Romanian Biotechnological Letters *Vol. 15, No.2, 2010*

ORIGINAL PAPER

Production of a Novel Pectin Lyase From Bacillus pumilus (P9), Purification and Characterisation and Fruit Juice Application

Received for publication, September 27, 2009
Accepted, April 14, 2010

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Abstract

Extracellular pectin lyase (EC 4.2.2.10) was produced by Bacillus pumilus (P9) in solid state fermentation. Pectin lyase enzyme was purified 36.36 fold by using DEAE-cellulose anion exchange column chromatography and characterized. Molecular weight of the enzyme was determined as 25 kDa by using Sephadex G-100 gel filtration chromatography.

*Purification of enzyme was controlled by SDS-PAGE. The optimum pH and temperature of enzyme was determined as pH 6.0 and 60°C, respectively. Pectin lyase was mostly stable at 40°C. Its' activity deceased in 50% for 1h at 60°C and 40% for 4 h at 50°C. V_{max} and K_M were calculated respectively as 0.298 mg/mL and 132.6 □mol/L*min. The presence of 10 mM concentration of Ca^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+}, Fe^{2+} and EDTA, L-cystein, ascorbic acid and □-mercaptoethanol significantly enhanced the pectin lyase of the purified enzyme.*

The purified pectin lyase enzyme was used for getting fruits juices. It was determined that yields of fruits juices significantly improved when it was compared with control.

Keywords: Pectin lyase, Purification, *Bacillus pumilus* (P9), Pectin, Juice Clarification

Introduction

Pectic substances are glycosidic macromolecules with high molecular weight. They form the major components of the middle lamella and primary plant cell wall [1]. Pectic substance consists of protopectins, pectinic acids, pectins and pectic acids. The main chain of pectin is partially methyl esterified 1, 4-D-galacturonan. Demethylated pectin is known as pectic acid (pectate) or polygalacturonic acid [2].

Pectic substances are naturally degraded by pectinases. The classification of pectic enzymes is based on their attack on the galacturonan backbone of the pectic substance molecule. Basically, there are three types of pectic enzymes; de-esterifying enzymes (pectin esterase), depolymerizing enzymes and protopectinases [1].

Pectin esterases catalyze the hydrolysis of methyl to produce pectic acid and methanol. Depolymerizing enzymes consist of hydrolases and lyases. Lyases are also called trans-eliminases, which split the glycosidic bonds of either pectate (polygalacturonate) or pectin (polymethylgalacturonate) [3].

Pectinases are produced by a large number of organisms, such as bacteria [4-6], fungi [7,8], actinomycetes [9] and yeast [10].

Pectinases have been used in processes and industries where the elimination of pectin is essential; fruit juice processing, coffee and tea processing, macerating of plants and vegetable tissue, degumming of plant fibers, treatment waste water, extracting vegetable oil, bleaching of paper, adding poultry feed and in the textile, alcoholic beverages and food industries [11].

Pectinolytic enzymes are commonly used during processing of fruits and vegetables for juices and wine. The pectic substances account for about 0.5–4% of the weight of fresh material. The raw pressed juice is rich in insoluble particles mainly made up of pectic substances. When the tissue is ground, the pectin is found in the liquid phase (soluble pectin) causing an increase in viscosity and the pulp particles. It is difficult to extract this juice by pressing or using other mechanical methods. With the addition of pectinases the viscosity of the fruit juice drops, the press ability of the pulp improves, the jelly structure disintegrates and the fruit juice is easily obtained with higher yields [3, 12].

Commercial pectinase is a mixture composed of three different enzymatic activities: polygalacturonase, pectin esterase and pectin lyase (E.C.: 4.2.2.10, polymethylgalacturonate lyase) [13]. Commercial preparations with pectin lyase are preferable as the major component in juices and wine processing because it avoids the production of methanol, the precipitation of pectin partially de-esterified with endogenous calcium, and the damage of volatile ester content responsible for the specific aroma of various fruits [14]. Furthermore, pectin lyase is the only enzyme which is able to cleave the α-1, 4 glycosidic bond of highly esterified pectins such as fruit pectin without the prior action of other enzymes [14-16].

