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Silva, Jônatas de Carvalho; de França, Pedro Renann Lopes; de Melo, Allan Henrique Felix; Neves-Petersen, Maria Teresa; Converti, Attilio; Souza Porto, Tatiana

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Optimized production of *Aspergillus aculeatus* URM4953 polygalacturonases for pectin hydrolysis in hog plum (*Spondias mombin* L) juice

Jônatas Carvalho Silva[1], Pedro Renann Lopes de França[2], Allan Henrique Felix de Melo[2], Maria Teresa Neves-Petersen[3,4], Attilio Converti[5], Tatiana Souza Porto[1,2]*

[1] Northeast Biotechnology Network, Federal Rural University of Pernambuco - UFRPE, Rua Manuel de Medeiros, s/n - Dois Irmãos, Recife, Pernambuco, Brazil, 52171-900

[2] Federal Rural University of Pernambuco - Academic Unit of Garanhuns, Avenida Bom Pastor, s/n - Boa Vista, Garanhuns, Pernambuco, Brazil, 55292-270.

[3] Department of Clinical Medicine, Aalborg University - AAU, Søndre Skovvej 15, 9000 Aalborg Ø, Denmark.

[4] Department of Health Science and Technology, Aalborg University - AAU, Frederik Bajers vej 7E, 9220 Aalborg, Denmark.

[5] Department of Civil, Chemical and Environmental Engineering, Pole of Chemical Engineering, Genoa University, via Opera Pia 15, Genoa, Italy, 16145

*Corresponding authors: Tel.: +55 81 33206345. E-mail address: portots@yahoo.com.br (T. S. Porto)

Graphical abstract
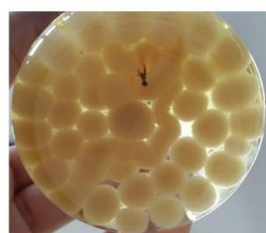
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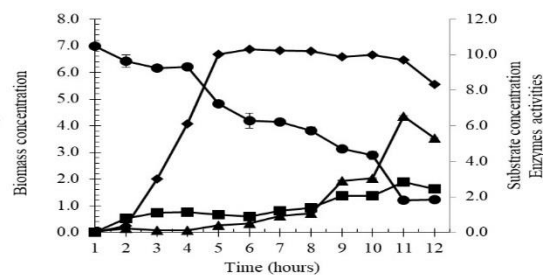
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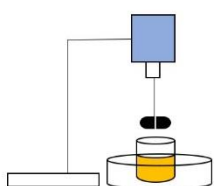
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design for pectin hydrolysis

Hog plum juice processing
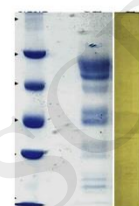
Biochemical characterization,
SDS-PAGE and novel zymography

Highlights

- Production of endo and total polygalacturonases (PGs) by *A. aculeatus* was optimized;

- Fermentation kinetics was studied, and the highest PG activity detected after 96 h;

- Optimized pectin hydrolysis reduced the viscosity of hog plum juice by almost 100%;

- Mathematical models performed by central composite design showed high adjustments;

- A novel zymography method was successful to determine the protein size.

Abstract

Production of total polygalacturonase (PG) and endo-polygalacturonases (endoPG) from *Aspergillus aculeatus* URM4953 was optimized in submerged fermentation using passion fruit peel as substrate for pectin hydrolysis in hog plum juice. The highest activities of PG (2.92±0.12 U/mL) and endoPG (6.51±0.04 U/mL) were obtained using 3% substrate and 0.1% of yeast extract after 96 h. Under these optimized conditions, the maximum specific growth rate of the microorganism was 0.06 h^{-1}, saturation constant 9.9 mg/mL, yield of biomass on consumed substrate 1.44 g/g, yields of PG and endoPG on consumed substrate 0.33 and 0.81 U/mg, and yields of PG and endoPG on biomass 0.45 and 0.95 U/mg, respectively. EndoPG, which is responsible for reduction of fruit juice viscosity, displayed an optimum temperature of 60°C and two optimal pH values under acidic (5.0) and neutral (7.0) conditions. A novel zymogram method showed PG activity in correspondence to a protein band of 31.7 kDa. The enzyme mixture was used for pectin hydrolysis in hog plum juice, which took place optimally at 40°C, achieving a juice yield of 35.3% and reducing fruit viscosity by 96.8% within 88 min. *A. aculeatus* polygalacturonases demonstrating great industrial potential for pectin hydrolysis in fruit juices.

