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Production of pectin lyase from *Geobacillus pallidus* p26, purification, characterization and fruit juice application

Safinur Yildirim Çelik[a], Nazan Demir[b], Yaşar Demir[b], Ahmet Adiguzel[c], Medine Gulluce[d]

[a]College of Education, Bayburt University, 69000, Bayburt, Turkey
[b]College of Art and Science, Muğla University, 48000, Muğla, Turkey
[c]Atatürk University, Faculty of Science, Molecular Biology and Genetics Department, 25240, Erzurum, Turkey
[d]Atatürk University, Faculty of Science, Biology Department, 25240, Erzurum, Turkey
DEMIRN@YAHOO.COM

Abstract: A bacterial strain was isolated from Pasinler hot spring, Erzurum, Turkey. The purified thermophilic isolate was identified as *Geobacillus pallidus* P26 and used to produce extracellular pectin lyase (EC 4.2.2.10). Pectin lyase enzyme was purified 34 fold by using DEAE-cellulose anion exchange column chromatography and characterized. Molecular weight of the enzyme was determined as 56 kDa by using Sephadex G-100 gel filtration chromatography. Purification of enzyme was verified by SDS-PAGE. The pH- and temperature optima of enzyme were determined (pH 9.0 and 60 °C, respectively). Pectin lyase was mostly stable at 50 °C for 24 hours. Its' activity decreased to 50 % for 24 h at 60 °C. K_M and V_{max} were calculated as 24.8 mg/mL and 2.28 μmol/L min, respectively. Purified pectin lyase was inhibited by Fe^{3+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+} and Hg^{2+} but not by Mg^{2+}. The purified pectin lyase enzyme was used for getting fruits juices. It was found that yields of fruits juices increased when they were compared with control.

Keywords: Pectin lyase, purification, characterization, *Geobacillus pallidus* P26

Introduction

Pectinases have been used in processes and technologies where the elimination of pectin is essential. Among those are 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 the textile, alcoholic beverages, and food industries (Jayani et al. 2005).
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 (Kashyap et al. 2001, Rai et al. 2004).
Pectinases are produced by a large number of organisms, such as bacteria (Kim et al. 1998), fungi (Alana et al. 1991, Obi et al. 1985), actinomycetes (Beg et al. 2000) and yeast (Blancoa et al. 1999).
Commercial pectinase is a mixture composed of three different enzymatic activities: polygalacturonase, pectin esterase and pectin lyase (Sunnotel and Nigam 2002). 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. Furthermore, pectin lyase is the only enzyme which is able to cleave the α-1,4-glycosidic bond of highly esterified pectin's such as fruit pectin without the prior action of other enzymes (Taragano and Pilosof 1999).
In this paper, the extracellular pectin lyase was produced from *G. pallidus P26* which is isolated from Pasinler Hot Spring, Erzurum, Turkey and, then 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 *G. pallidus P26*.

Materials and Methods

Isolation of Strain

The water sample was obtained from Pasinler hot spring, Erzurum, Turkey and transferred into laboratory under aseptic conditions. The sample

was streaked and inoculated into Nutrient Agar (NA) plates and then, incubated in aerobic incubator with an adjusted temperature of 55—60 °C for 24—48 hours. After incubation period, different colonies developed in the media were selected and purified on Nutrient Agar. Isolated and purified bacterial strain was stored in Nutrient Broth containing 15 % glycerol at –86 °C until being used.

Physiological and Biochemical Characterization of Isolate

The temperature range for growth was determined by incubating the isolate from 30 to 80 °C with 5 °C interval. The effect of NaCl on the thermophilic bacterial growth was studied in NB medium containing 2.0, 3.0, 4.0, 5.0, 8.0, 10.0 % (w/v) NaCl. The pH dependence of growth was tested in the pH range 4.0—11.0 in nutrient broth medium. Gram reactions, the presence of catalase, oxidase and amylase reduction were investigated according to the methods described by Harley and Prescott (1983).

DNA Extraction from Pure Culture

Total genomic DNA was extracted from bacteria samples using a modified method previously described by Adiguzel (2006).

