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An Isolate of *Bacillus clausii* Appears to Possess Four Distinct Pectinolytic Activities

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ABSTRACT: For the bacterial source, Koyulhisar (Sivas, Turkey) apple orchard soil samples were used. Obtained isolates were identified at the species level by partial protein- and 16S rRNA gene sequence homology All of them were determined to be *Bacillus clausii*. Cultures were prepared with these isolates and after removing the cells, the proteins in the upper fluid were precipitated with the help of an organic solvent. While performing pectinase screening, it was noticed that an existing protease digested other proteins. This proteolytic activity was eliminated by increasing the incubation temperature to 55ºC. Four different pectinase activities, polygalacturonase, pectin lyase, pectate lyase, and pectin methylesterase, were then demonstrated. Four protein bands corresponding to these activities visualised by SDS-PAGE were 80 kDa, 58 kDa, 46 kDa and ~ 28 kDa, respectively. Spectroscopic methods were employed for the identification of the enzymes. The smallest protein band (28 kDa) was found to be pectin methylesterase. Km and Vmax values of this enzyme were 0.4 and 217U, respectively.

Keywords: *Bacillus clausii*, pectate lyase, pectin lyase, pectin methylesterase, polygalacturonase.

Bir *Bacillus clausii* İzolatı Dört Farklı Pektinolitik Aktiviteye Sahip Görünmektedir

ÖZET: Bakteri kaynağı olarak Koyulhisar (Sivas, Türkiye) elma bahçesi toprak örnekleri kullanıldı. Elde edilen izolatlar tür düzeyinde kısmi protein- ve 16S rRNA gen dizisi homolojisi ile belirlendi. Hepsinin *Bacillus clausii* olduğu belirlendi. Bu izolatlar ile kültürler hazırlandı ve hücreler uzaklaştırıldıktan sonra üst sıvıdaki proteinler organik bir çözücü yardımı ile çöktürüldü. Pektinaz taraması yapılırken, mevcut bir proteazın diğer proteinleri sindirdiği fark edildi. Bu proteolitik aktivite, inkübasyon sıcaklığının 55ºC'ye çıkarılmasıyla engellendi. Dört farklı pektinaz aktivitesi, poligalakturonaz, pektin liyaz, pektat liyaz ve pektin metilesteraz bundan sonra saptanabildi. SDS-PAGE'de görüntülenen ve bu aktivitelere karşılık gelen dört protein bandı, sırasıyla 80 kDa, 58 kDa, 46 kDa ve ~ 28 kDa idi. Enzimlerin tanımlanması için spektroskopik yöntemler kullanıldı. En küçük protein bandının (28 kDa) pektin metilesteraz olduğu bulundu. Bu enzimin K_m ve V_{max} değerleri sırasıyla 0.4 ve 217U idi.

Anahtar Kelimler: *Bacillus clausii*, pektat liyaz, pektin liyaz, pektin metilesteraz, poligalakturonaz.

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INTRODUCTION

Pectinases consist of a unique group of enzymes that catalyze the degradation of pectic polymers found in the plant cell wall. They belong to the polysaccharidase family, also known as pectolitic or pectic enzymes, which contribute to the breakdown of pectins from various plants. In the current biotechnological period, pectinase is one of the increasingly used enzymes (Kavuthodi and Denoj Sebastian, 2018). Pectinases, used since 1930, make up a significant part of industrial enzymes (Rebello et al., 2017). They are applicable in the production of functional foods (Khan et al. 2013; Prathyusha and Suneetha 2011), stacking and degumming of fibers in the textile industry (Cao et al., 1992), quality paper production (Ahlawat et al., 2008), fermentation of coffee and tea, oil extraction and pectic wastewater treatment, and in bioethanol production (Kashyap et al., 2001; Rebello et al., 2017). Microbial pectinases are estimated to have a 25% share in the world food enzymes market (Sharma et al., 2012).

