

# Production, Purification of Pectinase from *Bacillus* sp. MBRL576 Isolate and its Application in Extraction of Juice

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**Abstract:** A bacterium isolated from orchard soil, was selected out of 109 bacterial isolates because of maximum pectinase production at acidic pH and 45° C temperature. The isolate was characterized morphologically, biochemically and culturally and was identified as *Bacillus* sp. MBRL576 based on nucleotide homology and phylogenetic analysis. Conditions for pectinase production under submerged fermentation from *Bacillus* sp. MBRL576 were optimized. Enzyme was purified using ion exchange chromatography and SDS PAGE. Crude pectinase was used for extraction and clarification of juice from fruits and vegetable. It was found to increase the clarification and yield of juice.

**Keywords:** pectin, *Bacillus* sp. MBRL576, pectinase, purification, juice extraction

## 1. Introduction

Pectinase is a general term used for a set of several enzymes, which breaks down pectin. Pectin is a high molecular weight polysaccharide, primarily made up of  $\alpha$  (1- 4) linked D-galacturonic acids and are found in the middle lamella and primary cell wall of higher plants. Pectinolytic enzymes are commonly used in processes involving the degradation of plant material and have a share of over 25% in the global scale of the food enzymes (Hoondal & Tiwari. (2002) and Naidu et al (1998). These enzymes have numerous applications in industries like food, paper and pulp, textile, etc. Several microorganisms including bacteria and fungi are known to produce different types of pectinolytic enzymes. However, selection of a particular organism remains a tedious task and the choice gets tougher when commercially competent enzyme yields are to be achieved. The present investigation was carried out to isolate and identify a potential pectinase producing bacterium and to optimize the conditions for enzyme production and to test its possibility for application in fruit juice extraction.

## 2. Material and Method

### 2.1 Chemicals and Reagents

Pectin, D-galacturonic acid monohydrates were obtained from Sigma Chemicals Co. All the other chemicals and reagents used for the study were of analytical /microbiological grade.

### 2.2 Isolation and identification

The pectinase producing bacteria were isolated from soil, decaying fruits and vegetables from different locations of Kurukshetra, Haryana (India) using pectin agar medium at pH 7.2. The isolate NV53 isolated from soil of orchard, was producing maximum zone of clearance after addition of 1% cetyl trimethyl ammonium bromide (CTAB) and was selected for further study. The NV53 was characterized by performing various morphological, cultural and biochemical

tests based on standard methods and identified as *Bacillus* sp. MBRL576.

### 2.3 Production of pectinase

Isolate NV53 was cultivated in basal medium (Reda2008) at pH-7 and incubated at 37°C for one day on a rotary shaker (NSW- 256) at 150rpm.

### 2.4 Optimization of cultural conditions for pectinase production

Isolate NV53 was cultivated by changing the various cultural conditions viz. pH, temperature, incubation time, agitation, inoculums size and age, sources of carbon and nitrogen and their concentration. The inoculated broth after incubation was centrifuged at 10,000g for 10mins at 4°C and the clear supernatant was used as crude enzyme for enzyme assay.

### 2.5 Assay of Pectinase Activity

Pectinase activity was assayed by the colorimetric method of Miller (1959) and absorbance of the colour developed was measured at 540 nm. One unit of enzyme was defined as the amount of enzyme which catalyses the formation of 1 $\mu$ mol of galacturonic acid/min.

### 2.6 Purification of pectinase

Isolate NV53 was cultivated in production broth under optimized condition and crude enzyme was precipitated with 80 % ammonium sulphate fractionation. The precipitates were dissolved in the minimum amount of acetate buffer (50mM, pH 4) and dialysed against the same buffer. The dialyzed enzyme was used for further purification by chromatography.

## 2.7 Ion Exchange Chromatography

Dialyzed enzyme, 3.0 ml, was loaded on DEAE-Sephacel, packed into a glass column (25x1 cm, 10 ml-bed volume). The column was equilibrated with acetate buffer (50 mM, pH 4). The column was washed with acetate buffer containing 0.0 to 0.5M NaCl concentrations. Fractions of 2.5 ml volume were collected. The protein content of the fractions was measured spectrophotometrically at 280 nm and the pectinase activity was determined by the method of Miller (1959). The fractions showing pectinase activity were pooled, concentrated and saved for further analysis.