In this paper, we report the production of extra cellular pectin lyase (E.C. 4.2.2.10) produced by *Bacillus pumilus* (P9) using solid state fermentation. Pectin lyase (PL) from *Bacillus pumilus* (P9) was purified and characterized. We also determined the action of pectin lyase in fruit juice. This is the first report on the production, purification and characterization of a pectin lyase from *Bacillus pumilus* (P9).

Materials and methods

Materials

Pectin (ED % 9.4) from citrus fruit, DEAE-cellulose, phenol, dithothreitol (DTT), thiobarbituric acid (TBA), Sephadex G100 and G150, the electrophoresis equipment and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). pGEM-T vector system was maintained from Promega Corp., Southampton, United Kingdom and ABI PRISM cycle sequencing kit was maintained from Macrogen, Korea. The other reagents were of analytical grade.

Production of Pectic Enzyme by Solid-State Fermentation

Bacillus pumilus (P9) was isolated from soil, and identified using Sherlock Microbial Identification System (MIS).

SSF was carried out using 250 ml Erlenmeyer flasks containing 5 g wheat bran, 1% citrus pectin (DE 9.4%, Sigma Chemical Co) and 10 ml salt mixture that composed of 0.14% $(NH_4)_2SO_4$, 0.02% $MgCl_2$ and 0.02% K_2HPO_4. The final moisture content of the medium was approximately as 67%. The flasks were sterilized for 20 min at 115^oC, inoculated, and then incubated at 27^oC for 5 days. The fermented material was mixed with 60 mL of 1% NaCl and centrifuged (4^oC, 10.000 rpm and 30 min). The supernatant was used to measurement of enzyme activity [17].

Purification of Pectin Lyase

The supernatant was dialyzed three times for two hours at 4^oC against 0.01 M sodium phosphate buffer, pH 8. After this dialysis, the supernatant was adjusted to pH 8 by the addition of NaOH and was loaded onto a DEAE-Cellulose column (20x2 cm inner diameter),

which had been pre-equilibrated with 0.01 M sodium phosphate buffer, pH 8.0. Adsorbed proteins were eluted with a linear gradient of 0 to 0.4 M NaCl (200 mL total volume) in 0.01 M sodium phosphate buffer, pH 8.0. Fractions exhibiting pectin lyase activity were pooled [18].

After scanning at 280 nm, the tubes with significant absorbance were pooled and protein was determined quantitative by the Coomassie Blue G-250 method [19].

Enzyme and Protein Assays

Pectin lyase activity was determined by using thiobarbituric acid (TBA) method, as this method described by Nedjma [20]. A 250 µl aliquot of suitably diluted enzyme solution was incubated in the presence of 250 µl of substrate (1.5% (w/v) pectin (DE 93%) / 0.05 M phosphate, pH: 8) for the duration 10 minutes.

NaOH (1 N, 0.05 ml) was added to 0.5 ml of the sample. The mixture was shaken briefly. The solution was heated at 80°C in a water bath for 5 min and cooled. After the addition of 0.6 ml of HCl (1 N) to acidify the medium, the solution was then shaken and 0.5 ml of 0.04 M TBA (in aqueous solution) was added. The tube was then heated at 80°C for 5 min in a water bath. The solution was briefly cooled in an ice-water bath before measuring the absorbance at 550 nm. A control was prepared by addition 1% NaCl instead of enzyme solution in the reaction mixture. One unit of PL activity (EU) was defined as the amount of enzyme which produces 1 µmol of unsaturated galacturonide per minute.

SDS-PAGE Electroforesis

SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme. It was carried out in 10% and 3% acrylamide concentrations for the running and the stacking gel, respectively, containing 0.1% SDS [21]. A 20 µg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye. The electrophoretic pattern was photographed (Figure 1).

Calculation of K_M and V_{max} for the Pectin Lyase

V_{max} and K_M values were determined by increasing concentration of substrate from 0.75 g/L to 3.75 g/L (0.75% pectin (DE 93%)/0.05 M phosphate, pH: 8). 50, 100, 150, 200 and 250 µL of each substrate solution was added in a cuvette containing 0.25 mL enzyme. Its total volume was making up 0.5 ml with buffer solution (0.05 M phosphate, pH: 8). Cuvettes were incubated 10 minute at 60°C. The activity of pectin lyase was determined as described above. V_{max} and K_M values were determined using a Lineweaver-Burk plot.

