Physicochemical Factors Influencing Pectinolytic Enzyme Produced by *Bacillus licheniformis* under Submerged Fermentation

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Abstract: Agricultural wastes containing pectin can be considered as an alternate substrate for the production of pectinase. In this study different agricultural wastes as well as pure chemicals were used for pectinase production. *Bacillus licheniformis* isolated from cassava waste dump site was used to produce pectinase by submerged fermentation. The levels of the enzyme production detected in culture media varied with the type of carbon source used. Effects of different nitrogen sources revealed that a combination of yeast extract and casein increased the enzyme yield compared to other nitrogen sources. The maximum enzyme activity was obtained under optimum conditions at an incubation period of 48 h, temperature of 40°C and pH of 9.0 with orange bagasse as carbon source. The pectinase identified in this study could have potential application in industrial processes.
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1. Introduction

Enzymes are among the most important products required to meet human needs in the areas of industrial, environmental and food biotechnology (Aehle, 2008; Chaudhri and Suneetha, 2012). Pectinase are among the major enzymes required in extraction of fruit and vegetable juices to increase yield; controlling clarity of juices; enzymatic peeling of fruits; improving the texture of fruits and vegetables; wine production; extraction of pigments and food colorings (Tochi *et al.,* 2009). They have also been applied in textile industry as well as coffee and tea fermentation (Kobayashi *et al.,* 2001; Jayani *et al.,* 2005). It is also used for waste water treatment containing pectin (Chaudhri and Suneetha, 2012). This enzyme account for approximately 25% of the world enzyme market (Jayani *et al.,* 2005). In recent years, interest in its microbial production has increased (Beg *et al.,* 2001; Viikari *et al.,* 2001; Jayani *et al.,* 2005).

The wide spread use of pectinases have placed it among the most important enzymes of great significance for biotechnology (Celestino *et al.,* 2006). Microbial pectinases are the main sources for pectinase production and serve as a preferred source because of rapid growth, limited space required for microbe cultivation, low production cost and more predictable and controllable enzyme content. Selection of the right organism therefore, plays a key role in yield of desirable enzymes (Rao *et al.,* 1998).

Several microorganisms have been used to produce different types of pectinolytic enzymes (Li *et al.,* 2005; Jayani *et al.,* 2010; Kumar and Sharma, 2012). Pectinases from fungal sources are known to produce best under acidic pH and low temperature which makes them more applicable to industrial processes which operate optimally at acidic to neutral pH (Jayani *et al.,* 2005). Bacteria are however, known to produce alkaline pectinases and therefore, are best applied to industrial processes operating in the alkaline pH and could withstand high temperatures exceeding 45°C (Hoondal *et al.,* 2002 Li *et al.,* 2005). Selection of a particular strain remains a tedious task and the choice gets tougher when commercially competent enzyme yields are to be achieved (Lotfi *et al.,* 2011).

To meet the growing industrial demands for pectinase, it is necessary to improve yield without increasing the cost of production. The optimization of fermentation conditions could enhance enzyme production without increasing cost. This is because the growth and enzyme production of organisms are strongly influenced by medium composition thus optimization of media components and cultural parameters is essential (George-Okafor and Mike-Anosike, 2012; Sharma *et al.,* 2013).

The present investigation aimed at optimizing the physicochemical factors required for the production of pectinase by *Bacillus licheniformis.*

2. Materials and Methods

2.1 Isolation of Microorganisms

Soil samples were collected from cassava waste heap at the Teaching and Research Farm Obafemi Awolowo University, Ile-Ife, Nigeria. The samples were collected into sterile Mac-Cartney bottles and transported to the laboratory for analysis. One gram of soil sample from the collection site was pooled and

homogenized in 10 ml sterile distilled water (Raju and Divakar, 2013). The suspension was streaked unto sterile nutrient agar plates in duplicates and were incubated at 35ºC for 24 h. Thereafter, colonies were subsequently subcultured for pure isolates.

2.2 Screening of the Bacterial Isolates for Pectinolytic Activities

The screening of the isolates for pectinase production was carried out by pectin agar plate method (Al-Ajlani *et al.,* 2012). Plates were incubated at 35°C for 48 h. Thereafter, the plates were flooded with Lugol's iodine and pectinase-producing colonies were detected by the appearance of a clear halozone around them.