PCR amplification and cloning of 16S rRNA gene fragments

The 16S rRNA gene was selectively amplified from purified genomic DNA by using oligonucleotide primers designed to anneal to conserved positions in the 3´ and 5´ regions of bacterial 16S rRNA gene. The forward primer, UNI16S-L (5´-ATTCTAGAGTTTGATCAT GGCTCA-3´), corresponded to positions 11 to 26 of *Escherichia coli* 16S rRNA, and the reverse primer, UNI16S-R (5´-ATGGTACCGTGTGACGGGCGGTGTGTA-3´), corresponded to the complement of positions 1411 to 1393 of Escherichia coli 16S rRNA (Adiguzel et al. 2009). PCR reaction conditions were carried out according to Beffa et al. (1996) and the PCR product was cloned to a pGEM-T vector system (Promega Corp., Southampton, United Kingdom). 16S rRNA gene sequence was determined with Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, Korea).

Nucleotide sequence submission

The nucleotide sequence was deposited in the GenBank under the accession number EU935591.

Production and Purification of Pectin Lyase

The strain *G. pallidus* P26 was grown in NB in 250 mL culture flasks. The media were incubated at 56 °C for five days. After incubation media were centrifuged (4 °C, 10000 rpm and 30 min). The supernatant was used for measurement of enzyme activity. The supernatant was dialyzed three times for two hours at 4 °C against 0.01 mol/L sodium phosphate buffer, pH: 8. After dialysis, the supernatant was adjusted to pH 8 by the addition of NaOH and was loaded onto a DEAE-Cellulose column (20 × 2 cm inner diameter), which had been pre-equilibrated with 0.01 mol/L sodium phosphate buffer, pH: 8.0. Adsorbed proteins were eluted with a linear gradient of 0 to 0.4 mol/L NaCl (200 mL total volume) in 0.01 mol/L sodium phosphate buffer, pH: 8.0. Fractions exhibiting pectin lyase activity were pooled (Afifi et al. 2002). After scanning at 280 nm, the tubes with significant absorbance were pooled and protein was determined quantitative by the Coomassie Blue G-250 method (Bradford 1976).

Enzyme Assays

Pectin lyase activity was determined by using thiobarbituric acid (TBA) method, as this method described by Nedjma (Nedjma et al. 2001). 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 %) in 0.05 mol/L phosphate, pH 8, (Sigma Chemical Co, USA) for 10 minutes. NaOH (1 mol/L, 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 mol/L) to acidify the medium, the solution was then shaken and 0.5 mL of 0.04 mol/L 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

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 (Laemmli 1970). 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.

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 %) in 0.05 mol/L 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 mol/L 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 mol/L Na_2HPO_4 containing 1 mmol/L 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) (Whitaker 1963).

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 mmol/L sodium phosphate buffer, pH: 8 at temperatures ranging from 0 °C 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 mol/L sodium acetate (pH 4—5), 0.05 mol/L sodium phosphate (pH 6—8) and 0.05 mol/L 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 mmol/L 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 Inhibitors on Pectin Lyase Activity

The effects of $HgCl_2$, $ZnCl_2$, $CoCl_2$, $CuSO_4$, $CaCl_2$, $MgCl_2$, and $FeCl_3$ on pectin lyase activity were investigated. Each inhibitor solution was prepared at three different concentrations (2×10^{-2}, 2×10^{-4} and 2×10^{-6} M) and 500 µL of each solution was added in a cuvette containing of 0.25 mL enzyme. After 10 min incubation of enzyme and agents, 0.25 mL substrate was added. Then, the effect of each agent was determined by measuring enzyme activity using the thiobarbituric acid (TBA) method (Nedjma et al. 2001).

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 *Geobacillus pallidus* 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, carrot, peach 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 8. Then, 10 g 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 40 °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 degree of fruit juice's yield was determined by comparing control. Mash treated with 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 (Soares et al. 2001).

Results and Discussion

The bacterial strain (P26) isolated in this study was subjected to various morphological, physiological and biochemical tests. The results showed that the strain was Gram-, catalase-, oxidase-, and amylase-positive, endospore forming, and motile rod. The optimum pH and temperature for this strain was determined as 7.5 and 55 °C, respectively. The strain was able to grow in the range of salt concentration of 2—5 %. These met the criteria of thermophilic bacteria, which grew at temperatures above 50 °C (Perry and Staley 1997). To verify the systematic

position of this bacterium, 16S rRNA analysis was undertaken. The sequence of this thermophilic isolate was most similar to that of *G. pallidus*, having 99 % sequence similarity. This similarity value (99 %) was retrieved in agreement with Zeigler (2005) and Meintanis et al. (2008) who confirmed that the 16S rRNA gene sequences similarity of *Bacillus* and *Geobacillus* type strains are higher than 98.5 %. Although the 16S rDNA gene is used as framework for modern bacterial classification, it has often been seen that its usage shows limited variation for the discrimination of closely related taxa and strains (Nübel et al. 1996; Meintanis et al. 2008; Adiguzel et al. 2009).