Key-words: Polygalacturonases; Hog plum juice (*Spondias mombin* L); Pectin hydrolysis; Optimization; *Aspergillus aculeatus.*

1. Introduction

Polygalacturonases (PGases) are the most important pectinolytic enzymes, whose great commercial interest is related to their ability to hydrolyze the α-1,4 glycoside bonds of polymeric chain of pectin [1], a polysaccharide component of vegetable cell wall that confers tissue rigidity [2]. In particular, total polygalacturonase activity is the sum of exo-polygalacturonase activity (EC 3.2.1.15), responsible for hydrolysis of glycoside bonds from the non-reducing pectin end chain releasing monomers of galacturonic acid, and endo-polygalacturonase activity (EC 3.2.1.67), which hydrolyzes pectin chain randomly releasing oligo-galacturonic acids, hence reducing pectin solution viscosity [3].

PGases are produced by higher plants [4], insects and protozoa [5], some phyto-parasitic nematodes [6] and microorganisms, i.e., bacteria, yeasts and filamentous fungi [7]. Strains belonging to the *Aspergillus* genus have been widely used for PGases industrial production [8].

Many studies have been reported on the use of submerged (SmF) and solid-state (SSF) fermentations to produce PGases. Although SSF are cheaper [9] and allow for higher enzyme activity [10], about 90% of industrial pectinases are produced by SmF [11] due to easier control of process parameters and scale-up [12].

PGases production requires special attention because it is directly influenced by several factors, i.e., the strain, carbon source, medium composition, cultivation conditions, type of fermentation, pH, temperature, oxygen supply, agitation and incubation time [13,14,15]. Agroindustry wastes such as wheat bran [16], sugarcane bagasse [9], citrus peel [17], apple [18], beet marc [8], have gained much attention for pectinase production in the last years, because they are cheap and rich in organic carbon and nitrogen supplements [19].

PGases, which together with other pectinases account for 25% of the global sales of food enzymes [20], are used mainly to extract and clarify wines and fruit juices [21,22], reduce viscosity [23] and enhance the filtration process and juice yield [24].

Hog plum (*Spondias mombin* L) is a seasonal and perishable fruit from the American continents, which, despite being widely consumed raw, needs being processed as fruit jellies, juices or ice creams to be found during the whole year [25,26].

Based on this background, the present work aimed at optimizing the production of PGases from *Aspergillus aculeatus* URM4953 in submerged fermentation using passion fruit peel as substrate and determining the kinetic parameters of fermentation. The goal was to use these polygalacturonases to carry out and optimize pectin hydrolysis in hog plum juice.

2. Materials and methods

2.1 Microorganism

Thirty-seven *Aspergillus* strains were obtained from the URM culture collection of the Federal University of Pernambuco (UFPE), Brazil, and grown in Czapek medium by incubation at 30ºC for 7 days.

2.2 Screening of fungi by submerged fermentation

Fungal screening for total PG production was performed in submerged fermentation using a medium made up of 10% passion fruit peel (granulometry from 0.5

to 2.0 mm) in a nutrient solution, pH 5.5, consisting of 0.1% yeast extract, 0.7 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4.7H_2O$ and 5.0 mM K_2HPO_4. After sterilization in autoclave, 50 mL of the medium were added in 250-mL Erlenmeyer flasks, inoculated with 10^5 spores/mL of each one of the *Aspergillus* strains. Fermentations were carried out at 30ºC for 72 h and 100-rpm orbital agitation, model TE-424 (Tecnal, Piracicaba, SP, Brazil). The crude extracts were obtained by centrifugation (Sorvall ST16R, Thermo Fisher Scientific, Osterode, Germany) of the extract at 5000 rpm for 15 min at 4°C. Polygalacturonase productions were evaluated using the one-way analysis of variance (ANOVA) and Tukey's test with a confidence level of 95% ($p < 0.05$). *Aspergillus* strains, which statistically (Tukey's test) displayed the highest total PG production, were submitted to mycotoxin test. Aflatoxin production was detected by the method described by Lin and Dianese [27].