Molecular Mass Determination Using Gel Filtration

Sephadex G-100 was soaked in with distilled water at 90°C for 5 hours and placed into a column (3×70 cm). The column was equilibrated for 24 h with 0.05 M Na_2HPO_4/1 mM dithioerythritol, pH 7.0. The eluent exhibited no absorbance at 280 nm. A protein standard solution was added to the column and the calibration curve was obtained. The concentration of the protein solution was 0.2 mg/ml. The standard proteins and pectin lyase enzyme were eluted under the same conditions (room temperature, flow rate of 20 mL/h) [22].

Effect of pH and Temperature on the Activity of Pectin Lyase

The optimum temperature for PL activity was determined by carrying out the standard assay in 50 mM sodium phosphate buffer, pH 8 at temperatures ranging from 0 to 90°C. In each case, the substrate was pre-incubated at the desired temperature for 5 min.

The pH optimum of the PL was measured at a fixed assay temperature of 50°C at various pH values between pH 4 and 11, using different buffers. Buffers used were 0.05 M sodium acetate (pH 4-5), 0.05 M sodium phosphate (pH 6-8) and 0.05 M sodium carbonate (pH 9-11).

Effect of Temperature on the Enzyme Stability

In order to study the thermal stability of the enzyme, buffered enzyme samples (50 mM sodium phosphate buffer, pH 8) were incubated for 1-24 h time periods at 40, 50, 60,

70 and 80°C. Enzyme samples were taken periodically and assayed to determine the residual enzyme activity as described above.

Effect of Metallic Salts and Some Chemicals on Pectin Lyase Activity

The effects of $HgCl_2$, $ZnCl_2$, $MnCl_2$, $CuSO_4$, $CaCl_2$, $MgCl_2$, $FeCl_3$, EDTA, L-cystein, ascorbic acid, β-mercaptoethanol and SDS on pectin lyase activity were investigated. Each inhibitor solution was prepared at 10 mM concentration) and 100, 200, 300, 400 or 500 µL of each solution was added in a cuvette containing of 0.25 mL substrate and 0.25 mL enzyme. Its total volume was making up 1 ml with buffer solution. The effect of each agent was determined by measuring enzyme activity using the thiobarbituric acid (TBA) method [20].

Juice Applications

The actions of purified pectin lyase, extract of crude enzyme and Pectinex 100 L Plus were used in process fruit juice. The extract of crude enzyme and purified pectin lyase were obtained from fermented *Bacillus pumilus* (P9) as described above. Pectinex 100 L Plus was bought from Novozyme. The obtained results were compared.

Pectinex 100 L Plus and crude extract was diluted and thus protein quantity of Pectinex 100 L Plus and crude extract was equalized to purified pectin lyase.

Juice Extraction

Fruits (apple, orange, carrot, and banana) were purchased from local markets. Fruits were peeled, deseeded and blended for 2-3 min, until a homogenous fruit pulp was obtained. The pH of the pulp was adjusted to 6. Then 5 grams of material were incubated with 2 ml of enzyme preparation (purified pectin lyase, crude extract and Pectinex 100 L Plus) for 5 h in a shaking water bath in 50°C.

Later the samples were incubated in a boiling water bath for 5 min to inactivate the enzyme. After cooling to room temperature, the juice was filtrated by vacuum through filter paper and the volume of obtained juice was measured. The purification degree of fruit juice's yield was determined by comparing control. Mash treated distilled water was used as a control. Mash residues were dried at 105°C until constant weight was achieved. The reduction in the dry weight of the treated mash was expressed as a percent relative to the control [23].

Results

Production of pectic enzyme by solid-state fermentation and purification and characterisation

In this study, pectin lyase was produced by *Bacillus pumilus* (P9) using solid state fermentation. Pectin lyase activity was produced from per gram of wheat bran as 1572 EU. By using DEAE-Cellulose anion exchange chromatography, pectin lyase was purified as 36.36 fold from bacteria extract (Table 1). The purified enzyme was characterized.