After isolation and identification of bacterial strain (*G. pallidus* P26*)*, this thermophilic isolate was used to produce of pectin lyase. The pectin lyase was produced by *G. pallidus P26* in NB at 56 ºC for five days. By using DEAE-Cellulose anion exchange chromatography, pectin lyase was purified as 34 fold from bacteria extract. The purified enzyme was characterized.

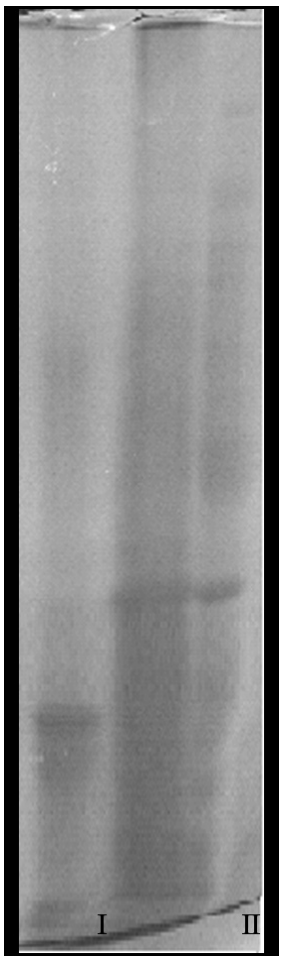

Fig. 1. SDS-polyacrylamide gel electrophoresis of (I) crude extract and (II) pectin lyase purified from *G. pallidus P26*. Experiment was done as described in Materials and Methods.

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.

V_{max} and K_M values were calculated by using Lineweaver-Burk graphs. K_M and V_{max} values of purified PL are 24.8 mg/mL ve 2.28 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 56000 Da. As this result is compared to other studies, it is approximately similar to molecular weight of PL (52000 Da monomer) from *Bacillus sp.* PN33 (Kim et al. 1998), more than (34000 Da) from *Penicillium italicum* (Alana et al. 1991) and (30900 Da) from *Aspergillus niger* (Obi and Moneke 1985).

The properties of purified enzyme from *G. pallidus P26* were determined. For this purpose, 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 ºC and 90 ºC with 10 degree increments (Figure 2) and the optimum temperature was found to be 60 ºC. Enzyme has activity between 0 ºC and 80 ºC. Optimum temperature of pectin lyase was identical to PLs from *Bacillus sp.* DT7 (60 ºC) (Kashyap et al. 2000), Bacillus GK-8 (Dosanjh and Hoondal 1996) and was higher than *Rhizopus oryzae* (50 ºC) (Hamdy 2005) and *Bacillus sp.* PN33 (40 ºC) (Kim et al. 1998).

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 9, and it was active between pH 5 and 11. Optimum pH of pectin lyase was identical to PL (pH: 9) from *Penicillium sp* EGC5 (Martin et al. 2004). This result is higher than the optimum pH of PL (pH: 5) produced from *Curvularia inaequalis* NRRL 13884 (Afifi et al. 2002) and *Aspergillus niger* (pH: 5) (Obi and Moneke 1985). But it is less than the optimum pH of PL (pH: 10) from *Moniliella* SB9 (Martin et al. 2004).