## 2.8 Gel Filtration chromatography

A glass column was packed with Sephadex G 150 (35x1:5 cm, bed volume 60 ml). The concentrated sample was loaded on to this column and elution of the proteins was done using acetate buffer (50 mM, pH 4). After the void volume (20 ml), fractions of 3 ml volume were collected. The absorbance of the samples for protein content and pectinase activity was done by the methods Miller (1959). The pectinase-positive fractions were pooled, concentrated and saved for further analysis.

## 2.9 SDS-Polyacrylamide gel electrophoresis (PAGE)

Ten percent SDS-PAGE was performed on the purified pectinase-positive sample by the method described by Laemmli (1970) using Bio-Rad electrophoresis apparatus. The gel was run on a constant voltage of 50 V. The gel was stained by the silver staining method of Merrill et al. (1981).

## 2.10 Application of pectinase

Ten grams of fruit (banana and apple) and vegetable (carrot) pulp was incubated with 1 ml of crude enzyme for 2h in a water bath at 40 °C followed by incubation in a boiling water bath for 5 min to inactivate the enzyme. Juice was extracted by centrifugation at 3000 rpm for 25 min. After cooling to room temperature, the supernatant was filtered and the volume of filtrate and dry weight of residue obtained were measured. Transmittance (%) of all types of filtered juices were measured at 650 nm and used as initial reading for clarification of juice. Pulp without enzyme treatment was used as control and commercial pectinase enzyme of HIMEDIA chemicals was used as standard and run simultaneously. The reduction in dry weight of the treated mash was calculated against control. For further clarification of these juices were again treated with enzyme by the method given by Baker and Bruemmer(1972) and transmittance was measured at 650 nm. Pectin content of juice was measured in terms of percent anhydrogalacturonic acid and determined after clarification step by carbazole method (McComb and McCready, 1952).

## 3. Results and Discussion

At present almost all pectinolytic enzymes used for industrial applications are produced by fungi. There are a few reports of pectinase production by bacterial strain. Out of various pectinolytic isolates, one isolate NV53 was

selected on the basis of its highest pectinase production detected both qualitatively and quantitatively.

## 3.1 Identification and characterization of Isolate NV53

NV53 was nonmotile Gram positive rods occurring singly, in pairs or in short chains with sub-terminal and swollen endospore. On solid medium of pectin agar colonies were smooth, shiny, opaque, raised, irregular, producing red pigmentation around the margin on second day of incubation. On liquid medium of nutrient broth containing pectin the organism forms pellicle and a red colored ring on the surface. Biochemical characterization was done using HI MEDIA Kit. Results of which are given in table 1. 16S rRNA gene of the NV53 isolate was amplified and partial sequencing of 1166bp was carried out. The isolate NV53 was identified as *Bacillus sp. MBRL576* by Xcleris Lab Ltd Ahmedabad based on BLAST analysis with the nr database of NCBI genbank database.

**Table 1:** Metabolic characterization of isolate NV53

Biochemical test	Result	Carbohydrate fermentation	Result
Indole	+	Glucose	+
Voges Proskauer	-	Mannitol	+
Citrate Utilization	-	Xylose	-
Lysine Utilization	+	Inositol	-
Ornithine Utilization	-	Sorbitol	-
Arginine Utilization	-	Rhamnose	+
Nitrate Reduction	+	Sucrose	+
Malonate	-	Lactose	+
Urease	+	Arabinose	+
Phenyl alanine deamination	-	Adonitol	+
H <sub>2</sub> S Production	+	Raffinose	+
ONPG	+	Salicin	+
Catalase	+	Trehalose	+

