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REGULAR ARTICLE

PARTIAL CHARACTERIZATION OF ENZYMATIC ACTIVITIES PRODUCED BY A WILD STRAIN OF *A. NIGER*

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ABSTRACT

Aspergillus niger, isolated from decay citrus peels in the province of Misiones, was able to produce pectinases by submerged fermentation. The enzymatic extract exhibited polygalacturonase, pectinesterase and lyase activities. Others enzymes capable of degrading cell wall polymers were also detected in the enzymatic extract such as cellulases and xylanases. Polygalacturonase was an endo-polygalacturonase. The enzyme exhibited a maximal activity at pH range between 4.5 to 5.0, was stable in the pH range from 2.5 to 5.5 and remained unchanged when was incubated at temperatures lower than 50 ºC. The fungi produced three PG isoenzymes. The enzymatic extract was able to clarify apple juice.

The results observed make the pectinolytic enzymes produced by *A. niger* appropriate for future application in fruit juice processing industries.

Keywords: *Aspergillus niger*, pecinolytic enzymes, polygalacturonase

INTRODUCTION

Enzymes hydrolyzing pectic substances, which contribute to the firmness and structure of plant cells, are known as pectinolytic enzymes or pectinases. Based on their mode of action, these enzymes include polygalacturonase (PG), pectinesterase (PE), pectinlyase (PL), pectatelyase (PAL). PG, PL and PAL are depolymerizing enzymes, which split the α-(1,4)-glycosidic bonds between galacturonic monomers in pectic substances either by hydrolysis (PG) or by β-elimination (PL, PAL). PG catalyzes the hydrolytic cleavage of the polygalacturonic acid chain while PL performs a trans-eliminative split of pectin molecule, producing an unsaturated product. PE catalyzes the de-esterification of the methoxyl group of pectin, forming pectic acid **(Jayani *et al*., 2005; Tari *et al*., 2007)**.

Pectinolytic enzymes play an important role in food technology, mainly in the processing of fruit juices and wines. In the clarification of juice, these enzymes produce a rapid drop viscosity as well as the flocculation of the micelles, allowing these particles to be separated by sedimentation or filtration **(Costa *et al*., 2007; Croak and Corredig, 2006; Nighojkar *et al*., 2006).**

Industrial pectolytic enzyme preparations have been widely used for many years, most being derived from cultures of *Aspergillus niger* which is a species accepted as GRAS (Generally Recognised As Safe) **(Rogerson *et al*., 2000)**.

In most industrial applications, fungal pectinases prove to be the most useful because they exhibit an optimum activity at a lower pH and temperature range, suited for most fruit and vegetable processing applications **(Favela-Torres *et al*., 2006; Ortega *et al*., 2004; Teixeira *et al*., 2000)**.

In the present work, enzymes produced by a wild strain of *A. niger*, isolated from citrus fruit peels, were characterized and PG physicochemical properties were determined by the study of the effect of temperature and pH on its activity and stability, in order to evaluate its application in the clarification of apple juice.

MATERIAL AND METHODS

Microorganism

A. niger, isolated from citrus fruit peels in the province of Misiones **(Martos *et al*., 2007)**.

Production medium

The liquid medium used contained (g/L): KH_2PO_4, 4; $NaHPO_4$, 6; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2\ SO_4$, 2; H_3BO_3, 10 µg/L; $MnSO_4$, 10 µg/L; $ZnSO_4 \cdot 7H_2O$, 70 µg/L; citrus pectin (Parafarm), 20; pH 4.1 **(Maldonado *et al.*, 1998)**.

Production of pectolytic enzymes

The inoculum was prepared by stirring 1-week-old slants with a sterile solution of Tween 80 in water to obtain a 1.25 10^7 spores/cm^3 suspension. Five hundred millilitre Erlenmeyers flasks with 95 mL of medium were inoculated with 5 mL of the inoculum and then incubated at 30ºC for 94 h on a rotary shaker at 200 rpm. The biomass was separated by filtration through a paper filter (Whatman # 1). The filtrate was stored at 18ºC until assayed. This culture medium supernatant was used as source of extracellular enzymes. The assay batch cultures were run in triplicate **(Martos *et al.*, 2009)**.