2.3 Identification of the Bacterial isolates

The bacterial isolates which hydrolyzed pectin were characterized by morphological and biochemical tests. The identity of the bacterial isolates was determined with reference to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

2.4 Production of Pectinase

The inocula were standardized to optical density 0.3 (Demirkan, 2011) and were inoculated at 1% (v/v) into the defined enzyme production medium containing 1 g citrus pectin, 0.2 g $(NH_4)_2SO_4$, 0.6 g K_2HPO_4, 0.2 g KH_2PO_4 and 0.01g $MgSO_4.7H_2O$ in 100 ml of distilled water. The initial pH was adjusted to 8.0 and sterilized under pressure at 121°C for 20 min. The medium inoculated in duplicates were incubated at 35ºC for 48 h in an orbital shaker incubator at 150 rpm. Thereafter, the cultures were centrifuged at 12000 rpm for 20 min and the cell free supernatants were used to evaluate pectinase activity (Kumar and Sharma, 2012).

2.5 Pectinase Assay

The pectinase activity of the crude culture filtrate was assayed according to the modified method of Miller (1959**)** as reported by Wang *et al.* (1997). The reaction mixture consisted of 0.80 ml of 1.0% w/v polygalacturonic acid in 100 mM Na_2CO_3−$NaHCO_3$ buffer (pH 9.0) and 0.20 ml of supernatant (enzyme solution). Control tubes (Enzyme blank) contained the same amount of substrate and 0.2 ml of the crude filtrate (enzyme solution) boiled for 10 min. Both the experimental and control tubes were incubated at 40 °C for 30 min. The reaction was terminated by the addition of 1.5 ml of 3,5-Dinitrosalicyclic acid (DNSA) reagent and the absorbance was taken at 540nm. One unit of pectinase activity was defined as the amount of enzyme that liberated reducing sugar equivalent to 1 µmol galacturonic acid per minute under the specified assay conditions (Karthik *et al.,* 2011). A standard calibration curve of galacturonic acid was constructed and used for the estimation of the polygalacturonic acid equivalent in µmol/ml/min.

2.6 Effects of Carbon Sources Used for Pectinase Production

Pectin, glucose, sucrose and agro wastes such as banana peels, orange bagasse, pineapple peels, yam peels and plantain peels were used as carbon sources. Agro wastes were collected from the local market and agro waste dumpsite in Ile-Ife. The wastes were oven dried at 80°C for 48 hours, pulverized to the powdered form. These substances were used at a concentration of 1% in place of the pectin in the production media other components were as described in the production medium.

2.7 Effects of Nitrogen Sources on Pectinase Production

The nitrogen sources utilized include peptone, urea, casein, yeast extract, $(NH_4)_2SO_4$, and Malt extract to test for the effect of nitrogen sources on pectinase production, other components of the media were as described in the production medium.

2.8 Effect of Incubation Time on the Production of Pectinase

The inoculated media were subjected to different incubation time for pectinase production. Aliquots were taken from the medium at time intervals of 12, 24, 36, 48 and 72 h for pectinase estimation.

2.9 Effect of Temperature on the Production of Pectinase

The inoculated production medium was incubated at selected temperatures: room temperature (25±2), 30, 35, 40 and 45°C and incubation time was 48 h.

2.10 Effect of pH on the Production of Pectinase

Effects of pH was studied at pH 6.0-10.0 using different buffers whose buffering capacity is within the required pH, the initial pH of media were adjusted to the appropriate pH and incubated at 35ºC for 48 h.

3. Results

The isolate was that possess the ability to hydrolyse pectin was identified as *Bacillus licheniformis.* No pectinolytic activity was observed when glucose was used as the sole carbon whereas the highest pectinase activity was observed with orange bagasse as a carbon source as shown in Figure 1. Figure 2 shows the result for the effect of nitrogen sources. Yeast extract as well as a combination of yeast extract and casein resulted high pectinase production. Figure 3 shows the effect of temperature on enzyme production. The optimal production temperature obtained was 40ºC and declined after this temperature by more than 40%. Figure 4 shows the effect of incubation time on pectinase production. The optimal incubation time obtained varied with different carbon sources; maximum activity was obtained at 48 hours of incubation using orange bagasse while other sources had optimum incubation time of 36 hours.

