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A comparative study of pectinolytic enzyme production by *Bacillus* species

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The economic and ecological function of pectinase enzymes in industries is gaining much attention with the need of highly productive strains of microorganisms to reduce production cost. The present investigation is a comparative evaluation of *Bacillus* sp. pectinases. The three isolates isolated from agro-waste, *Bacillus stearothermophilus, Bacillus cereus* and *Bacillus subtilis*, showed very high pectinase activities with pectin as the substrate. The three isolates also showed varying degree of preference for banana peels and wheat bran but their activity towards orange peel was very low compared to other substrate. The effect of temperature on the pectinase of the three isolates showed that *B. stearothermophilus* had optimum temperature at 60°C while *B. cereus* and *B. subtilis* both showed optimum activity at 50°C. On the effect of pH, *B. stearothermophilus*, *B. cereus* and *B. subtilis* showed optimum pectinase activities at pH 7.5, 8.0 and 9.0, respectively. Metal ions enhanced the activity of pectinase produced by the three isolates. The study exhibited that *B. stearothermophilus* gave the most pectinase activity, optimum temperature and a moderate alkaline pH, possibly making it a better source for industrial purpose.

Key words: Pectinase, enzymes, pectin, bacteria, *Bacillus species*.

INTRODUCTION

Pectin, a major component of plant cell wall occurs primarily in the non-woody parts of plants which are parts that are most likely to be consumed. It also contributes to the firmness and structure of plant tissues (Sathyanarayana and Panda, 2003). During ripening process, plants generally use pectinase enzymes to hydrolyse some of the pectin in and between cell wall making the cell weaker and therefore soft and edible (Sakai, 1992). Pectinases are a complex heterogenous group of different enzymes that act specifically on pectic substances by decreasing intracellular adhesivity and tissue rigidity (Tatiana da Costa and Flevo, 2005). They have also been reported to be responsible for spoilage of fruits and vegetables (Collmer and Keen, 1986; Whitaker, 1990). However, pectinase has varied important uses in the industries especially in the juice and food industry. Reports have shown that the treatment of fruit pulps with pectinases increase juice volume from fruits (Kashyap et al., 2001; Kaur et al., 2004) with a 50% reduction in filtration time (Blanco, 1999). Pectinase has been reported to be useful in the paper and pulp industry (Beg et al., 2001; Viikari et al., 2001) as well as in the decomposition and recycling of plant wastes (Jayani et al., 2005).

Pectinolytic enzymes can be derived from different sources such as plants, animals and microorganisms (Whitaker, 1990; Banu et al., 2010; Namasivayam et al., 2011). Different types of microorganisms have been

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exploited for the production of pectinase because micro-organisms are not influenced by climatic and seasonal factors and can be subjected to genetic and environmental manipulations to increase yield (Vibha and Neelam, 2010). Microbial pectinases has been reported to account for 25% of the global food enzymes sales and majority of these are from fungal sources (Jayani et al., 2005). Pectinases from fungal sources produce best under acidic pH and low temperature conditions and can therefore not be used in varied industrial processes where neutral to alkaline pH with high temperatures exceeding 45°C are required. It has been shown that bacteria require high pH and temperature to produce pectinase (Chesson and Cordner, 1978; Silley, 1986). *Bacillus spp* have been reported to produce as high as 20-25 g/L of pectinase as compared to other bacterial isolates (Soares et al., 1999; Schallemey et al., 2001; Namasivavam et al., 2011). Based on the economic and ecological roles pectinase enzymes play, highly productive strains are required in the industries to reduce production cost. The present investigation is aimed at comparing *Bacillus spp* that produce high level pectinase under various physico-chemical conditions.