Enzyme assays

Qualitative cup-plate assay: Petri dishes were filled with 20 mL of a medium composed by 1 % polygalacturonic acid (PGA, Sigma) and 1.5 % (w/v) agar solution in a 0.2 M sodium acetate buffer (AcB), pH 5.0. Cups 5 mm in diameter were cut and filled with 45 µL of the enzymatic extract. The plates were incubated at 37°C for 24 h. The pectolytic activity was recognized by a clear halo after the medium was flooded with 5 N HCl **(Blanco *et al.*, 1999; Souza *et al.*, 2003)**.

Total pectinolytic (TP) activity: TP activity was considered as a sort of global enzymatic degradation of pectin, in which different enzyme activities were involved (PG, PE, PL) **(Cavalitto *et al.*, 1996)**. TP activity was determined at 37ºC by viscometry. The reduction in viscosity of a 1.5 % citrus pectin (Sigma) solution by the enzymatic extract was followed with a Cannon-Fenske viscosimeter **(Tuttobello and Mill, 1961; Acuña-Argüelles *et al.*, 1995)**.

Polygalacturonase (PG): PG activity was assayed by measuring the reducing groups released from 0.2 % PGA (Sigma) solution in AcB (0.2 M, pH 5.0) by dinitrosalicylic acid method **(Miller, 1959)**. The reaction was carried out at 37ºC, 10 min. A calibration curve was

made using galacturonic acid (GA, Sigma) as standard. One unit of PG was defined as the amount of enzyme which releases 1 μmol of GA per minute.

Xylanase and cellulase: xylanase and cellulase activity were assayed as was PG activity except for the use of xylan (Sigma) and carboxymethylcellulose (Sigma), respectively, as substrates.

Pectinlyase (PL): PL, was assayed by monitoring the increase in absorbance at 235 nm and 37ºC of 0.5 % citrus pectin (Sigma) solution in AcB (0.2 M, pH 5.0), as described by **Albersheim *et al.* (1966)**. One unit of PL activity was defined as the amount of enzyme which produces an increase of one unit of absorbance in the conditions of the assay.

Pectatelyase (PAL): PAL was assayed as was PL activity except for the use of PGA as the substrate.

Pectinesterase (PE): PE activity was determined by color change of a pH indicator (bromocresol green) added to the reaction mixture, due to carboxyl groups being released during the reaction. As substrate it was used 0.5 % citrus pectin (Sigma) in water, pH 5.0 **(Villariño *et al*., 1993).**

Mode of action of PG

The endo or exo mode of action of PG was determined by measuring the formation of reducing groups together with changes in viscosity of a 0.5 % PGA (Sigma) solution in AcB (0.2 M, pH 5.0), at 37ºC. An endo-PG is characterized by a strong reduction in viscosity (e.g. 50 %) with a concomitantly low release of reducing groups (e.g. 1- 3 %) whereas an exo-PG has to hydrolyze greater than 20 % of the glycoside linkages to obtain an equivalent viscosity reduction **(Blanco *et al*., 1994; Schwan *et al*., 1997)**.

For TLC analysis of PGA degradation products, heat inactivated samples were spotted (10 μL) on aluminium sheets (silica gel 60 F254, Merck), and the chromatography performed by using the ascending method with n-butanol:acetic acid:water (9:4:7, v/v/v) as the solvent system. Detection was accomplished by spraying the dried plate with 3 % phosphomolybdic acid dissolved in 10 % sulfuric acid in ethanol followed by heating at 105ºC for 5 min. GA was used as standard **(Contreras Esquivel and Voget, 2004)**.

Effect of pH on polygalacturonase activity and stability

The effect of pH on PG activity was determined by incubating the reaction mixture at pH values rangin from 4.0 to 6.0, under standard enzyme assay conditions.