## 3.2 Production of pectinase

*Bacillus sp. MBRL576* was cultivated in enzyme production medium (Reda, 2008) for pectinase production under submerged fermentation. All the experiments were performed in an incubator shaker with agitation of 150 rpm. Conditions for pectinase production were optimized. Maximal pectinase production (168.83 IU/ml) was observed at pH 4 (fig 1), when pH of the production medium was adjusted between pH 3.0-12.0. Effect of incubation temperature viz. 25°C, 30°C, 35°C, 37°C, 40°C, 45°C and 50°C was studied by incubating the *Bacillus* in production medium at pH4.0 for 24h. The optimum temperature was found to be 45°C and further increase in temperature reduces the pectinase production (fig 2). Maximal pectinase production was found to be at 24 hours of incubation period when incubated at different time intervals of 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 96 h, and 120 h, 144 h, 168 h, 192 h and 216 h (fig 3). Aeration by shaking increases pectinase production when speed of agitation was increased from 120 rpm to 160 rpm with maximum production at 150rpm (fig 4). To study the effect of inoculum size and age, production medium was inoculated with 1.0% to 10.0% inoculums (fig 5) of 0h, 6 h, 12 h, 18 h, 24 h, 30h, 36h and 48 h age (fig 6). The maximal pectinase production was observed when 6.0% inoculum of 24 hours age was used for production. Supplementation of

production medium with 1% sucrose as C-source resulted in maximum production of pectinase followed by starch, cellulose and trehalose as compared to all other carbon sources used however it was suppressed greatly when supplemented with maltose, xylose and mannose (fig 7, 8). Production medium without any carbon source was used as control in enzyme production of the various nitrogen sources used maximal pectinase activity was observed when 0.2% of sodium nitrate was added to the production medium. Potassium Nitrate and gelatin also supported the production of pectinase whereas ammonium sulphate, ammonium dihydrogen phosphate inhibited the enzyme production (fig 9, 10).