Table 1. The purification of pectin lyase from *Bacillus pumilus.*

Enzyme Fraction	Volume	Activity	Total Activity		Protein	Specific	Purification
	mL	EU/mL	EU	%	(mg/ml)	EU/mg	Fold
Crude extract	65	1.583	102.9	100	0.912	1.736	-
DEAE-Cellulose	50	1. 376	68.8	81.4	0.285	4.83	3.39
Sephadex G 150	30	2.195	54.9	79.8	0.0125	175.6	36.36

The purity of the enzyme was checked with SDS-PAGE (Figure 1). As result, a single band indicated purity of the protein was obtained on 10% SDS–PAGE.

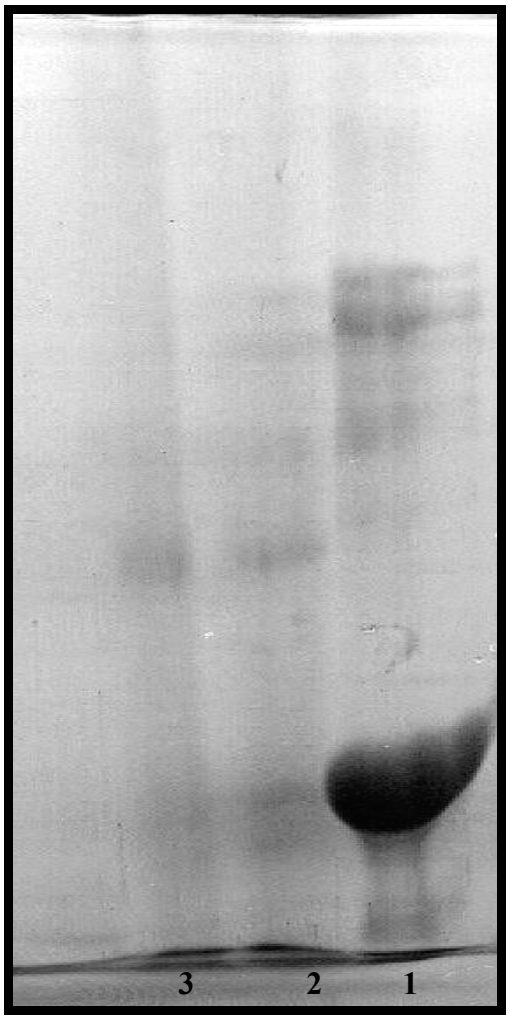

Figure 1. SDS-poliacrilamide gel electrophoresis pectin lyase from *Bacillus pumilus;* (1) standart proteins, (2) purified pectin lyase by DEAE cellulose ion exchange chromatography, (1) purified pectin lyase by gel filtration chromatography.

V_{max} and K_M values were calculated by using Lineweaver-Burk graphs. K_M and V_{max} values of purified PL are 0.298 mg/mL and 132.6 μmol/L.min., respectively. The molecular weight of the enzyme was determined by Sephadex G 100 gel filtration chromatography. For PL, the molecular weights were 25 000 Da. As this result is compared to other studies, it is approximately similar to molecular weight of PL (52 000 Da monomer) from *Bacillus sp*. PN33 (24), more than (34 000 Da) from *Penicillium italicum* [25] and (30 900 Da) from *Aspergillus niger* [26].

Effect of pH and Temperature on the Activity of Pectin Lyase

The properties of purified enzyme from *Bacillus pumilus* (P9) were determined. For this aim, optimum pH, optimum temperature and stability of the enzyme was investigated. In addition, the effects of some substances on enzyme activity were found out. Enzyme inhibition and stability are considered to be the major constraints in the rapid development of biotechnological processes. Stability studies also provide valuable information about structure and function of enzymes. The results of this study can give valuable, for more productive applications of enzyme in industry, implications.

The stability of pectin lyase is affected by both physical parameters (pH and temperature) and chemical parameters (inhibitors or activators). The enzymatic hydrolysis of pectic substances also depends on several physicochemical factors. It depends on contact time, enzyme concentration, temperature of incubation and pH.

The effect of temperature was investigated between 0°C and 90°C with 10 degree increments (Figure 2) and the optimum temperature was found to be 60°C. Enzyme has activity between 20°C and 80°C. Optimum temperature of pectin lyase was similar to PLs from *Bacillus sp.* DT7 [27, 28] and was higher than *Rhizopus oryzae* [29], *Curvularia inaequalis* NRRL 13884 [18], *Bacillus sp*. PN33 [24].