The pH stability of the enzyme was evaluated by measuring the residual activity, under standard enzyme assay conditions, after incubating the enzyme for 24 h at 4ºC at various pH from 2.5 to 7.5. The buffers employed in these measurements were citrate/phosphate buffer (pH 3.0 and 6.0 - 9.0) and AcB (pH 4.0 - 5.0). All the experiments were conducted in triplicate and the results show the mean values of the activities.

Effect of temperature on polygalacturonase stability

The thermostability of the enzyme was determined by measuring the residual activity, under standard enzyme assay conditions, after incubating the enzyme solution for 60 min at various temperatures from 5ºC to 70ºC, at pH optimum. All the experiments were conducted in triplicate and the results show the mean values of the activities.

Protein analysis by liquid chromatography

The enzymatic extract was freeze-dried, resuspended (10 ×) in AcB (0.2 M, pH 5.0) and centrifuged (6.000 × g, 10 min, 5 ºC). This enzyme solution was desalted by passage through PD-10 column (Amersham Pharmacia Biotech) equilibrated with AcB (0.2 M, pH 5.0). The sample (1 mL) was loaded on an anion exchange column, MonoQ HR 5/5 (Amersham Pharmacia Biotech), coupled to an FPLC system, equilibrated with AcB (0.2 M, pH 5.0). The proteins were eluted with 0-0.5 M NaCl gradient in the same buffer, at a flow rate of 1 mL/min. Fractions of 1 mL were collected and analyzed for enzymatic activity.

Enzymatic clarification of apple juice

Juice extraction process: apple fruits were purchased from a local market, washed with water to remove any adhering substances, peeled, cut into small peaces and then crushed by a blender. The juice was extracted by strainig the pulp through a cheese cloth. The juice obtained was heated at 80ºC for 2 min to inactivate the native enzyme and then it was

centrifugated at 4000 rpm, during 10 min to remove the suspended particles **(Chatterjee *et al.*, 2004)**.

Enzymatic treatment: for clarification process, Erlenmeyer flasks with 100 mL of apple juice, at its natural pH (4.59), and 20 mL of the enzyme preparation were incubated within the range of enzyme stability on a rotary shaker at 150 rpm. Juice with 20 mL of water, instead of enzymatic extract, was used as reference. It was taken 5 mL of fruit juices from each flask at different time intervals up to 30 min. The samples were heated at 90°C for 5 min, to inactivate the enzyme. The juice treated was centrifugated at 4000 rpm for 10 min and the supernatant was collected. The experience was run by triplicate **(Chatterjee *et al.*, 2004)**.

Physical analysis: Viscosity of the juice was measured using a Cannon-Fenske viscosimeter; turbidity using a turbidimeter Model 250 Parsec and clarity by measuring the transmittance at 540 nm, using a Shimadzu UV-Vis spectrophotometer. Results are the average of three replication experiments.

RESULTS AND DISCUSSION

Pectinolytic activity of the enzymatic extract

A. niger was able to produce pectinolytic enzyme by submerged fermentation. The enzymatic extract formed clear halos in qualitative cup-plate assay (figure1).

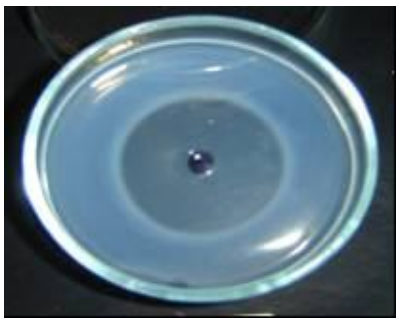

Figure 1 Hydrolysis halos on plates with polygalacturonic acid

The enzymatic extract was able to reduce pectin solution viscosity, reaching ~ 41 % of VR at 30 min (table 1).

Table 1 Viscosity reduction of a pectin solution by the action of pectinases of *A. niger*

Time (min)	VR (%)
5	15 ± 0,438
10	23 ± 0,452
15	29 ± 0,461
20	34 ± 0,596
25	38 ± 0,625
30	41 ± 0,838

VR: viscosity reduction.