Figure 5 shows the effect of initial pH of media on enzyme The maximal production of the enzyme by the organism was at pH 9.0 an indication that bacteria produces well under alkaline conditions.

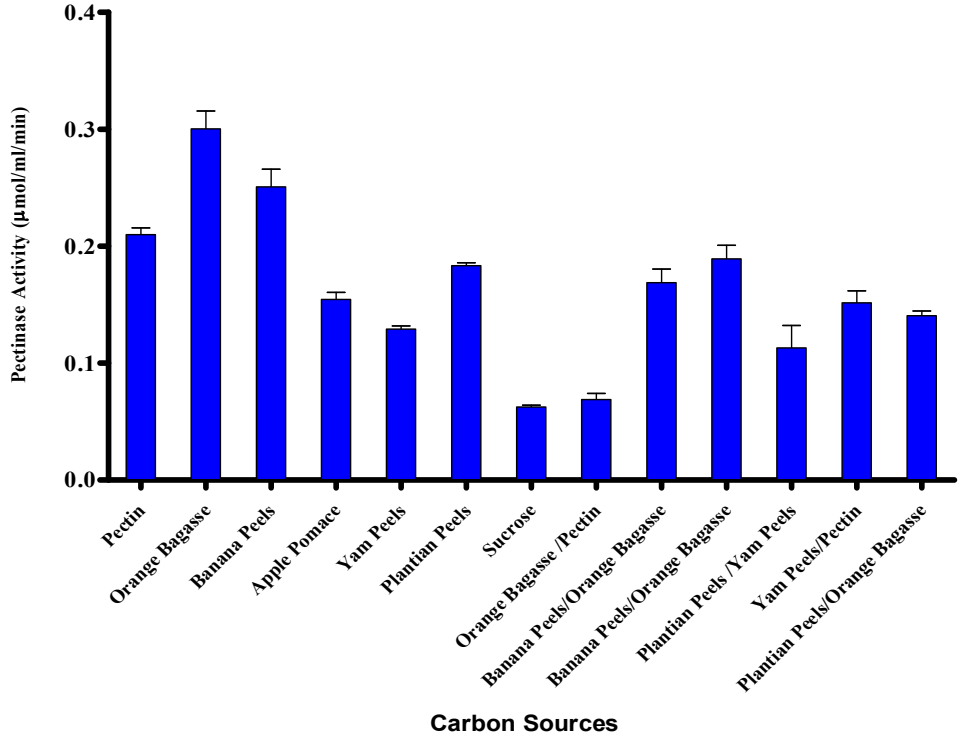

Figure 1: Effects of Carbon Sources on Enzyme Production.