The term pectinase signifies a heterogeneous group of hydrolyses, including homogalacturonane-degrading polygalacturonases (PG) or pectin depolimerase; polymethylgalacturonases (PMG); it is also used for lyases or heterogeneous enzymes containing transeliminases and pectin esterases (PE), also known as pectin methyl esterases (PME). These enzymes can break down glycosidic bonds, which are abundant in fruit, or modify pectin (Urena, 2016; Rebello et al., 2017).

Pectin acts as a cross-linker polysaccharide in the primary cell wall and middle layer of fruits and vegetables and cross-links hemicellulose fibers. The use of pectinases therefore facilitates the access of cellulases to their substrates (Giacobbe et al., 2014).

Pectinases can be broadly classified into acidic and alkaline groups based on pH requirements for optimum enzymatic activity (Li et al., 2012). Pectin hydrolases are mainly produced by fungi and are more active at temperatures between 40 and 60°C in an acid or neutral environment, while bacterial pectinases are more active in acidic conditions (Pedrolli et al. 2009). Carbohydrolases constitute the second largest group of the industrial enzymes (Priya and Sashi 2014; Rebello et al., 2017).

Members of *Erwinia, Bacillus, Saccharomyces, Kluyveromyces, Aspergillus, Penicillium, Fusarium and Rhizopus* have been the good sources of pectinolytic enzymes. The choice of microbial sources in pectinase production is usually made on the basis of culture conditions, the number and type of pectinase produced, pH and thermal stability of the enzymes, and on the genotypic features of the organism (Khairnar et al., 2009).

This study includes the identification of relatively infrequent alkaline pectinases in bacteria compared to acidic pectinases. In the literature, research on this type of pectinases is quite inadequate. Therefore, the main original message of this study is the detection of four different pectinase activities in one bacterium.

MATERIALS AND METHODS

Collection of Soil Samples

Samples were collected from apple orchards in Koyulhisar district of Sivas province. The upper layer was removed and underneath soil was then collected in sterile glass jars.

Identification of bacteria by mass spectrometry

Colonies grown in Horikoshi-I agar at 37^{o}C by 24 h incubation were used in the analysis. The colonies grown in the plate were treated with the solution used in the MALDI-TOF device and compared with the partial protein profiles in the library of the device and the original spectrum results were recorded.

Genomic DNA preparation

Bacterial colonies were inoculated in liquid Horikoshi I medium and grown overnight at 37ºC. Cells were collected by centrifuging for 5 min at 5,000 rpm at +4ºC. Sucrose solution, 200µl, (25% sucrose, 30 mg/ml lysozyme in 1xTE, pH 8.0) was added onto the pellet and cells were suspended. Cell lysis was achieved by one-hour incubation at 37º. Samples were deproteinized by adding a proteinase K solution (1mg/ml in 370µl 1xTE) and 30 µl 10% SDS, and by incubation at 37 ºC. Afterwards, 100µl 5M NaCl and 80µl CTAB/NaCl solution were added samples were incubated for 10 min at 65ºC. Phenol/chloroform extraction was performed for 3 min at 10,000 rpm. The aqueous phase was taken into new Eppendorf tubes and DNA was precipitated by adding 1 volume of 99% isopropanol. DNA wools were taken and washed with 70% ethanol. The liquid was discarded and DNA wool was dried at 37ºC for 10 min and stored at -20 ºC (Akbalik, 2003).

Isolates were identified to be *Bacillus clausii* by sequence homology of 16S rRNA gene. The nucleotide sequence was submitted to GenBank (Accession Number: MT524963).

Enzyme Production and Identification

Horikoshi-I sıvı besiyerine ekim yapılarak 37ºC'de bir gece inkübe edildi. Supernatant was used for the extracellular enzyme production (Table 1). Protein content was precipitated by using one equal volume of ethanol.