Enzyme activities

The enzymatic extract presented PG activity (7.08 UE/mL), PE, lyase activity. Lyase activity was pectinlyase (1.147 UE/mL), no absorption was observed with PGA substrate which indicated that the enzyme concerned was not a pectatolyase.

The enzymatic extract exhibited PE activity, as it was evidenced by the color change (blue to yellow) of the mixture reaction after 4 h of incubation.

Others enzymes capable of degrading cell wall polymers were also detected in the enzymatic extract such as cellulases (0.308 UE/mL) and xylanases (3 UE/mL).

It was reported that fungi, in general, produce a complex mixture of pectic enzymes (PG, PL, PAL; PE) and enzymes that degrade other cell wall polysaccharides such as cellulases and xylanases **(Rogerson *et al.*, 2000; Singh *et al.*, 2002; Hasunuma *et al.*, 2003)**. This enzymatic pool allows to increase the overall yield of the extraction or clarification process in the fruit juice industry **(Tari *et al.*, 2008).**

Mode of action of PG

Figure 2 shows the decrease in viscosity and the increase in reducing groups as a function of time of PGA solution caused by PG from *A. niger*. The products of hydrolysis of PGA by TLC are shown in figure 3.

The crude extract rapidly reduced the viscosity of the PGA solution. The viscosity of the substrate decreased more than 50 % in 10 min when only 9 % of the glycosidic bonds of the substrate were hydrolyzed (figure 2).

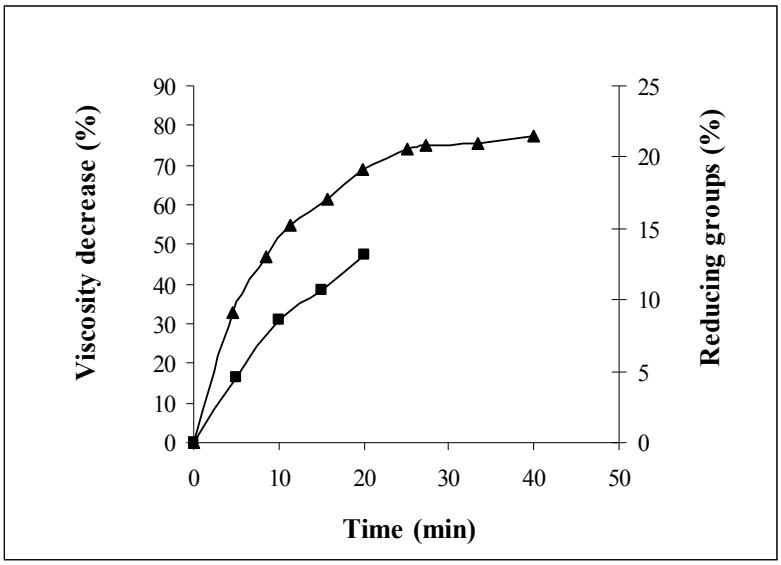

Figure 2 Degradation of polygalacturonic acid with enzymatic extract of *A. niger*. Symbols: (-▲-) viscosity decrease, (-■-) Reducing groups

TLC analysis indicated that the first products were oligomers and the monosaccharide appeared after 30 min of hydrolysis (figure 3).

These behaviors are characteristics of polygalacturonases which act by an endo-splitting mechanism (endo-PG) **(Call *et al*., 1985; García *et al*., 2002)**.

Endo-PGases are widely distributed among fungi, bacteria and yeasts and are also found in higher plants. In contrast, exo-PGases occur less frequently being report mainly in bacteria **(Pedrolli and Carmona, 2010)**.

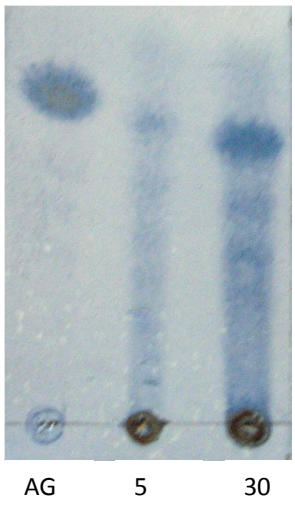

Figure 3 TLC of the degradation products during enzymatic digestion of PGA solution. Numbers below each line indicate the reaction time

Effect of pH on PG activity and stability

Figures 4 and 5 show the pH dependence on the activity and stability of PG produced by *A. niger*, respectively.