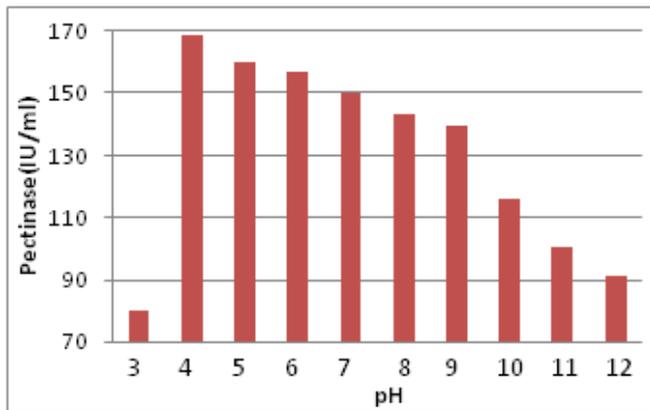


Figure 1: Effect of pH on pectinase production

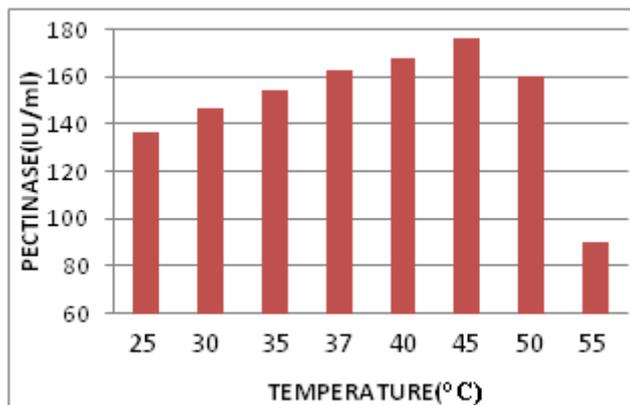


Figure 2: Effect of temperature on pectinase production

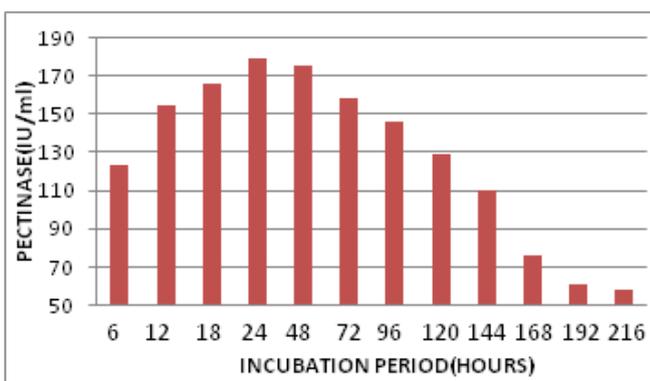


Figure 3: Effect of Incubation period on pectinase production

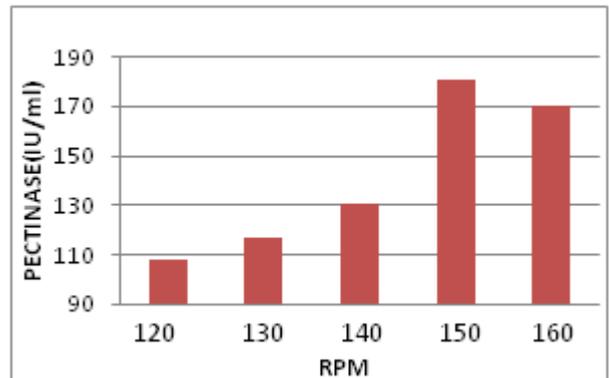


Figure 4: Effect of shaking condition on pectinase production

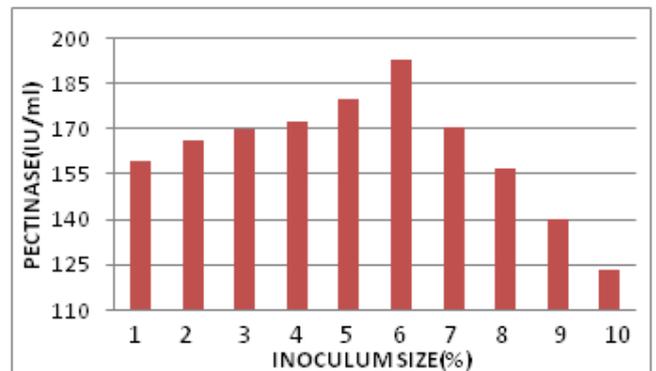


Figure 5: Effect of inoculum size on pectinase production

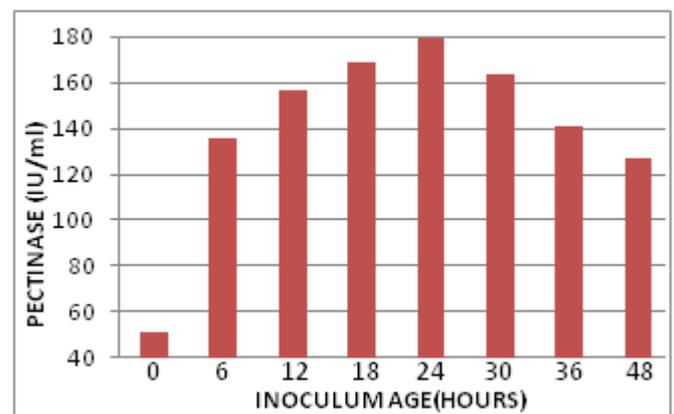


Figure 6: Effect of inoculum age on pectinase production

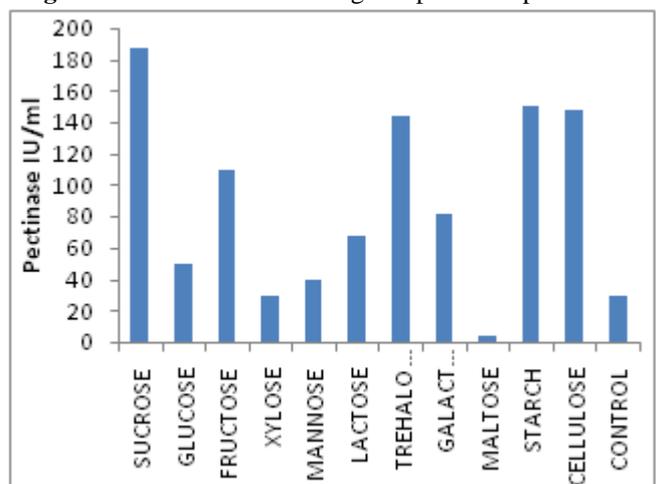


Figure 7: Effect of carbon sources on pectinase production

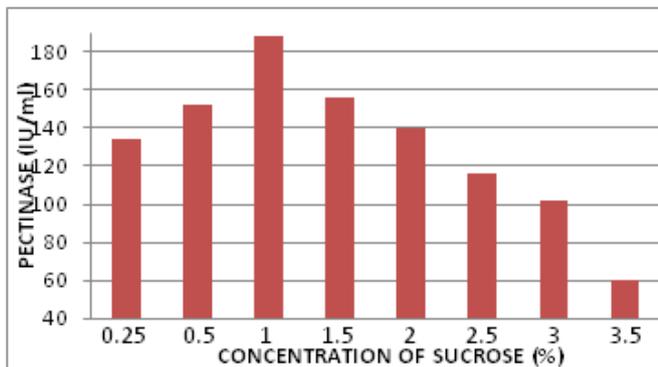


Figure 8: Effect of sucrose conc. on pectinase production

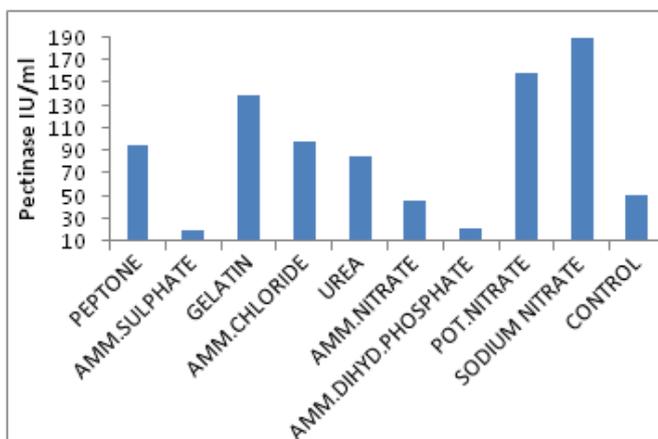


Figure 9: Effect nitrogen sources on pectinase production

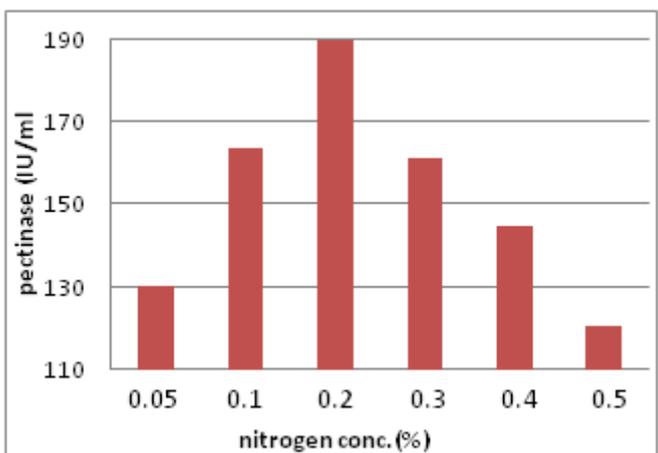


Figure 10: Effect of sodium nitrate conc. on pectinase production

### 3.3 Purification of Pectinase

The pectinase from *Bacillus sp. MBRL576* was purified in different steps. Initially crude enzyme was precipitated by ammonium sulphate at 80% saturation, dialyzed, purified in DEAE- Sephacel and Sephadex G150 column. At each stage pectinase activity and protein were measured. Results of purification are given in table2. Enzyme was purified 2.86 fold after Sephadex G150 step. When this purified pectinase was electrophoresed on 10% SDS-PAGE, a single band was observed, indicating the complete purification of the enzyme. Using standard protein markers the size of the purified enzyme was found to be 66 kDa (Figure11)

Table 2: Purification of pectinase from *Bacillus sp. MBRL576*

Purification step	Total volume (ml)	Total pectinase units( IU)	Protein (mg)	Specific activity (IU/mg)	Purification fold	% yield
Crude enzyme	500	94500	56	1687.5	1	100
0-80% Ammonium sulphate fractionation	500	70000	25	2800.0	1.65	74.074
DEAE Sephacel	500	45000	14.71	3059.1	1.81	47.61
Sephadex G150	500	14500	3	4833.3	2.86	15.34