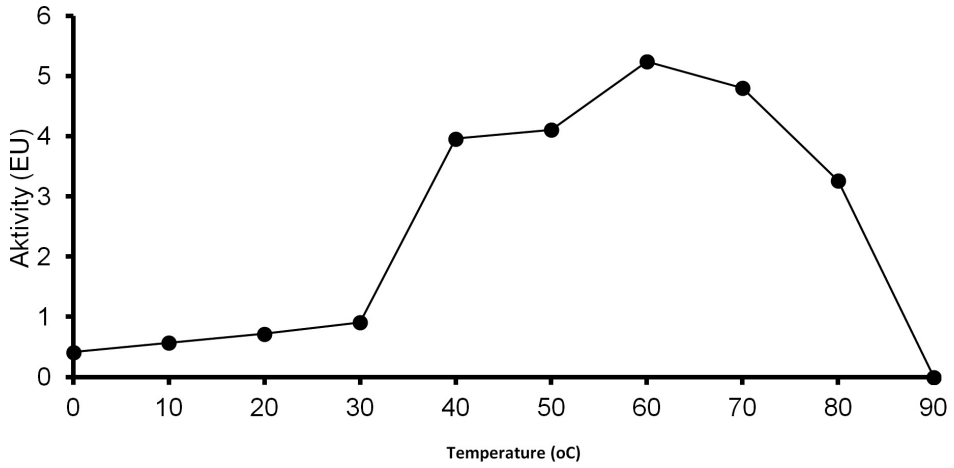

Fig. 2. The effect of temperature on activity of the purified pectin lyase from *G. pallidus P26*. Experiments were done as described in Materials and Methods.

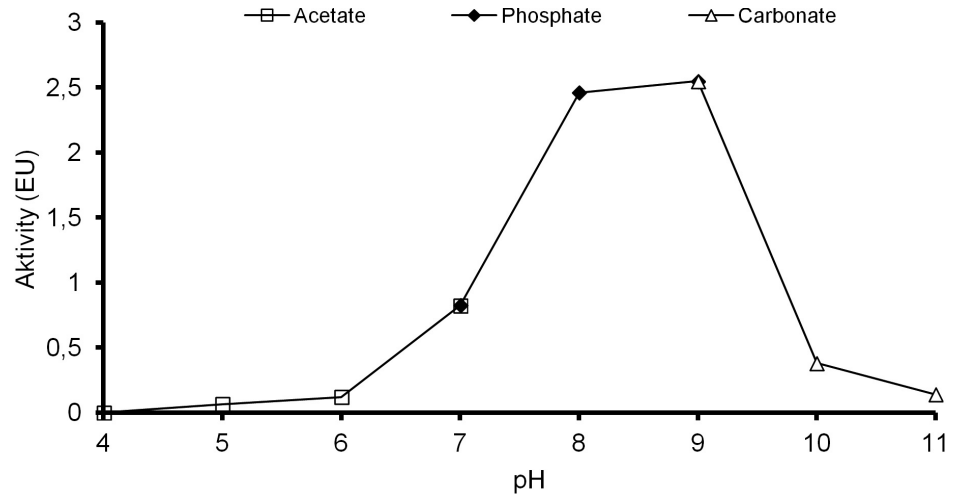

Fig. 3. The effect of pH on activity *o*f the purified pectin lyase from *G. pallidus P26*. Experiments were done as described in Materials and Methods.

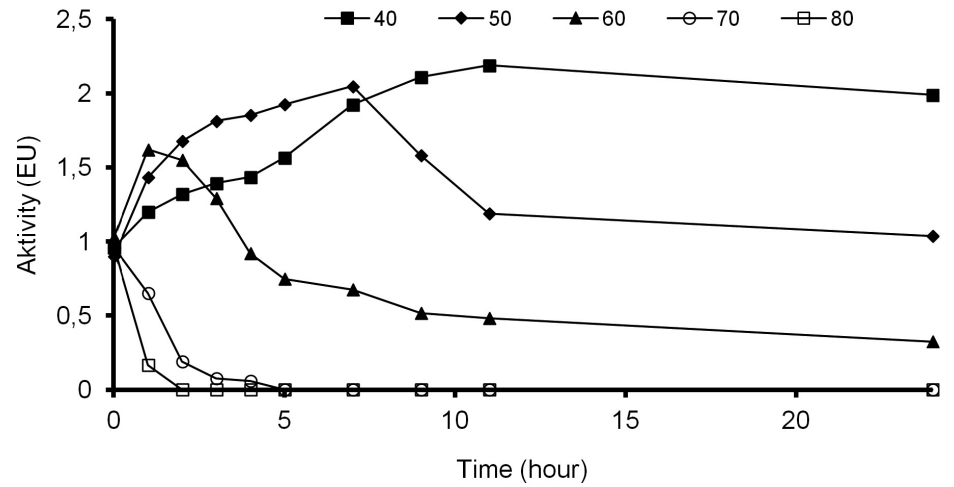

Fig. 4. The temperature stability of the purified pectin lyase from *G. pallidus P26*. Experiments were done as described in Materials and Methods.