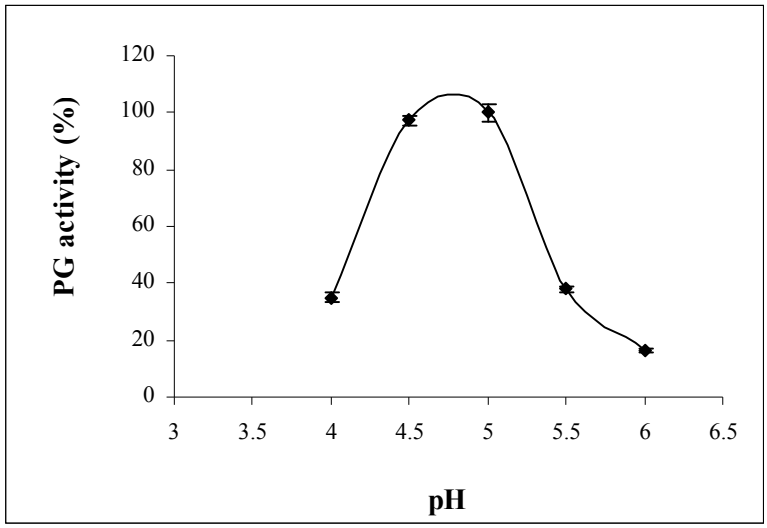

Figure 4 Effect of pH on PG activity from *A. niger*

PG secreted by *A. niger* exhibited maximal activity at a pH range between 4.5 to 5.0. At pH 4.0 and 5.5, PG activity values were 35 % and 38 % respectively (figure 4).

The optimum pH of this enzyme is very close to that reported for PG from *Rhizopus* spp **(Elegado and Fujio, 1993)**, *A. niger* CH4 **(Acuña-Argüelles *et al.*, 1995)**, *Lentinus edodes* **(Zheng and Shetty, 2000)**, *A. awamori* and *A. japonicus* **(Jayani *et al.*, 2005)**. The commercial enzymes Rapidase C80 (Gist-Brocades) and Pectinase CCM (Biocon) exhibited maximal PG activity at pH 4.0, while PG in Pectinex 3 XL (Novozyme) reached a maximum at pH 4.7 **(Ortega *et al.*, 2004)**. The optimum pH for PG activity of *Rhizopus microsporus* var. *rhizopodiformis* was 3.5 **(Damásio *et al.*, 2011)**.

The enzyme was stable at 4ºC for 24 h over a pH range between 2.5 to 5.5. At pH 7.5, the residual activity was 54 % (figure 5).