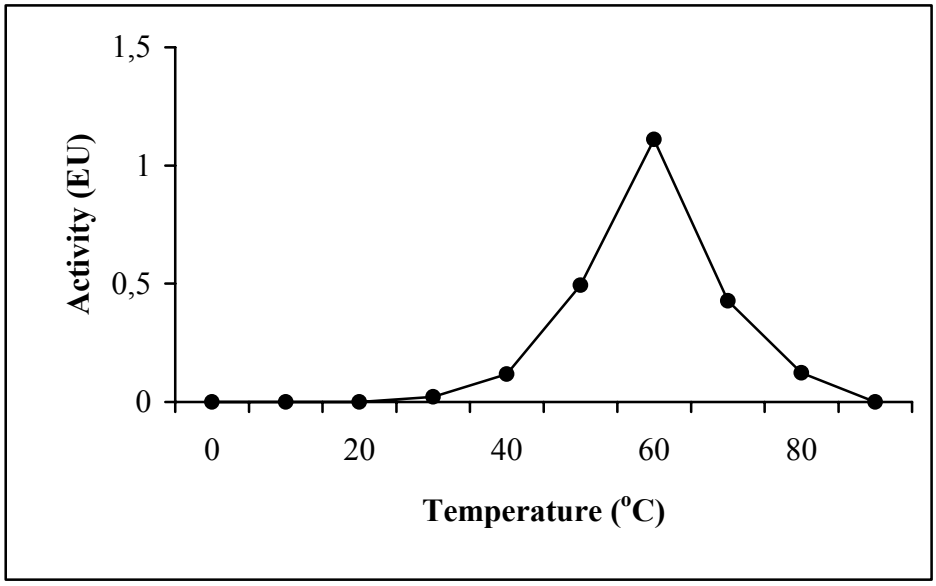

Figure 2. The effect of temperature on the activity of purified pectin lyase from *Bacillus pumilus* (P9).

Similarly, optimal pH studies were investigated for pectin lyase with 1 pH unit increments between 4 and 11 (Figure 3). The optimal reaction pH for pectin lyase was 6, and it was active between pH 5 and 10. Optimum pH of pectin lyase was identical to PL from *Bacillus sp.* DT7 [28]. This result is higher than the optimum pH (pH: 5) of PL produced from *Curvularia inaequalis* NRRL 13884 [18] and (pH: 5) of *Aspergillus niger* [26]. But it is less than the optimum pH (pH: 9) of PL from *Moniliella* SB9 and (pH: 10) from *Penicillium sp* EGC5 [30].

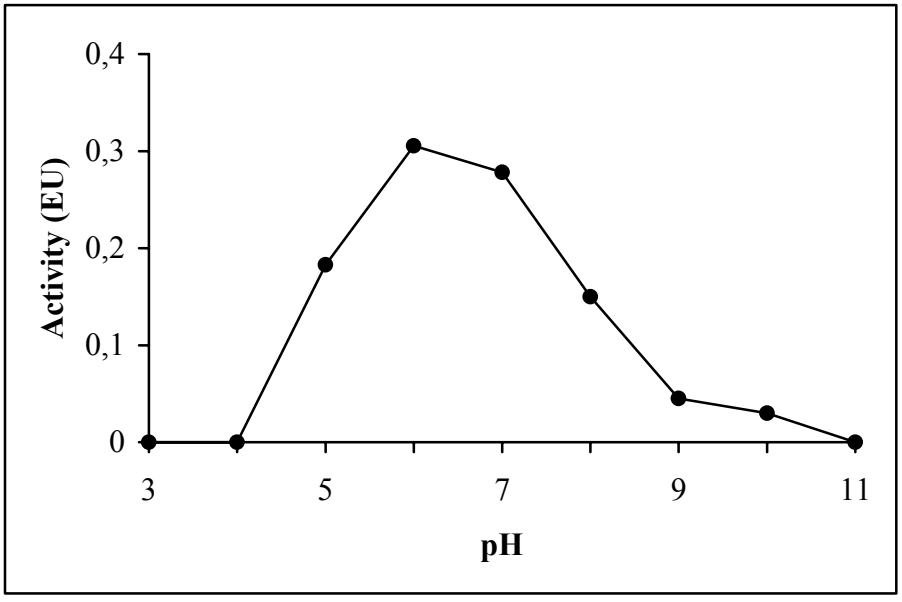

Figure 3. The effect of pH on the activity of purified pectin lyase from *Bacillus pumilus* (P9).