Table 1. Pectinolytic enzymes and their substrates

Enzyme	Substrate
Polygalacturonase	Polygalacturonic acid
Pectin lyase	Pectin
Pectate lyase	Polygalacturonic acid
Pectin methylesterase	Pectin

Analysis of the protein precipitate by SDS-polyacrylamide gel electrophoresis

For 8% SDS-PAGE, 30% acrylamide-bisacrylamide, 1.5M Tris (pH 8.8), 10% SDS were taken into a beaker in the desired volume. TEMED and APS were then added at the same time and mixed gently without causing foaming. With the help of a Pasteur pipette, the separating gel was poured between the glass plates. N-butanol was added on the gel to obtain a flat line. The gel was allowed to solidify for 10 minutes. Then, alcohol was removed, washed with distilled water and the remaining water was removed with the help of blotting paper. The loading gel was prepared in the same way. Combs were placed after pouring the stacking gel with a Pasteur pipette. After waiting 10 min for the gel to solidify, the combs were removed and samples were loaded. Samples were run for one and a half hours at 80V. Protein bands were visualized by staining with Coomassie brillant blue R-250 overnight.

Polygalacturonase (PG) activity

DNS method was used for PG activity measurement (Berber, 2018). Enzyme activity was calculated as µmol galacturonic acid released per minute. Preparing polygalacturonic acid (1% 200µl) and enzyme solution (200µl) at different pH (pH 3.6-5.6 acetate buffer, pH 6.0-7.6 phosphate buffer, pH 8.0-9.0 Tris-HCl buffer, pH 10.0-11.0 carbonate buffer, and pH 12.0-13.0 KCl-NaOH buffer) at 50ºC and at different temperatures (4ºC, 20ºC, 37ºC, 45ºC, 50ºC, 55ºC, 60ºC, 70ºC) were incubated for 10 minutes. The values read at 540 nm against blanc were recorded.

Pectin lyase (PL)

Pectin lyase (PL) activity was determined on the basis of the absorbance at 235 nm of the double bonds formed in the substrate pectin. The activity was calculated as the change of absorbance measured at 235 nm per minute. For pectin lyase activity, 200µl 1% pectin solution and 200µl enzyme were incubated at different temperatures at 37ºC and at different temperatures (4ºC, 20ºC, 37ºC, 45ºC, 50ºC, 55ºC, 60ºC, and 70ºC) for 10 min. After incubation, 400µl of 0.01M HCl was added to them and the activity was stopped by incubation for 5 min in boiling water and the samples were cooled on ice for 5 min. Sample absorbance values were recorded by reading against the corner at 235 nm.

Pectate lyase (PGL)

The pectate lyase (PGL) activity was assessed at 235 nm and on the basis of the double bonds formed in polygalacturonic acid. 200µl 1% polygalacturonic acid solution and 200µl enzyme for pectate lyase activity at different pH (pH 3.6-5.6 acetate buffer, pH 6.0-7.6 phosphate buffer, pH 8.0-9.0 Tris-HCl buffer, pH 10.0-11.0 carbonate buffer, pH 12.0-13.0 KCl-NaOH buffer) were incubated at 50oC and at different temperatures (4ºC, 20ºC, 37ºC, 45ºC, 50ºC, 55ºC, 60ºC, and 70ºC) for 10 minutes. After incubation, 400µl of 0.01M HCl was added to them and the activity was stopped by incubation for 5 min in boiling water and the samples were cooled on ice for 5 min. Sample absorbance values were recorded by reading against the corner at 540 nm.

Pectin methylesterase (PME)

Determination of pectin methylesterase (PME) activity was based on the pH change. The activity was calculated by measuring the amount of NaOH required to keep the reaction pH constant. Pectin solution (5 ml 1%) and 100µl enzyme solution were prepared at different pH (pH 3-13 range) and incubated at 50ºC and different temperatures (20ºC, 37ºC, 45ºC, 50ºC, 55ºC, 60ºC) for 10 min. The amount of 0.1N NaOH required for the pH to remain constant in ten minutes was determined. Activity was calculated according to the amount of NaOH used.