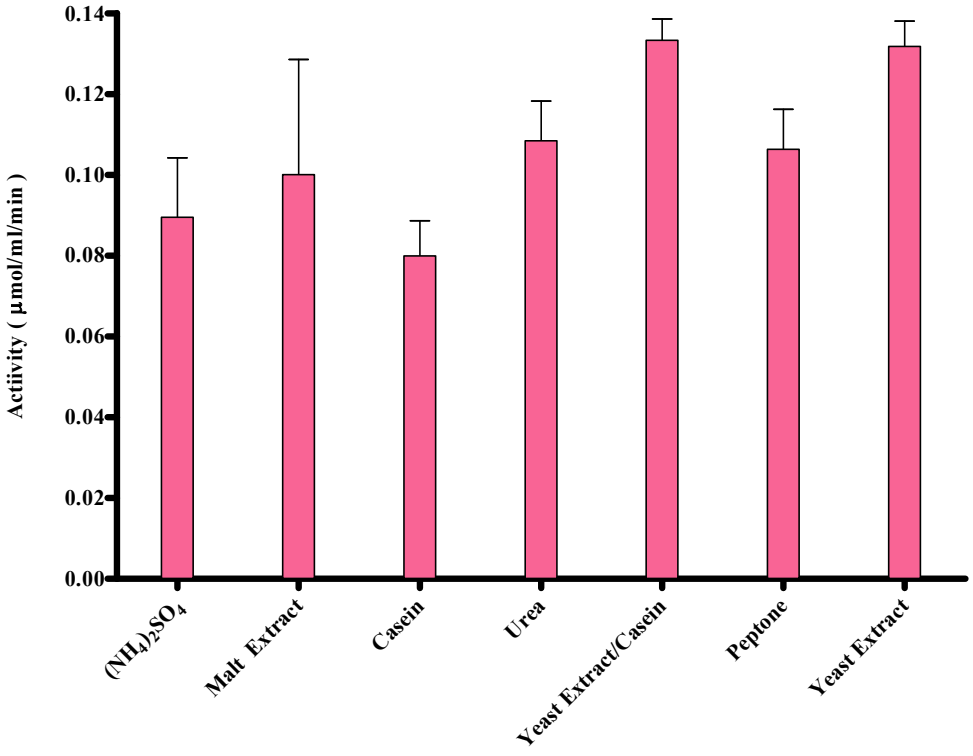

Figure 2: Effects of Various Nitrogen Sources on Enzyme Production.

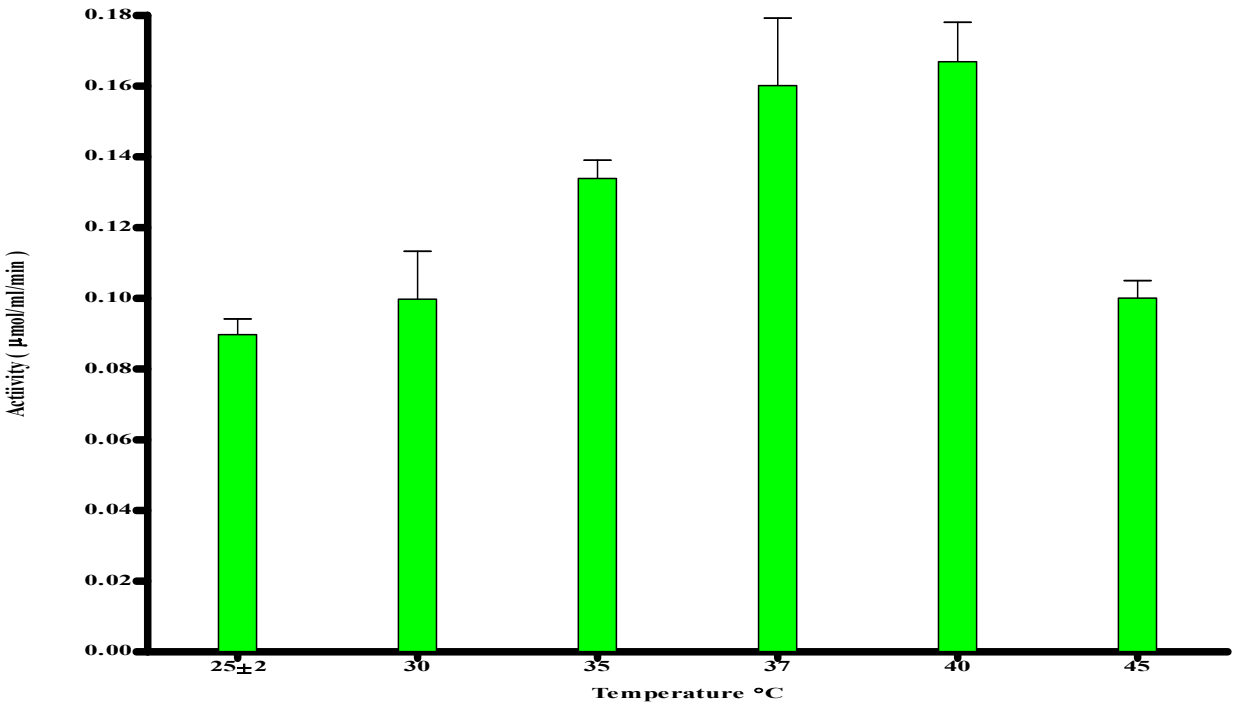

Figure 3: Effect of Temperature on Enzyme Production.