2.3 Optimization of polygalacturonases production

Total polygalacturonase and endo-polygalacturonase were produced in submerged fermentation by the *Aspergillus* URM4953 strain selected by the above fungal screening. To prepare the fermentation media as described later, it was used passion fruit peel flour with smaller granulometry than that used for the screening medium (< 0.5 mm) to increase the surface area [28].

Fermentations were carried out according to a central composite design (CCD) where yeast extract (nitrogen source) and substrate (carbon source) concentrations were selected as the independent variables. Suspension of 3.4% (w/v) passion fruit peel flour was prepared in deionized water and autoclaved for 20 min at 121°C to extract the pectin from flour. After extraction, the mixture was filtered to remove suspended solids. The

supernatant was collected, and the pectin material extracted was quantified as described in section 2.5 and diluted to 2.0, 3.5, 7.0, 10.5 and 12.0 mg/mL. Thereafter, salts 0.7 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4.7H_2O$ and 5.0 mM K_2HPO_4 and yeast extract (0, 0.1, 0.2, 0.3 and 0.34%) were added into the pectin solutions. The fermentation medium was finally sterilized in autoclave as described above.

Fermentation medium were inoculated with 10^5 spores/mL. The fermentation was carried out at 30°C and shaking at 130 rpm for 72 h. After this time, the fermented broth was filtered and centrifuged for 5 min at 4000 rpm. The supernatant was called crude enzyme extract and stored frozen (-20°C). Enzyme activities were determined in triplicate. The regression model and individual linear, quadratic, and interaction terms were determined using the Statistic 8 program (StatSoft Inc., Tulsa, OK, USA) with $p < 0.05$ and used to generate the response surface plots and Pareto Charts.

2.4 Pectinase activities

Total polygalacturonase activity was determined by the method of Miller [29]. Briefly, 500 µL of the crude extract incubated with 500 µL of 10 mg/mL citrus pectin in 1.0 M acetate buffer, pH 4.5, at 50°C for 40 min in a water bath. A 100-µL aliquot was removed and added to 1.0 mL of a 0.1 mg/mL dinitrosalicylic acid (DNSA) solution in 0.4 M NaOH and 1.55 M sodium tartrate. The mixture was boiled for 5 min and cooled in an ice bath. Distilled water (5.0 mL) was added, and the mixture homogenized. The absorbance was measured with a UV-Vis spectrophotometer, model SP-1105, (Spectrum, Curitiba, Brazil), at 540 nm. Data were plotted on a standard curve of optical density versus concentration of α-D-galacturonic acid as a reducing sugar. One unit of pectinase activity was defined as the amount of enzyme required to One unit of pectinase activity

was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute.

Endo-polygalacturonase activity was determined incubating 250 µL of the enzymatic extract with 5.5 mL of 25 mg/mL citrus pectin in 1.0 M acetate buffer, pH 4.5. This mixture was incubated at 50ºC for 10 min and then cooled in an ice bath. The viscosity reduction of pectin solution was measured using an Ostwald's viscometer. A viscosimetric unit (U/mL) was defined as the amount of enzyme required to decrease the initial viscosity per min by 50%, under the conditions previously described Marciel et al [30].