The thermo stability study of pectin lyase was carried out at temperatures ranging from 40°C to 80°C (Figure 4).

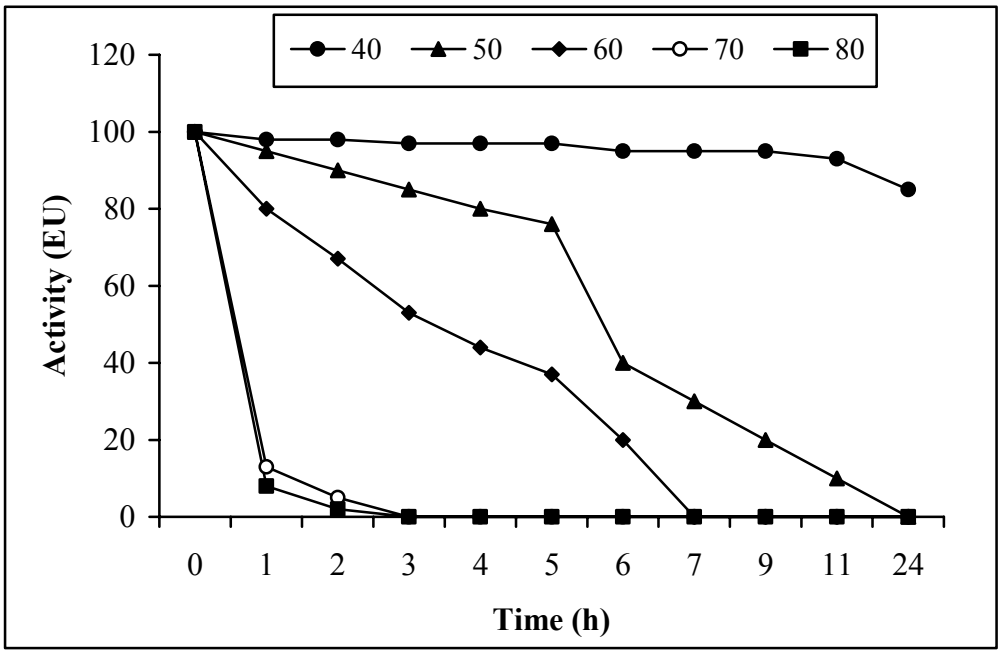

Figure 4. The temperature stability of the purified pectin lyase from *Bacillus pumilus*.

The purified enzyme appeared to be stable and retained its full activity after 1 h incubation from 40°C to 50°C, but the activity was reduced to 20% after 1 h at 60°C. Pectin lyase activity decreased dramatically when the temperature increased above 70°C with only 13% and 8% activity remaining 70°C and 80°C, respectively. Thermo stability of PL was 40°C, because it remained nearly full active at 40°C for at least 24 h. The thermo stability of PL from *Aspergillus niger* was determined as 40-50^{o}C [26]. It was determined that PL from *Rhizopus oryzae* inactivated after 45 minutes at 70^{o}C [29]. Even if, it was found that pectin lyase from *Pythium splendens* was stable at 4-50°C, but its activity decreased rapidly beyond 50°C [31].

Effect of Metallic Salts and Some Chemicals on Pectin Lyase Activity

The effect of various metal ions on pectin lyase activity was tested at 10 mM (Table 2). Purified pectin lyase was completely inhibited by 10 mM of Hg^{2+}, $Mn^{2+,}$ EDTA, β-mercaptoethanol and SDS. A slight activation effect was observed in the presence of Ca^{2+}, $Mg^{2+,}$ L-Cystein, Ascorbic acid (10 mM) on the purified pectin lyase. While Mg^{2+} (10 mM) was stimulated the activity of PL 10 mM Zn^{2+}, Cu^{2+} and Fe^{2+} was inhibited. In the presence of 10 mM of Ca^{2+} decrease in activity were observed.