The thermal stability study of pectin lyase was carried out at temperatures ranging from 40 ºC to 80 ºC (Figure 4). The purified enzyme appeared to be stable and retained its full activity after 24 h incubation from 40 ºC to 50 ºC. Pectin lyase activity decreased when the temperature increased above

Tab. 1. Effects of some metallic salts on the activity of pectin lyase from *G. pallidus P26*. Experiments were done as described in Materials and Methods.

	% Activity		
Concentration	**10^{-2} mol/L**	**10^{-4} mol/L**	**10^{-6} mol/L**
Control	100	100	100
Ca^{2+}	2	39	137
Mg^{2+}	163	150	119
Co^{2+}	13	41	51
Fe^{3+}	6	44	60
Zn^{2+}	19	89	92
Hg^{2+}	0	2	11
Cu^{2+}	0	38	40

70 °C. But, because of purified PL from the thermophilic bacteria, the purified enzyme can keep its' activity for 2 h and 5 h at 80 °C and 70 °C, respectively. In addition, the activity of purified PL only decreased 50 % after 24 h at 60 °C. Thermo-stability of PL was 40—50 °C, because it remained nearly full active at 40—50 °C for at least 24 h. The thermo stability of PL from *Aspergillus niger* was determined as 40—50 °C (Obi and Moneke 1985). It was determined that PL from *Rhizopus oryzae* inactivated after 45 minutes at 70 °C (Hamdy 2005). 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 (Chen et al. 1998).

The effects of various metal ions on pectin lyase activity were tested at 10, 0.1 and 0.001 mmol/L concentrations (Table 1). Purified pectin lyase was inhibited by 10, 0.1 and 0.001 mmol/L of Co^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+} and Hg^{2+}. While Ca^{2+} (0.001 mmol/L) stimulated the activity of PL, 0.1 and 10 mmol/L Ca^{2+} inhibited it. An activation effect was only observed in the presence of Mg^{2+} (10, 0.1 and 0.001 mmol/L) on the purified pectin lyase.

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 2 and 3. There was an increase, compared to the control, in the volume of juice of apple, banana, carrot and peach caused by the treatment of fruit pulps with purified enzyme, crude extract and Pectinex 100 L Plus (Table 2). These results obtained for purified enzyme were by 50 % less than those for Pectinex 100 L Plus (fruit juice of bananas and carrot). The most accepted results for peach and apple was achieved in

Tab. 2. 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. Experiments were done as described in Materials and Methods.

Amount (100 g)		**Control**	**Purified pectin lyase**	**The crude extract**	**Pectinex 100 L Plus**
Apple	Juice (mL)	123	131	148	171
	Increasing in yield as %	–	7	20	39
Banana	Juice (mL)	10	112	50	196
	Increasing in yield as %	–	1020	400	1860
Carrots	Juice (mL)	150	164	168	178
	Increasing in yield as %	–	9	12	19
Peach	Juice (mL)	94	102	115	160
	Increasing in yield as %	–	9	22	70

Tab. 3. Percent decrease dry weight solid residue (D.W.S.R.) from different fruit pulps treated with purified pectin lyase, crude extract and Pectinex 100 L Plus for 5 h. Experiments were done as described in Materials and Methods.

Amount (100 g)		**Control**	**Purified pectin lyase**	**The crude extract**	**Pectinex 100 L Plus**
Apple	D.W.S.R (g)	4.0	3.1	2.9	2.7
	Decrease %		22.5	27.5	32.5
Banana	D.W.S.R (g)	16.1	12.8	14.7	7.6
	Decrease %		20.5	8.7	52.8
Carrot	D.W.S.R (g)	3.6	3.4	3.3	2.2
	Decrease %		5.6	8.3	38.9
Peach	D.W.S.R (g)	4.6	4.5	4.4	1.4
	Decrease %		2.2	4.3	69.6

commercial Pectinex 100 L Plus. Highest yield was obtained in bananas and was eleven times higher than this in the control.

The material was pressed more easily than the control and the residual dry weight of solid residue decreased to 25 % (Table 3). As a result, the productivity yield of fruit juice was increased. Banana with a high level of soluble pectin (Pilnik and Voragen 1993) 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.

In this paper, this is the first report on the production of a pectin lyase from *G. pallidus P26*. We purified and characterized extracellular pectin lyase (E.C. 4.2.2.10) produced by *G. pallidus P26*. We were also determined the action of pectin lyase in fruit juice. It was concluded that purified pectin lyase from *G. pallidus P26* can be used in processes of fruit juice.

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