MATERIALS AND METHODS

Microorganisms

In 100 ml of sterile distilled water, 1 g of soil collected from agro-waste dumpsite was added. Isolation was carried out using 10 fold serial dilution and pour plate technique in triplicate. The cultures were incubated at 35°C for 48 h. Thereafter, the distinct bacteria colonies were streaked on nutrient agar (LAB M USA, UK) and incubated at 35°C for 24 h. When Gram-stained films were examined, the isolates that appeared as Gram-positive rods were identified as *Bacillus subtilis, Bacillus cereus and Bacillus steareothermophilus* based on result from biochemical test as enumerated (Breeds et al., 1957; Olutiola et al., 1991). The Bacillus isolates were maintained in nutrient agar slants at 4°C.

Chemicals and reagents

Pectin, ammonium chloride, sodium dihydrogen phosphate and potassium chloride were obtained from BDH Chemical Limited, Poole, England. Glucose, 3, 5- dinitrosalicylic acid (DNSA), sodium potassium tartarate, sodium borate, boric acid, sodium citrate, and citric acid were obtained from Sigma Chemical Company, St. Louis, Mo., USA. Sodium hydroxide, Trizma base, Trizma-HCl, ethylene-diamine tetraacetic acid (EDTA) were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Other reagents were either purchased from Sigma Chemical or BDH Chemical.

Pectinase production by submerged fermentation

Pectinase production by submerged fermentation was conducted as described (Kumar and Sharma, 2012). Conical flasks containing 100 ml pectin broth containing (0.5 g pectin, 0.1 g yeast extract, 0.715 g NH_4Cl, 0.45 g $Na_2HPO_4.12H_2O$, 0.63 g KH_2PO_4, 0.075 g KCl, and 0.025 g $MgSO_4.7H_20$) were inoculated with a colony of the test isolates in triplicate. The cultures were incubated in a rotary shaker incubator at 30°C for 36 h at 120 rpm. The cultures were thereafter centrifuged at 4,000 rpm for 10 min and the cell free supernatants were stored at -4°C.

Determination of total protein concentration

The total protein content of the supernatants was determined according to the method described by Bradford (1976) using bovine serum albumin (BSA) as standard protein.

Pectinase assay

The crude pectinase enzymes produced by the three isolates were assayed using the colorimetric method as outlined by Miller (1959). Five milliliter (5 ml) of cell free supernatant was incubated with 2% pectin in 0.1 M acetate buffer at pH 6.0 and the reaction mixture was incubated at 40°C for 10 min. After adding 1.0 ml of DNSA reagent (without sodium potassium tartarate), the mixture was boiled for 5 min at 90°C. The reaction was stopped by adding 1 ml of Rochelle's salt (sodium potassium tartarate- Sigma, USA). The mixture was further diluted by adding 2 ml of distilled water and later followed by reading the absorbance at 595 nm to estimate the reducing sugars released. A standard graph was generated using standard glucose solution. One unit of pectinase activity was defined as the amount of enzyme which liberated 1 µm glucose per min (Karthik et al., 2011).

Effect of substrate concentration

The effect of varying the concentrations of different substrates (banana peel, orange peel, sugarcane bagasse and wheat bran) was studied. The substrates were obtained by drying the peels of the various fruits in an oven maintained at 50°C till they were completely dried except for the wheat which was purchase dried. The dried peels and the wheat were then grinded to powder. The powder was used in the preparation of 2% solution of each of the substrate in 0.1 M acetate buffer, pH 6.0. The concentrations were varied between 0.05 and 0.25 mM for each substrate. The effect of varying the concentration of substrates followed the assay procedure earlier described (Miller, 1959). In place of the pectin in the assay, the other substrates were used.

Effect of temperature

The effect of temperature on the activity of the enzyme was carried out for each isolate. 0.25 ml of 2% pectin in 0.1 ml acetate buffer was added to the crude enzymes and assayed at varying temperatures between 40 and 70°C for the different isolates.

Effect of pH

The effect of pH optimum on pectinase activity was measured for each isolate at a fixed assay temperature of 40°C at various pH values ranging from 3.0 - 9.0. In each case, the following pH buffer solutions were used following the assay method described (Miller, 1959) 0.1 M citrate buffer (pH 3.0-5.0), 0.1 M phosphate buffer (pH 6.0-8.0) and 0.1 M borate buffer (pH 9.0). 0.5 ml of 2% pectin was added into each extract and 0.5 ml of different buffer was added in each case and assayed.