2.5 Fermentation kinetics

Kinetics of fermentation carried out under the best conditions pointed out by the CCD described in section 2.3 was investigated along 112 h. Biomass concentration was determined gravimetrically by filtering 50 mL of the fermented medium through a Whatman filter no. 1 and drying at 105°C in oven for 12 h and desiccator for 12 h. Pectin concentration was determined by the method of Mccomb and Mccready [31] with some modification. Briefly, aliquots (50 µL) of the fermented medium were taken at different times and dissolved in 0.05 M NaOH solution for de-esterification and then in 6 mL of concentrated H_2SO_4 previously cooled in ice bath. The reaction was carried out in boiling water bath for 10 min. After that, 500 µL of 1.5 mg/mL carbazole reagent were added, the resulting mixture was stabilized for 30 min at room temperature, and the absorbance measured at 520 nm using the same UV-Vis spectrophotometer described above. Pectin concentration was expressed as reducing sugars (mg/mL) by a calibration curve obtained using galacturonic acid as standard.

Due to the different *A. aculeatus* URM4953 growing phases, the specific rates of biomass growth (μ), substrate consumption (q_s) and enzyme productions (q_p) were plotted as functions of biomass concentration (X) at a given time (t), according to the equations:

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{1}$$

$$q_s = -\frac{1}{X}\frac{dS}{dt} \tag{2}$$

$$q_p = \frac{1}{X}\frac{dP}{dt} \tag{3}$$

where *S* and *P* are the concentrations of substrate (pectin) and of each enzyme (PG or endoPG), respectively.

Kinetic parameters such as the specific growth rate (μ_{max}) and saturation constant (k_s) were determined by the empirical Monod equation using the least square method:

$$\frac{dX}{Xdt} = \frac{\mu_{max}\,[S]}{k_s+[S]} \tag{4}$$

The yield of biomass on substrate ($Y_{X/S}$) was calculated from the curves of μ and q_s during the exponential growth rate using the equation:

$$Y_{X/S} = \frac{\mu}{q_s} \tag{5}$$

while, the yields of biomass on product ($Y_{X/P}$) and of product on substrate ($Y_{P/S}$) were calculated from the curves of μ and q_p and of q_p and q_s up to the achievement of the maximum enzyme activities (96 h) by the equations:

$$Y_{X/P} = \frac{\mu}{q_p} \tag{6}$$

$$Y_{P/S} = \frac{q_p}{q_s} \tag{7}$$

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2.6 Biochemical characterization

In previous study PG from *A. aculeatus* URM4953 was characterized in terms of biochemical features [32] therefore, only the endoPG was biochemically characterized in the present study as described below.

2.6.1 Effect of pH on endo-PG activity and stability

The effect of pH on endoPG activity was investigated at 50°C using 10 mg/mL pectin solutions in different 0.1 M buffers, namely sodium acetate (pH 3.5 − 5.0), sodium citrate (pH 5.5 − 7.0), and Tris-HCl (pH 7.0 − 9.0). For comparison purposes, the endoPG activity, determined as described in section 2.4, was expressed as percentage relative activity with respect to its maximum value. All experiments were performed in triplicate and the results expressed as mean values ± standard deviation. EndoPG stability was determined incubating the enzyme-containing samples in the buffers described above without substrate, determining the enzyme activity at different times (0, 12 and 24 h) and expressing it as percentage residual activity with respect to its initial value.

2.6.2 Effect of temperature on endoPG activity and stability

The influence of temperature on endoPG stability was assessed on aliquots of the enzyme previously submitted to exposition at different temperatures (from 30 to 70 °C) for up to 60 min and determining the residual endoPG activity using a 10 mg/mL pectin solution in 0.1 M sodium acetate buffer, pH 4.5. As for the effect of pH, for comparison

purposes the enzyme activity was expressed as percentage relative activity with respect to its maximum value.

On the other hand, the effect of temperature on the starting activity was investigated in the range 30-70 °C through triplicate experiments carried out using the same pectin solution as above, but without any previous incubation. Once again, for comparison purposes the results were expressed as percentage residual endoPG activity with respect to its initial value.