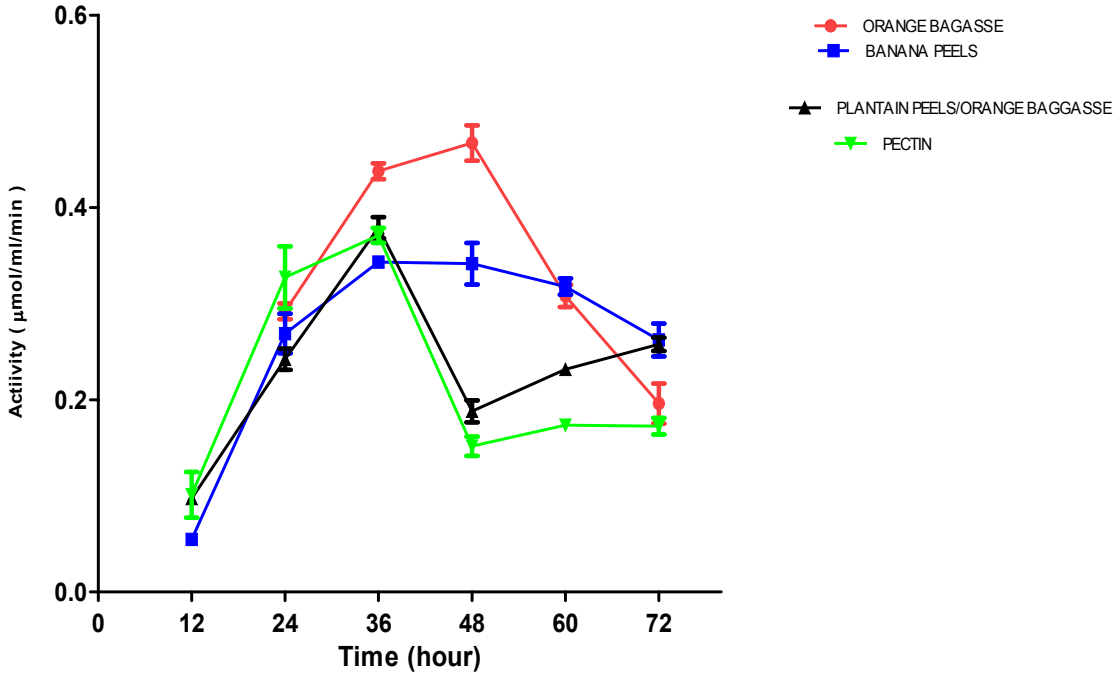

Figure 4: Effect of Incubation Time on Enzyme Production.

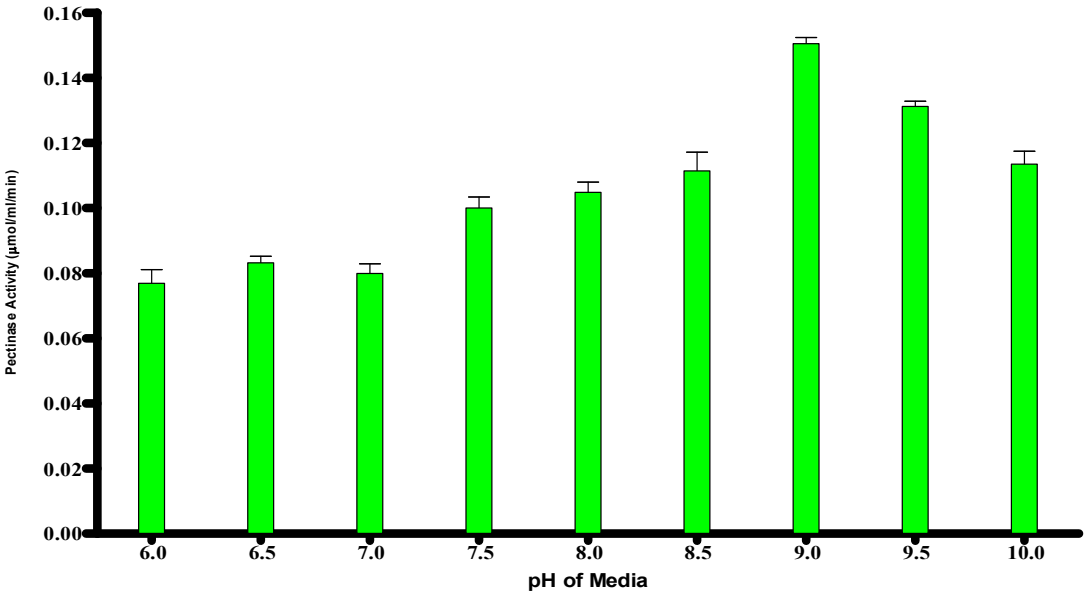

Figure 5: Effects of pH on Enzyme Production.