Table 1. Pectinase activities and Total protein concentration in the crude extracts of each test isolate.

Enzyme source	Activity (µmol glucose/min/ml)	Protein conc. (mg/ml)	Specific activity
B. cereus	6.589	35.27	0.184
B. subtilis	6.198	33.26	0.186
B. stearothermophilus	6.751	34.74	0.194

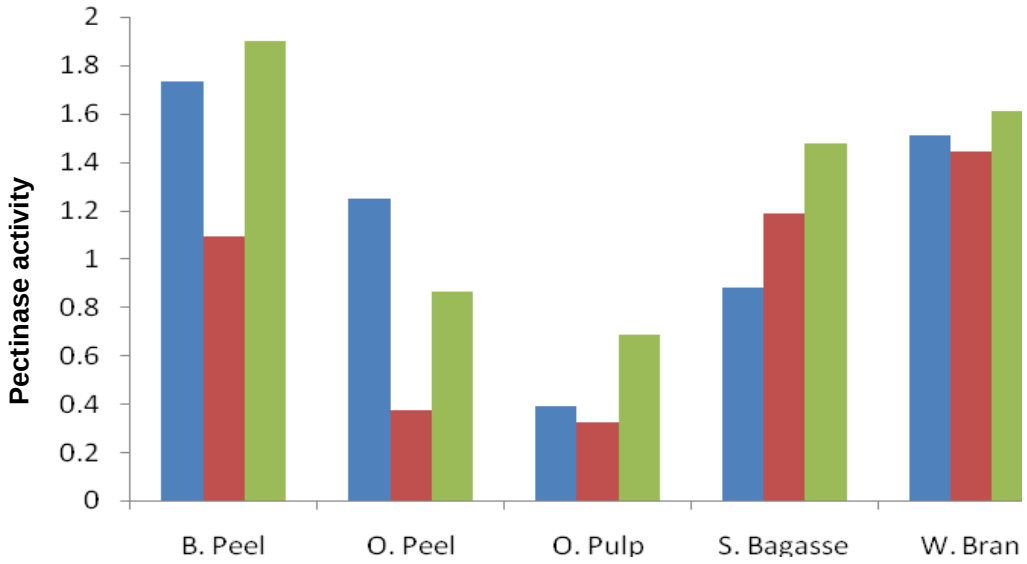

Figure 1. Effect of substrate (banana peel, orange peel, orange pulp, sugarcane bagasse and wheat bran) on pectinase activity. The blue bar: *B. cereus*; red bar: *B. subtilis*; green bar: *B. stearothermophilus*.

Effect of metal ion

The pectinase activity of each extract was determined with different metal ions using the chloride salt solutions of the following metals Na^{+}, K^{+}, Ni^{2+}, Mn^{2+} and Zn^{2+}. 0.1 ml of each metal ion was added into 0.25 ml of 2% pectin in 0.1 M acetate buffer, pH 6.0. The mixture was incubated at 40°C for 10 min and assayed.

RESULTS

All the experiments were conducted in triplicate and the result is the mean of the data derived. The results of the activity, protein concentration and specific activities of the different isolates are shown in Table 1. Figure 1 shows the effect of varying concentrations of substrates on the three isolates. The three isolates showed varying degree of preference for banana peels and wheat bran but activity towards orange peel was very small. The effect of temperature on pectinase activities of the three isolates is presented in Figure 2. *B. stearothermophilus* had opti0-mum temperature at 60°C while both *B. cereus* and *B. subtilis* showed optimum activity at 50°C. *B. stearothermophilus*, *B. cereus* and *B. subtilis* showed optimum pectinase activities at pH 7.5, 8 and 9 respectively (Figure 3). There was no significant pectinase inhibition by the metal ions amongst the isolates (Table 2)