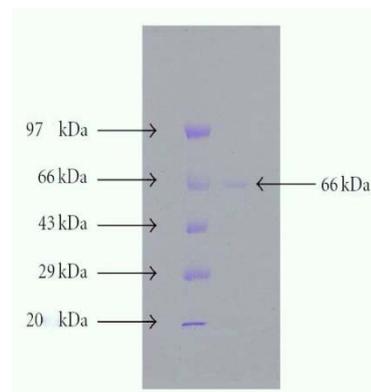


Figure 11: Single band of 66 kDa on SDS PAGE

### 3.4. Application of pectinase in juice extraction

Pectin is one of the components of plant cell wall and is found in many fruits and vegetables. It remains suspended after extraction of juice. The fruits and vegetables treated with crude pectinase enzyme from *Bacillus sp. MBRL576* and commercial pectinase were used for extraction of juice. The yield obtained was improved by enzyme treatment and material was pressed more easily than the control and the residual dry weight of solid residue decreased. Enzyme treatment of crushing and macerating apple, banana and carrot led to increase in juice volume. Banana is a soft fruit with a high level of soluble pectin (Pilnik & Voragen 1993) and after maceration resulted in a semi gelled mass which was very difficult to press. Pulp treated with pectinase from *Bacillus sp. MBRL576* showed better pressing characteristics and 133% higher juice yield (Table3). The juices obtained by enzymatic treatment had lower viscosity compared to those untreated, possibly due to reduction of pectin content. The difference among yields obtained with the fruits tested could be due to the pH value of the mash, the ripening state but mainly to pectic substances present and their level of esterification (Soares et al, 2001). Dry weight of solid residue decreased appreciably after enzyme treatment of apple pulp as compared to banana and carrot. Pectin content of all the enzyme treated juices was also reduced.

**Table3.** Extraction of juice from treated and untreated fruit and vegetable pulp

	Apple			Banana			Carrot		
	Un treated	<i>Bacillus sp. MBRL 576</i> pectinase	Commercial pectinase	Un treated	<i>Bacillus sp. MBRL 576</i> pectinase	Commercial pectinase	Un treated	<i>Bacillus sp. MBRL 576</i> pectinase	Commercial pectinase
volume of pulp (ml)	15	15	15	12	12	12	13	13	13
volume of juice (ml)	30	40	25	25	40	25	40	50	25
pH of juice	3.5	3.5	3.5	5.0	5.0	5.0	5.9	5.9	5.9
dry weight of residue(g)	1.593	0.837	0.512	1.505	0.913	1.244	0.910	0.653	0.861
% decrease in dry weight	-	47.46	67.86	-	39.34	17.34	-	28.24	5.38
Pectin* content	19.245	16.88	16.132	44.811	42.594	32.264	42.78	23.39	21.415

\*Pectin content determined after clarification of juice

**Table 4:** Clarification of juice

Transmittance (%)	Apple		Banana		Carrot	
	Initial	final	Initial	final	Initial	final
Untreated	1.01	-	3	-	0.79	-
<i>Bacillus sp. MBRL 576</i> pectinase	1.25	2.13	4	9.38	0.85	1.00
commercial pectinase	1.07	3.27	7	17	1.07	1.07

#### 4. Conclusion

This is probably the first report of pectinase production by *Bacillus sp. MBRL576*. The organism *Bacillus sp. MBRL576* has been isolated, characterized and identified. The culture conditions were optimized for pectinase production. *Bacillus sp. MBRL576* has shown best results at pH 4 and temperature of 45°C in 24 h of incubation period at 150 rpm under submerged fermentation. But it was producing pectinase at a wider range of pH and temperature. The pectinase was purified by ammonium sulphate precipitation and column chromatography using DEAE-Sephacel and Sephadex G150 column. Purified enzyme produced single band on SDS-PAGE and was found to be 66 kDa. Crude enzyme was used for extraction and clarification of juice.

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