4. Discussion

The demand for enzymes in food industry is rising, and about 25% of the overall enzyme demand in the food industry is pectinase (Jayani *et al.,* 2005). Naturally, pectinase can be obtained from various sources of microorganisms such as *Aspergillus niger, Penicillium spp* (Sarioglu *et al.,* 2001), *Bacillus spp.* (Soares *et al.,* 1999; Rehman *et al.,* 2012), *Clostridium spp.* and *Pseudomonas spp* (Prathyusha and Suneetha, 2011). However, increasing demand for pectinase requires further exploration of sources of pectinase with desirable characteristics.

In this study, the effect of physicochemical parameters on the pectinase produced by *Bacillus licheniformis* isolated from agriculture waste dump site was evaluated using submerged fermentation. Glucose as sole carbon source did not result in the production of pectinase is an indication that the production of pectinase in *Bacillus licheniformis* is inducible and not constitutive. Dosanjh and Hoondal (1996) reported that production of pectinase using 1% glucose as a sole carbon source is an indication of constitutive nature of pectinase production, pectinases are produced by inductive effects of pectin containing substances as seen in this study; some organism produce this enzyme both constitutively and inductively. Glucose is known to repress the transcription of genes encoding enzymes required for the utilization of alternative carbon sources; some of these genes are also repressed by other sugars such as galactose, sucrose, arabinose and the process is known as catabolite repression (Chellegatti *et al.,* 2000; Beg *et al.,* 2001).Orange bagasse gave the optimum yield which is in agreement with reports of other investigators that orange baggase could be an excellent carbon source for pectinase production (Giese *et al.,* 2008; Ahmed and Mostafa, 2013).

Effects of organic and inorganic nitrogen sources were evaluated in pectinase production by *Bacillus licheniformis*. An increase of about 21% yield in pectinase production was observed when yeast extract as well as a combination of yeast extract and casein was used as nitrogen source. However, casein alone resulted in a low pectinase production. Similar results have been reported by other workers on different forms of pectinase, Thakur *et al.* (2010) reported that yeast extract resulted in high yield of polygalacturonase activity from *Mucor circinelloides* ITCC 6025 and also a combination of casein hydrolysate and yeast extract gave high yield of polygalacturonase from *Mucor circinelloides* ITCC 6025. Jayani *et al.* (2010) working on *Bacillus sphaericus* (MTCC 7542) reported that a combination of yeast extract and casein hydrolysate also gave high polygalacturonase activity.

In this study, 40°C was observed to be the optimum temperature for the production of pectinase by *Bacillus licheniformis.* This result is in agreement with pectinase production by *Bacillus circulans* isolated from dump yards of vegetable wastes which also had the optimum temperature of 40°C (Raju and Divakar, 2013). However, it was observed that the enzyme production decreased as the temperature was increased to 45°C. About 40% of the enzyme produced was lost at 45ºC as compared to the production at 40ºC. This may be an indication that temperature above 40°C

is not suitable for pectinase production by *Bacillus licheniformis*.

Incubation time has been shown to have an impact on enzyme yield. Reports have shown that many bacteria and fungi produce pectinase maximally at incubation time of 72 hours and above (Kumar and Sharma 2012; Oyeleke *et al.,* 2012; Islam *et al.,* 2013). However, in this study it was observed that maximum yield of pectinase produced by *B. licheniformis* is 36-48 hours for the various substrates used. A decrease in yield was observed when incubation time was increased above 48 hours. The short incubation period for *Bacillus licheniformis* may suggest that production of pectinase from the microbe could be cost effective for commercial exploitation. Also, the effect of pH on pectinase production was studied at a pH range of 6.0-10. The optimum pH for pectinase production by *B. licheniformis* was 9.0 with enzyme activity of 0.1505µmol/ml/min. Most *Bacillus species* have been reported to produce pectinase maximally at alkaline pH ranging from 7.0 to 9.0 (Kobayashi *et al.,* 1999; Rehman *et al.,* 2012).

In conclusion, the study shows that pectinase production from *Bacillus licheniformis* requires short incubation time, it can be induced and inexpensive agrowaste substrates can be utilised.This may suggest that the cost effectiveness of using the microorganism for pectinase production may be exploited for commercial production of the enzyme.

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