Zymography

Chemicals, except TEMED and APS, were mixed in a beaker. Glass plates were prepared for electrophoresis. TEMED and APS were then added simultaneously and mixed gently. The gel was poured between the plates with a pipette tip and a comb was inserted. The gel was left on the bench for 10 min for the gel to solidify. The combs were then removed and the samples loaded into the wells. Electrophoresis was carried out at 80V for 90 min.

After electrophoresis, for zymogram, 0.1% pectin and polygalacturonic acid were added to the gels and the gels were incubated in optimum pH buffer for one hour at optimum temperature. Staining was performed overnight at +4ºC in ruthenium red (0.05%). With the help of distilled water, excess paint was removed and the zones were examined.

RESULTS AND DISCUSSION

Isolate Identification

Morphological and partial biochemical characterization showed that the isolates were Gram-positive, catalase-negative, and rod-shaped bacteria (results not shown). One of the strains was selected for further studies.

Partial protein analysis was performed by MALDI-TOF mass spectrometry in the Department of Microbiology, Faculty of Medicine, Sivas Cumhuriyet University, and it was determined that all the isolates were *Bacillus clausii*.

A phylogenetic analysis based on 16S ribosomal RNA (rRNA) gene sequence comparison also showed that this strain belonged to *B. clausii*. The rRNA gene sequence was submitted to GenBank (Accession number: MT524963, GenBank).

Studies on Enzyme Activity

Polygalacturonase (PG)

The optimum temperature was determined according to the activities obtained at different reaction temperatures (Figure 1A). The enzyme was found to be more active at 20ºC, 50ºC and 60ºC. Optimum pH scanning was then performed at these three temperatures. The highest enzyme activities were taken at 20ºC, pH 11.5, pH 5, and pH 7, respectively (Figure 1B). Similar activity profiles were obtained at pH9 and pH12 at 50ºC, and a relatively higher activity was observed at pH5 (Figure 1C). The highest enzyme activities were recorded at pH 12, pH 5, and pH 9 at 60ºC, respectively (Figure 1D).

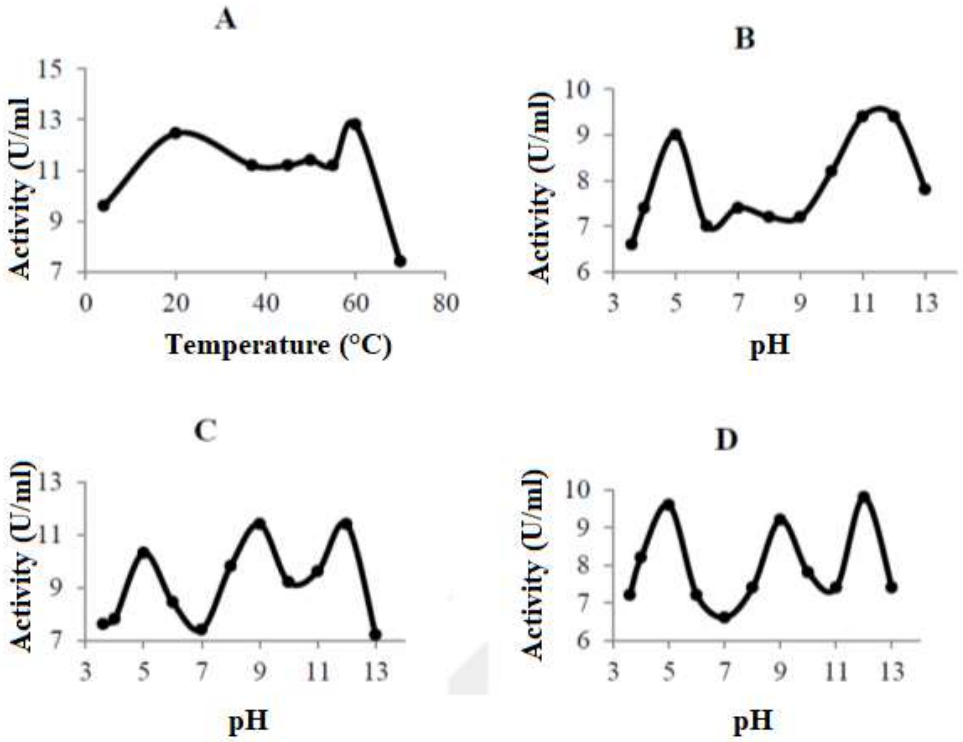

Figure 1. Polygalacturonase (PG) activity at different pH and temperature points

Pectin lyase (PL)