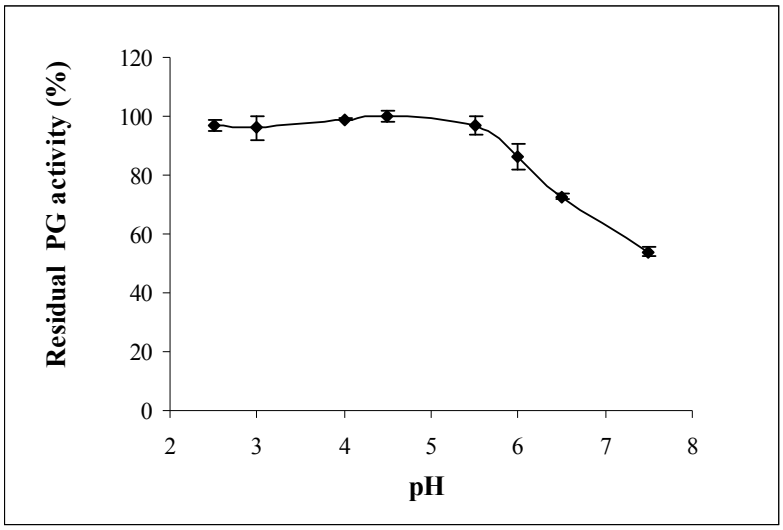

Figure 5 Effect of pH on PG stability from *A. niger*

PG from *A. sojae* ATCC 20235 was stable at pH 5.0 and retained 60 % and 70 % of its activity at pH 3.0 and 7.0 respectively **(Tari *et al*., 2008)**. Fungal PGs are usually stable in acid medium, nevertheless, PG *A. giganteus* proved to be more stable over a neutral and alkaline pH range **(Pedrolli and Carmona, 2010).**

Effect of temperature on PG stability

Studies of PG thermal inactivation are shown in figure 6.

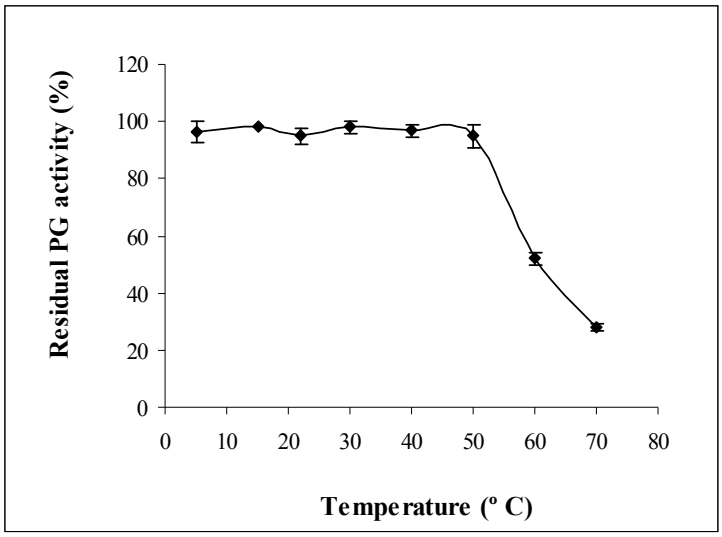

Figure 6 Effect of temperature on PG stability from *A. niger*

PG activity remained unchanged when the enzyme was incubated up to 50ºC during 60 min (pH 5.0). At 60°C the enzyme retained 52 % of its maximum activity (figure 6).

PG activity of *Rhizopus microsporus* var. *rhizopodiformis* was stable up to 55ºC **(Damásio *et al*., 2011)**. An endo-PG of *A. niger*, produced in solid state fermentation, was stable up to 40ºC and presented 60 % of its maximum activity after about 60 min of incubation at 50ºC **(Hendges *et al*., 2011)**.

Protein analysis by liquid chromatography

The chromatography showed that PG of *A. niger* could be separate into at least 3 bands (fractions: 1–3, 10–11 y 17–21) (figure 7). No other enzymatic activities were detected in these fractions. The results obtained leads to the proposition that multiple enzymes of PG (isoenzymes) were concerned.

It was reported that fungi usually produce two or more PG isoenzymes **(Pedrolli and Carmona, 2010)**.

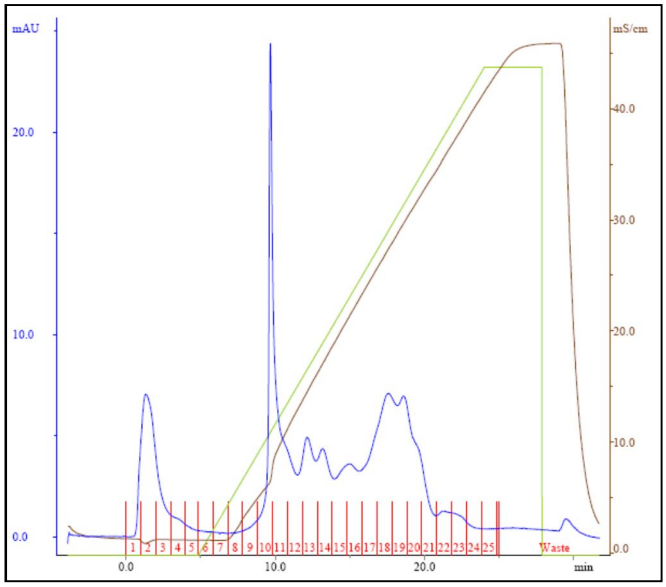

Figure 7 Elution profile of enzymatic extract of *A.niger* obtained in ion exchange chromatography