Table 2. The effect of some metal ions and chemicals on pectin lyase activity from *Bacillus pumilus*.

Chemical Compounds	Concentration (mM)	Pectin lyase Activity (%)
Control	-	100
$CaCl_2$	10 mM	132.1
$MgCl_2$	10 mM	119.2
$Hg(NO_3)_2$	10 mM	0
$MnCl_2$	10 mM	0
$ZnSO_4$	10 mM	87.9
$Cu(NO_3)_2$	10 mM	11.1
$FeSO_4$	10 mM	67.3
EDTA	10 mM	0
L-Cystein	10 mM	102.6
Ascorbic acid	10 mM	101.5
□-mercaptoethanol	10 mM	124.1
SDS	10 mM	0

Juice Applications and Juice Extraction

Removing solid substance and improving productivity was separately processed with purified enzyme, crude extract and Pectinex 100 L Plus and the results were presented in table 3 and 4. There was an increase, compared to the control, in the volume of juice of apple, banana, carrot, peach caused by the treatment of fruit pulps with purified enzyme, crude extract and Pectinex 100 L Plus (Table 3). These results obtained for purified enzyme, crude extract and Pectinex 100 L Plus were similar for fruit juice except for peach. The most accepted results for peach was achieved in commercial Pectinex 100 L Plus. Highest productive yield was gotten in bananas as eight times higher than the control.

Table 3. Increasing in yield as fold in juice yield from different fruit pulps treated with purified pectin lyase, crude extract and Pectinex 100 L Plus for 5 h.

	Apple (5 g)		**Banana (5 g)**		**Carrots (5 g)**		**Orange (5 g)**	
	Juice (mL)	**Increasing in yield% as fold**	**Juice (mL)**	**Increasing in yield% as fold**	**Juice (mL)**	**Increasing in yield% as fold**	**Juice (mL)**	**Increasing in yield% as fold**
Control	12	-	8	-	11.5	-	5.95	-
Purified pectin lyase	13	108.3	13	162.5	12.5	108.7	7	117.6
The crude extract	14	107.7	9.5	118.8	.12.5	108.7	6.95	116.8
Pectinex 100 L Plus	17	141.7	15	187.5	14	121.7	8	134.5

The material was pressed more easily than the control and the residual dry weight of solid residue decreased from 6 to 72% (Table 4). As a result, the productivity yield of fruit juice was increased. Banana with a high level of soluble pectin [32] resulted in a semi gelled mass which was very difficult to press after maceration. The juice obtained by enzymatic treatment had lower viscosity compared to those non-treated, possibly due to reduction of pectin content.

Table 4.

	Apple (5 g)		**Bananas (5 g)**		**Carrots (5 g)**		**Orange (5 g)**	
	D.W.S.R (g)	**% Decrease**	**D.W.S.R (g)**	**% Decrease**		**D.W.S.R (g)**	**% Decrease**	**D.W.S.R (g)**
Control	2,7	-	8,5	-	Control	2,7	-	8,5
Purified pectin lyase	1,9	29.6	2,38	72	Purified pectin lyase	1,9	29.6	2,38
The crude Extract	2,1	22.2	3,3	61.2	The crude Extract	2,1	22.2	3,3
Pectinex 100 L Plus	1,87	30.7	2,6	69.4	Pectinex 100 L Plus	1,87	30.7	2,6

In this paper, we report the production, purification and characterization of extra cellular pectin lyase (E.C. 4.2.2.10) produced by *Bacillus pumilus* (P9) in solid state fermentation. This is the first report on the production, of a pectin lyase from *Bacillus pumilus* (P9). We were also determined the action of pectin lyase in fruit juice. It was concluded that purified pectin lyase from *Bacillus pumilus* (P9) can be used in processes of fruit juice.

Discussion

Production, purification and characterisation of extra cellular pectin lyase (E.C. 4.2.2.10) produced by *Bacillus pumilus* (P9). This is the first report on the production of a pectin lyase from *Bacillus pumilus* (P9). We also determined the action of pectin lyase in fruit juice production. It was concluded that purified pectin lyase from *Bacillus pumilus* (P9) can be used in process of yielding fruit juice.

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