Pectin lyase activity was calculated by periodical readings of the absorbance at 235 nm. The reaction mixture was incubated at different temperatures and the activities were measured, and 37ºC was determined as the optimum temperature. Two pH optimums, 7 and 10, were obtained (Figure 2).

Pectate lyase (PGL)

Pektate lyase activity was based on absorbance readings at 235nm. Activities at different pH and temperatures were measured and recorded. Activity peaks at three different pH points (5, 9 and 11) were detected. For these three pH points, optimum temperature points were determined: 37ºC and 50ºC for pH 5; 60ºC for pH 9; and 20ºC, 45ºC, and 55ºC for pH 11 (Figure 3).

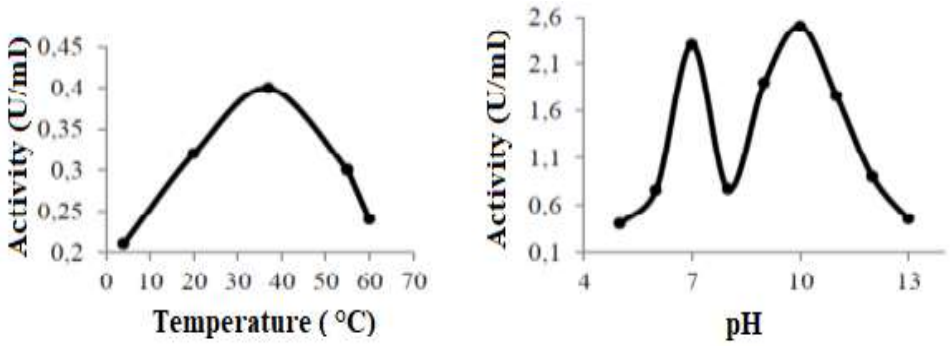

Figure 2. Pectin lyase activity at different pH and temperature points

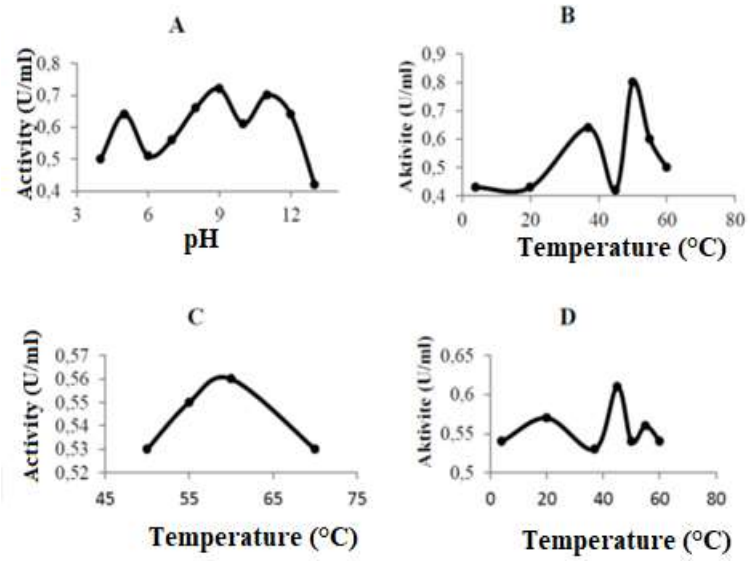

Figure 3. Pectate lyase (PGL) activity at different pH and temperature points

Pectin methylesterase (PME)

Pectin methylesterase activity was measured by the titration method, for which 0.01M NaOH was used. The total amount of NaOH used to keep the pH constant during the course of the reaction were taken as the measure of the methylesterase activity. Optimum reaction temperature was 55^{o}C at pH 8 (Figure 4).