DISCUSSION

Pectinolytic enzyme can be derived from different sources (Whitaker, 1990; Banu et al., 2010; Namasivayam et al., 2011). However, pectinase producing microorganisms have due advantage over other sources because they can be subjected to genetic and environmental manipulations to increase yield (Vibha and Neelam, 2010). It has been reported that most *Bacillus spp.* enhances the production of pectinase (Hirose et al., 1999). In this study, pectinase production by *B. cereus, B. subtilis and B. stearothermophilus* were compared. Our results in Table 1 show the three *Bacillus spp.* were able to produce pectinase. The results is in good agreement that selected *Bacillus sp.* can produce

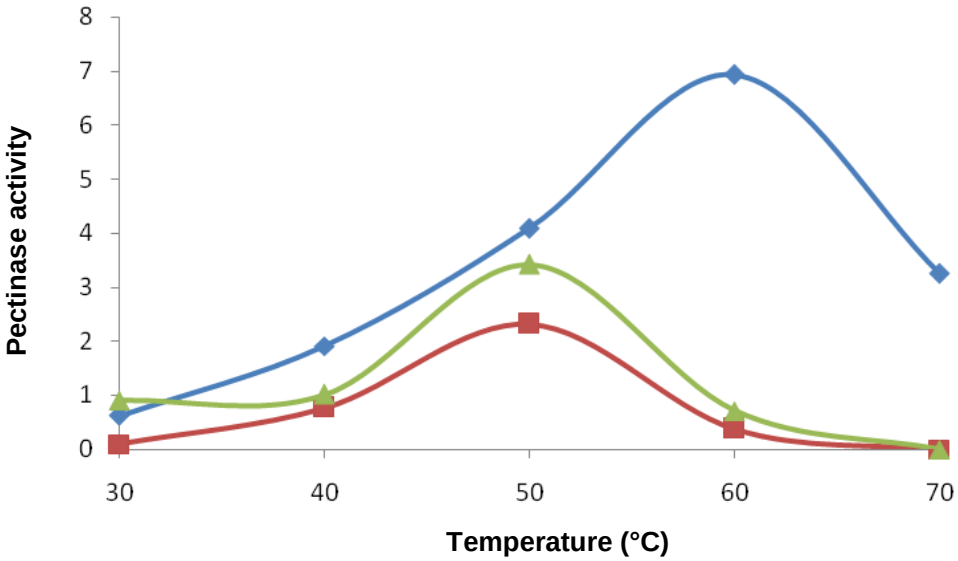

Figure 2. Effect of temperature on the pectinase activities of the isolates. Blue colour, *B. stearothermophilus*; red colour, *B. subtilis*; green colour, *B. cereus*.

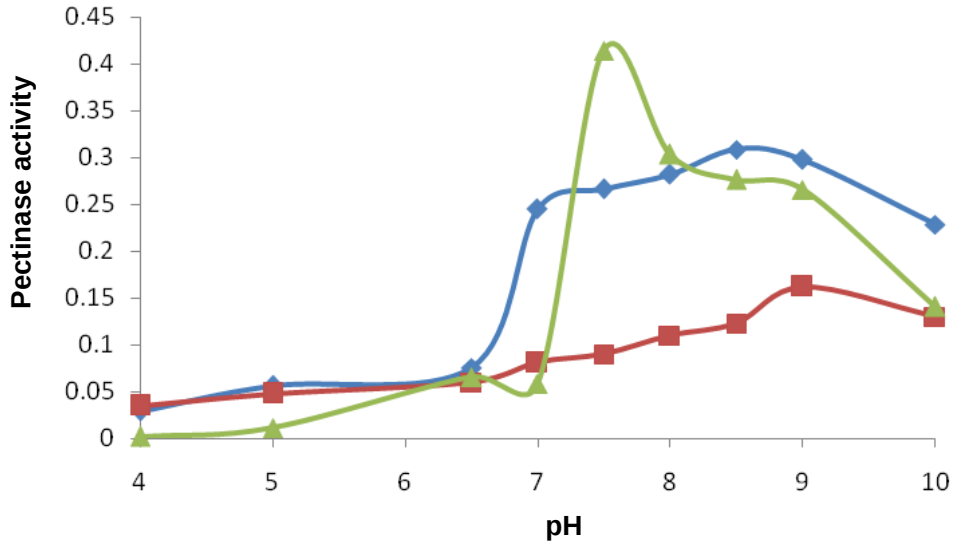

Figure 3. Effect of pH on the pectinase activities of the isolates. Blue, *B. cereus*; red, *B. subtilis*; green, *B. stearothermophilus*.