Enzymatic clarification of apple juice

Figure 8 shows a photograph of apple juice after enzymatic treatment with EE of *A. niger* at 45ºC (within the range of PG stability) and pH 4.59 (pH of apple juice). The effect of

the enzymatic treatment on the viscosity and turbidity of apple juice after 1 h of treatment is presented in table 2, and the transmittance at different time of clarification in figure 9.

Table 2 Effect of enzymatic treatment on different physical parameters of apple juice.

Physical parameters	Natural juice	Clarified apple juice	% Reduction
Viscosity	115 seg	68.6 seg	59.6
Turbidity	280 NTU	5.2 NTU	98.14

NTU: Nephelometric turbidity units

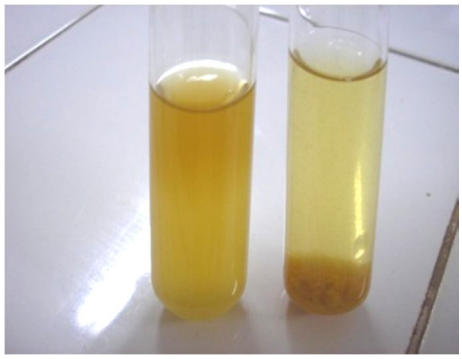

Figure 8 Clarified apple juice with *A. niger* enzymatic extract. Left: negative control

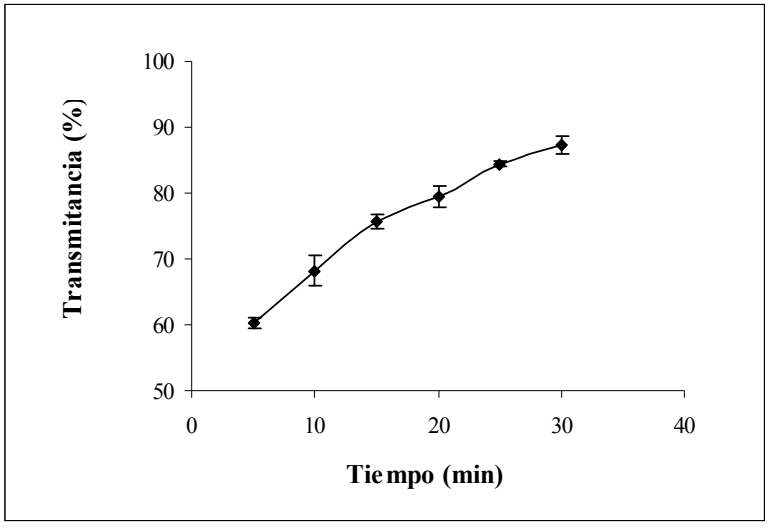

Figure 9 Transmittance of apple juice during clarification process with *A. niger* enzymatic extract

A. niger enzymatic extract was able to clear apple juice (figure 8). The enzymatic extract reduced the viscosity of the apple juice up to 59.6 % and the turbidity up to 98.14 % (table 2).

An increase in the transmittance of the juice was observed after the addition of the enzymatic solution and continued for 30 min (figure 9).

CONCLUSION

The pectinolytic strain of *A. niger*, isolated from citrus peels, was able to produce pectic enzymes during submerged fermentation. The enzymatic extract mainly exhibited PG activity. Others enzymes such as PE, PL, cellulases and xilanases were also detected. Polygalacturonase was an endo-polygalacturonase.

Pectolytic pools with high PG activity contents, as the one reported here, are useful to be used in clarification of fruit juice. Besides *A. niger* enzymatic extract presented high PG activity at a lower pH range, suited to most fruit and vegetable processing applications.

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