2.7 SDS-PAGE and zymogram analysis

Proteins from *A. aculeatus* URM4953 crude extract were detected by SDS-PAGE electrophoresis, which was performed using a 12% (v/v) polyacrylamide running gel. Protein weigh molecular markers were 97.0 kDa (phosphorylase b), 66.0 kDa (albumin), 45.0 kDa (ovalbumin), 30.0 kDa (carbonic anhydrase), 20.1 kDa (trypsin inhibitor) and 14.4 kDa (α-lactalbumin). Protein bands were then stained by using a Coomassie Brilliant Blue R-250 solution made up of methanol, acetic acid and water and destained with the same solution without Coomassie.

The total polygalacturonase activity was determined by a novel zymogram method using the same zymogram gel as that used for SDS-PAGE. Crude extract was deionized, lyophilized and resuspended in 10 µL of a sample buffer, which was made up with 10% (v/v) of 10 mg/mL SDS solution, 10% (v/v) of glycerol and 2.5% of 2 mg/mL bromophenol blue solution in a 0.5 M buffer Tris-HCl pH 6.8. The electrophoresis was carried out at a constant current of 300 V at 25°C for approximately 2 h. Afterwards, the gel was incubated for 30 min at room temperature with 2% (v/v) Triton X-100 under orbital shaking at 60 rpm, washed with deionized water several times and incubated for 1

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h at 60 rpm in a 0.5% polygalacturonic acid solution in 50 mM Tris–HCl buffer, pH 8, to incorporate it into the gel and allow for protein renaturation. The gel was then incubated at 50ºC for 40 min in a water bath to determine pectinase activity. Then, the gel was washed with deionized water, let to react with dinitrosalicylic acid solution (section 2.4), boiled for 5 min and cooled in an ice bath. The gel was washed with deionized water till the bands became visible.

2.8. Substrate specificity of total PG

Substrate specificity of pectinases from *A. aculeatus* URM4953 was studied by incubating the pectinolytic extract at 50°C, pH 4.5, for 40 min with different substrates at 10 mg/mL concentration, namely polygalacturonic acid, citrus pectin, xylan and carboxymethylcellulose as described in section 2.4.

2.9 Pectin hydrolysis of hog plum juice

Hog plum juice was obtained processing the fruits in a commercial fruit pulper, model DES-60 (Braesi, Caxias do Sul, RS, Brazil), followed by sieving to remove the remaining residues. Hog plum juice was stored at -20ºC until use. After mixing 1.0 U of crude extract per mL of hog plum juice, pectin hydrolysis was performed according to a preliminary 2^2-factorial design repeating three times the central point, where the hydrolysis time (20, 40 and 60 min) and temperature (40, 50 and 60°C) were selected as the independent variables and the percentage reduction of viscosity (RV), total soluble solids (TSS), pH and juice yield (Y_J) as the responses. To this purpose, RV (%) was determined from absolute viscosity data collected by an Ostwald viscometer, TSS level

(ºBrix) using a refractometer (RHB32, AKSO, São Leopoldo – RS, Brazil), pH using a digital pH meter (Tec5, Tecnal, Piracicaba, SP, Brazil), and Y (%) by difference of final and initial free volumes of processed and fresh juice, respectively. Aliquots of hydrolyzed juice were centrifuged for 10 min at 4000 rpm to separate the aqueous phase from the portion of undegraded pulp.

Such a preliminary factorial design allowed identifying RV and Y_J as the only statistically significant responses and 60 min and 40°C as the best hydrolysis time and temperature, therefore these conditions were set as central point of an additional central composite design (CCD) with the aim of optimizing pectin hydrolysis. For this purpose, the hydrolysis time was varied from 32 and 88 min and temperature from 25 and 54°C. The regression model and individual linear, quadratic, and interaction terms were determined using the Statistic 8 program (StatSoft Inc., Tulsa, OK, USA) with $p < 0.05$ and used to generate the corresponding response surface plots and Pareto Charts.

3. Results and discussion

3.1 Screening of Aspergillus strains with polygalacturonase activity

Fungal screening is the first step to select industrial microorganisms with high enzyme activities [30]. Of the 36 *Aspergillus* strains with potential total polygalacturonase activity available in the URM collection of UFPE institution, 27 sporulated after 7 days of incubation (Table 1). The ANOVA analysis followed by the Tukey's test evidenced statistically significant differences in their ability to produce PG; in particular, three of them, belonging to *Aspergillus niger* (URM5741 and URM5838)

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and *Aspergillus aculeatus* (URM4953) species, stood out showing the highest PG activities (3.8-4.06 U/mL) (Table 1).