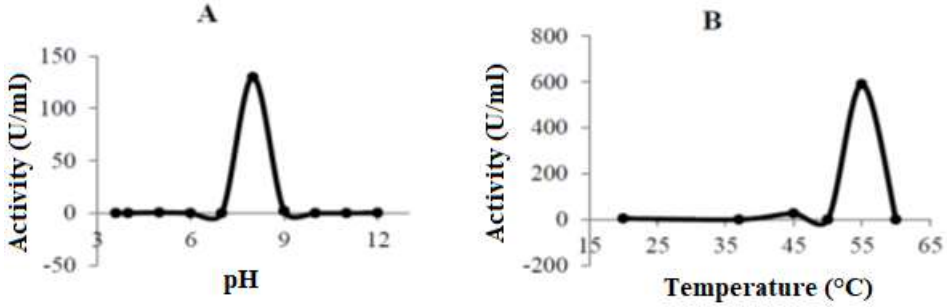

Figure 4. Pectin methylesterase (PME) activity at different pH and temperature points

Enzyme Identification by SDS PAGE

Proteins precipitated by adding two volumes of ethanol to the culture supernatant, were loaded onto the sodium dodecylsulfate polyacrylamide gel. Four distinct protein bands, 80 kDa, 58 kDa, 46 kDa, and about 28 kDa were obtained (Fig. 5). Each of the protein band was assigned to be one of the four pectinolytic enzymes in the light of zymogram results (Figure 6).

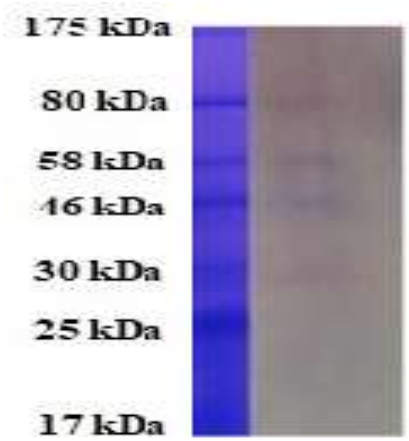

Figure 5. Four extracellular pectinolytic enzymes (SDS-PAGE gel image) First line on the left, protein marker; second line, extracellular proteins, precipitated in two sample volume of 95.5% ethanol

Identification criteria of the four distinct pectionlytic activities based on the zymograme results: (1) at 50ºC and at pH8, PGA yielded pectinolytic activity while pectin did not; (2) at 37ºC, pectin and PGA formed zones in different places; (3) and observation of a more distinct zone in pectin containing gel at 55ºC, compared to PGA, is parallel with the activity results obtained s at different temperatures; (4) after these results were evaluated together, it was thought that polygalacturonase, pectate- and pectin lyases, and pectin methylesterase (Figure 6) were responsible for the creation of four distinct activity zones. On the basis of the these zymogram results, the four protein bands were assigned to each of the pectinolytic activities: the lowest band, pectin methylesterase, approximately 28 kDa; the topmost band pectin lyase, 80 kDa; the second band from the top, pectate lyase, 58 kDa; and the third band, polygalacturonase, approximately 46 kDa (Table 2). Thus, it was shown that enzyme activities could be purified with the help of precipitation using different organic solvents, and zimograms performed at different temperatures.

