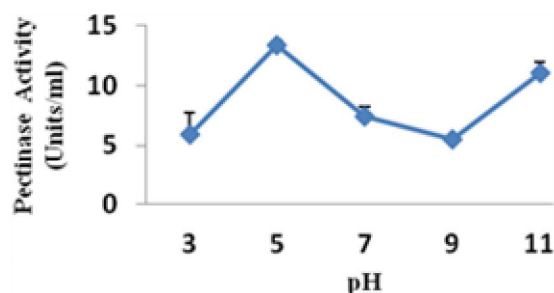


Figure 2. Effect of pH on extracellular *Bacillus subtilis* pectinase production

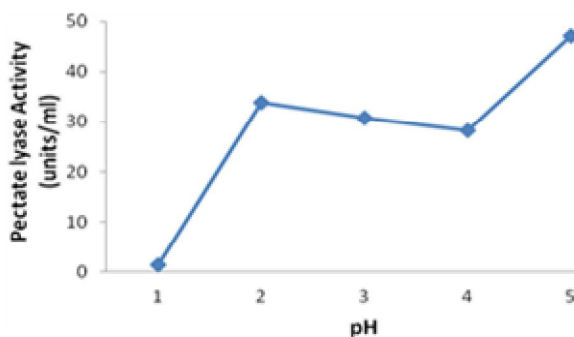


Figure 3. Effect of pH on extracellular *Bacillus subtilis* Pectate lyase production

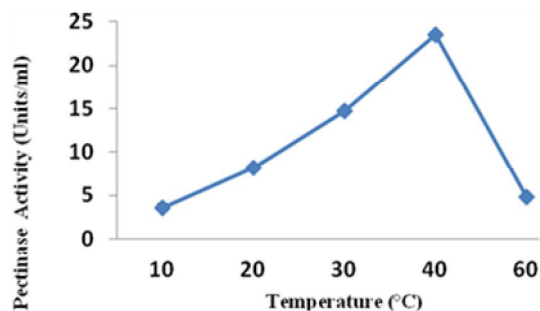


Figure 4. Effect of temperature on extracellular *Bacillus subtilis* pectinase production

The enzymatic activity measured at pH 11.0 accounts for both polygalacturonase and pectate lyase production. Hence marine *Bacillus subtilis* isolated produces two types of pectinases polygalacturonase and pectate lyases majorly at pH 5.0 and pH 11.0 respectively.

Further in this study the parameters for production of polygalacturonase were optimized as end use of polygalacturonase or pectinase enzyme in bioscouring of cotton. Bioscouring of cotton is also done using other hydrolytic enzymes such as lipase, cellulase and protease (Karapinar 2004, Kalantzi 2010). All of them are hydrolytic

enzymes and require the presence of water. Hence focus of study was on studying optimum production parameters for polygalacturonase hydrolase and not on pectinlyase.

Optimum temperature for the extracellular pectinase production was found to be at the temperature of 40°C as can be seen from Figure 4. If the temperature increased from 10°C to 40°C, pectinase activity was found to be increasing while at 60° C there was drop in the enzymatic activity. Optimum temperature of 37°C for pectinase production by *Bacillus sp.* DT7 has been already reported by Kashyap *et al.*, 2010.

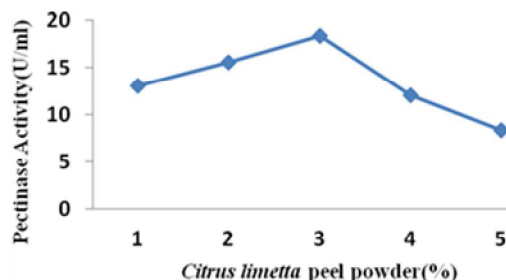


Figure 5. Effect of natural pectin substrate concentration on extracellular *Bacillus subtilis* pectinase production

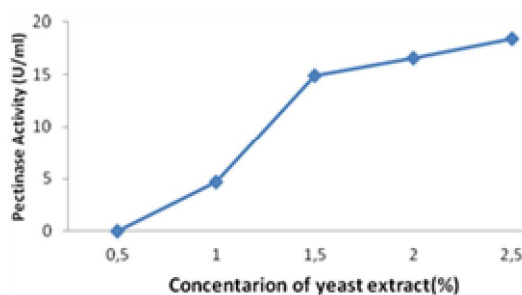


Figure 6. Effect of yeast extract concentration on extracellular *Bacillus subtilis* pectinase production

Figure 5 reveals that increase in the concentration of *Citrus limetta* peel powder showed increased production of extracellular pectinase. However, beyond 3% *Citrus limetta* peel powder used in the production medium the production of the pectinase was observed to be decreased. This can be attributed to the phenomenon of catabolite repression, where galacturonic acid or one of the metabolites produced is undergoing self catabolite repression (Tsuyumu, 1979). Similar result of enzyme activity

inhibition at higher pectin concentration in fermentative medium was reported in pectinase production by *Aspergillus niger* MTCC 281 by using natural substrates (Palaniyappan *et al.*, 2009).

Figure 6 indicates that the supplementation of the fermentative medium with yeast extract presents a positive effect on the pectinase activity. A concentration of 2.5% yeast extract was found to

be optimum for the extracellular pectinase production. Further increase in yeast extract concentration showed inhibition of enzyme activity. Similar results of inhibition of pectinase production from *Bacillus subtilis* EFRL 01 at higher yeast extract concentration are as well reported by Qureshi *et al.*, 2012. It can be inferred that supplementation of medium with nitrogen source leads to increase in pectinase production.

Conclusion

Marine *Bacillus subtilis* strain isolated from marine sediment sample was found capable to produce extracellular pectinase enzyme. For maximal yield of enzyme production the fermentation parameters were optimized. Use of sea water and *Citrus limetta* peels as ingredients in fermentative medium for submerged cultivation of bacterium and extracellular pectinase production makes process economically viable.

Based on the present study, it is concluded that marine *Bacillus subtilis* has wide scope for the industrial production of pectinase under submerged fermentation using *Citrus limetta* peels.

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