When these three strains were subjected to mycotoxin qualitative test, upon exciting the agar-coconut medium at 366 nm (data not shown), only *A. niger* URM5838 showed an intense fluorescent halo, which is characteristic of the intense aflatoxin fluorescence emission [33] associated to aromatic groups [34,35].

Although *A. niger* enzymes are generally recognized as safe by the United States Food and Drug Administration [36] and *A. niger* URM5741 was negative to the qualitative mycotoxin analysis, it has been reported that some *A. niger* strains, under stress conditions, can produce carcinogenic mycotoxins such as ochratoxin A and aflatoxin [37]. *A. aculeatus* URM4953 was then selected as PG producer in this study not only because it displayed an PG activity statistically coincident with that of *A. niger* URM5741 and URM5838 ($p > 0.05$), but also because there is no report in the literature about toxin production by the species to which it belongs and currently its pectinases are marketed all over the world.

3.2 Optimization of polygalacturonases production

Table 2 lists the activities of total polygalacturonase (PG) and endo-polygalacturonase (endoPG) produced by *A. aculeatus* URM4953 in submerged fermentations carried out according to the Central Composite Design (CCD) described in section 2.3. The best results were obtained, as a whole, using concentrations of passion fruit peel flour (substrate) and yeast extract (nitrogen source) of 3.0% and 0.1%, respectively, conditions around which the response surfaces pointed out optimum regions for both total PG (Fig. 1A) and endoPG (Fig. 1C) activities.

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The statistical analysis revealed that substrate concentration was the independent variable that most significantly influenced PG production (Pareto Chart of Fig. 1B). Its positive linear effect means that an increase in substrate concentration until 3.0% led to a progressive activity increase, while the negative quadratic one that this response decreased beyond that concentration threshold. The same trend was qualitatively observed for yeast extract concentration, whose increase till 0.2% improved PG activity, but beyond this value it exerted a strong negative quadratic effect on such a response. However, interaction of the two independent variables (1Lx2L) was significantly negative, thereby configuring an antagonistic effect [38]. Resuming, PG production was enhanced by increasing the level of variable with the stronger positive effect, i.e. substrate concentration, while decreasing that of the less-influencing or even negatively-influencing one, i.e. yeast extract concentration [39].

For endoPG, the statistical analysis showed a qualitatively similar behavior to that of PG, except for the statistical insignificance ($p > 0.05$) of the linear effect of yeast extract concentration and the interaction one (Fig 1D).

The optimal conditions for PG (A_{PG}) and endoPG (A_{endoPG}) activities were then predicted by the second order polynomial equations:

$$A_{PG} = -0.537 + 1.651\ x - 0.291\ x^2 + 6.628\ y - 11.981\ y^2 - 0.760\ xy \qquad (8)$$

$$A_{endoPG} = -6.133 + 6.664\ x - 1.059\ x^2 + 33.182\ y - 73.421\ y^2 - 3.533\ xy \qquad (9)$$

where x and y are the coded levels of substrate and yeast extract concentrations, respectively. The values of the determination coefficient (R^2) were very high (0.994 and 0.962, respectively), and the lack of fit value was not statistically significant ($p > 0.05$), evidencing good adjustment.

3.3 Fermentation kinetics

Fig. 2 illustrates the time behaviors of biomass and substrate (pectin) concentrations as well as PG and endoPG activities during *A. aculeatus* URM4953 fermentation carried out under the optimum conditions pointed out by the CCD. After a 12-h lag phase, the microorganism grew exponentially up to 38 h, with maximum specific growth rate (μ_{max}) of $0.06\ h^{-1}$ and saturation constant of 9.93 mg/mL, kept in the stationary phase up to 96 h and then suffered the typical decline phase owing to substrate starvation [40]. Malvessi and Silveira [41] observed higher μ_{max} (0.15-$0.30\ h^{-1}$) for pectinase-producing *Aspergillus oryzae* CCT3940 in wheat bran medium at different pH values.