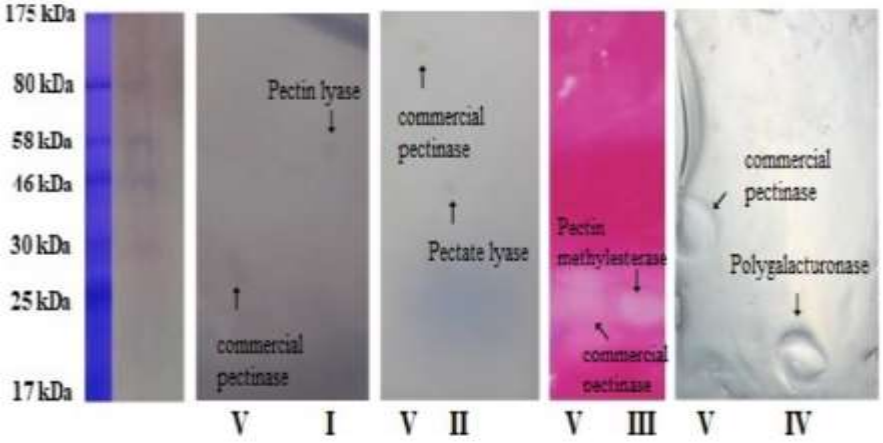

Figure 6. Zymogram results

Table 2. The isolated four pectinases, their molecular weights and substrate specificities

Symbol	Enzyme	Molecular weight (kDa)	Substrate	
			Pectin	Polygalacturonic acid
I	Pectin lyase	58	+	-
II	Pectate lyase	35	-	+
III	Pectin methylesterase	25	+	-
IV	Polygalacturonase	20	-	+

Support for these findings found in the literature could be summarised as follows: A pectin lyase purified from *Bacillus subtilis* has produced a zymogram zone at 38 kDa (Saharan and Sharma, 2019). An approximately 35-kDa protein band exhibiting pectinolytic activity has been detected using PGA. The enzyme has been purified from *Aspergillus luchuensis* mut (Kamijo et al., 2019). A 53 kDa endopolygalacturonase of *Bacillus paralicheniformis* has been identified by SDS-PAGE and pectic zimography (Khan et. al., 2018) (Table 3).

Table 3. Pectinolytic enzymes found in Literature

Symbol	Enzyme	Molecular weight (kDa)	Substrate	References
I	Pectin lyase	38	pectin	Saharan and Sharma, 2019
II	Pectate lyase	35	Polygalacturonic acid	Kamijo et al., 2019
III	Pectin methylesterase	--	--	--
IV	Polygalacturonase	53	pectin	Khan et. al., 2018

Bacillus clausii is a probiotic organism (Urdaci et al., 2004). This makes it more interesting to understand the extracellular enzyme content. Although studies with other species of *Bacillus* have frequently been encountered in the literature, there has been very limited information on *Bacillus clausii*, involving mostly pectin-and pectate lyases.

Commercial pectinases usually consist of pectinase mixtures. For example, Gonzalez and Rosso (2011) have studied pectin methylesterase activity in commercial pectinase (Pectinex 100L Plus) containing mixed pectinolytic enzymes. As a result of the study, the optimum pH and temperature of the enzyme were found to be 4 and 45^{o}C, respectively.

Raju and Divakar (2013) have purified pectinase from an isolate of *Bacillus circulans*. They have reported that the enzyme had optimum activity at pH 7 and 40^{o}C. Similar results have been shown for a pectinase of *Bacillus pumilus* (Rukmini et al., 2016).

A pectate lyase gene from *Bacillus clausii* S10 has been introduced into *E. coli* BL21 (Zhou et al., 2017). The optimum pH- and temperature points of the cloned enzyme activity have been shown to be 10.5 and 70^{o}C, respectively.

CONCLUSION

Commercial preparations often contain a mixture of related enzyme activities. During the study for comparative reasons, a commercial pectinase was used. Its SDS-PAGE image indicated the presence of more than one protein bands (data not shown). A similar observation was also made with a commercial

tRNA preparation which contained significant amounts of genomic DNA and high molecular weight RNA species (16S and 23S). In the light of the experiences with the commercial enzyme preparations, it could justly be argued that our pectinase preparation can be used as it is as a commercial enzyme preparation. For further investigations, however, each of the enzymes should be studied in pure form. These four enzymes will be purified and their primary structure will be determined in the near future.

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