Table 2. Effect of metal ion on pectinase.

Metal ions (10 mM)	Residual Activiy of pectinase (%)		
	B. cereus	***B. subtilis***	***B. stearothermophilus***
Na^{+}	96.56	99.14	99.87
K^{+}	98.68	105.31	100.0
Ni^{2+}	109.00	104.93	100.0
Mn^{2+}	106.83	103.57	103.60
Zn^{2+}	105.81	105.30	103.84

and secrete large quantities (about 20-25 g/L) of extracellular pectinase enzyme (Soares et al., 1999; Schallemey et al., 2001; Namasivavam et al., 2011). High pectinase activity was recorded for the three isolates using industrial pectin. The use of other substrates (wheat bran, orange peel, sugar cane peel and banana peel) revealed the preference of banana peels and wheat bran by the isolates for pectinase production. Namasivavam et al. (2011) working on *B. cereus* isolated from market solid waste reported that pectinase production was enhanced by wheat bran. Also, Phutela et al. (2005) reported the enhancement of pectinase by wheat bran and industrial pectin on the production of pectinase from thermophilic *Aspergilus fumigatus* isolated from decomposing orange peels.

In our study, optimum temperature between 50 and 60°C was obtained for the three *Bacillus spp*, with *B. stearothermophilus* showing highest activity at 60°C while *B. cereus* and *B. subtilis* both showed highest activities of pectinase at 50°C. Different optimum activities have been reported for different pectinases from different sources. In the presence of Ca^{2+}, the enzyme from strain of *Bacillus spp* KSM-P15 degraded polygalacturonic acid with an optimal activity around pH 10.5 and 50-55°C (Kobayashi et al., 1999). Namasivayam et al. (2011) reported an optimum temperature for maximum activity of pectinase from *B. cereus* to be 37°C. Similarly, 35°C was reported for pectinase from *Penicillin chrysogenum* (Banu et al., 2010). Phutela et al. (2005) reported an optimum temperature of 60°C for thermophilus *A. fumigatus* pectinase. Other thermophilic fungi have been reported to optimally produce enzymes at 50°C (Rubinder et al., 2002).

The effect of pH on *B. stearothermophilus*, *B. cereus* and *B. subtilis* showed optimum pectinase activities at pH 7.5, 8 and 9, respectively. Reports have shown the pectinase activity to be highest around alkaline pH (Namasivayam et al., 2011; Kumar and Sharma, 2012) but a slightly acidic pH of 6.5 was obtained for pectinase from *P. chrysogenum* (Banu et al., 2010). In our study, metal ions K^+, Ni^{2+}, Mn^{2+} and Zn^{2+}, enhanced the activity of pectinases from the three isolates, while N^+ had a slight inhibitory effect on the enzyme. Similarly, Ca^+, Mg^{2+}, Mn^{2+} and Zn^{2+} were reported to activate pectinase from *Penicillin italicum* but was inhibited by Cu^{2+} and Fe^{2+} (Alana et al., 1990). Banu et al. (2010) observed little effect of Mg^{2+} and Ca^{2+} on pectinase from *P. chrysogenum* while Ca^{2+} enhanced the production of pectinase by *B. cereus* obtained from market solid waste (Namasivayam et al., 2011).

Conclusion

The physicochemical characteristics of pectinase from these isolates make it an attractive tool for various industrial uses. However, *B. stearothermophilus* gave the most activity, optimum temperature and a moderate alkaline pH, possibly making it a better source for industrial purpose.

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