Pectin concentration progressively decreased from 10.5 to 1.8 mg/mL, corresponding to a yield of biomass on consumed substrate ($Y_{X/S}$) of 1.44 g_X/g_S. Reginatto et al. [42] observed lower $Y_{X/S}$ values (0.326-$0.989\ g_X/g_S$) for pectinase-producing *A. niger* LB-02-SF at different levels of glucose, ammonium sulfate and wheat meal extract in the medium.

Meanwhile, PG activity increased progressively, achieved a maximum value of 2.92 ± 0.12 U/mL after 96 h and then decreased concomitantly with the biomass concentration decay. The average yields of PG on consumed substrate ($Y_{P/S}$) and biomass ($Y_{P/X}$) were 0.33 U/mg_S and 0.45 U/mg_X, respectively. EndoPG activity also increased till 96 h achieving a maximum value of 6.51 ± 0.04 U/mL and then decreased, corresponding to $Y_{P/S}$ and $Y_{P/X}$ of 0.81 U/mg_S and 0.95 U/mg_X, respectively.

3.5 Pectinases characterization

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Enzyme biochemical characterization is of paramount importance in industrial processes because provide information on how to maintain their activity as longer as possible. In particular, pectinase characteristics depend on several factors, mainly the fungal species and the carbon source [4].

A. aculeatus URM4953 total PG was previously characterized by Silva et al. [43], in terms of optimum hydrolysis temperature (50**°**C), stability (90% of residual activity after 60 min at 30-40**°**C) and optimal pH under acidic (4.0) and neutral (7.0) conditions.

The results of endoPG biochemical characterization are summarized in Fig 3. The activity profile versus temperature shows that endoPG acts optimally at 60**°**C using citrus pectin as a substrate and is quite stable in the range 30-40**°**C, with more than 90% of activity retained after 60-min incubation (Fig 3A). EndoPG optimum temperature usually ranges from 30 to 55ºC [44]; to give only a few examples, Maciel et al. [30] and Yuan et al. [45] reported optimum temperature of 40ºC for endoPG from *A. niger* URM4645 and *Penicillium* sp. CGMCC 1669, respectively.

Likewise, endoPG showed two optimum pH values, the one under acidic (pH 5.0) and the other under neutral conditions (pH 7.0) (Fig. 3B), likely due to the occurrence of different endoPG isoforms, like the highly homologous ones secreted by *A. niger* (endoPG I, II and A, B, C, D and E) [46]. Consistently with the literature [44], endoPG was stable in a wide pH range (3.0-6.0), yielding more than 100% of residual activity after 24 h (Fig 3C). It has been reported that such a hyperactivation under acidic conditions may be the result of structural conformational changes due to the influence of medium dielectric constant on the polarization of enzyme molecules [47]. Indeed, interacting with enzyme molecules, polar solvent molecules generate pronounced dipoles on their surfaces, hence active sites may become more solvent exposed to react [48].

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Different substrates were also used to test the total PG activity, namely galacturonic acids, citrus pectin, xylan and carboxymethylcellulose (Fig 3D). One can see that citrus pectin was the best substrate for the activity of *A. aculeatus* URM4953 pectinase, thus confirming literature data [44].

SDS-PAGE revealed bands corresponding to crude extract proteins with molecular weight from 15.2 to 59.9 kDa, while the zymogram showed total PG activity in correspondence to 31.7 kDa (Fig. 4). Polygalacturonase zymograms are generally revealed with a solution of ruthenium red [44], a chemical compound that reacts with acidic substances. Since this reagent is expensive and potentially carcinogenic, we propose in this study a new alternative to reveal bands with pectinolytic activity using DNSA, i.e. the same reagent used to determine total polygalacturonase activity. Such a new method was able to correlate the activity band revealed by zymography with the protein one revealed by SDS-PAGE.

Ahmed et al. [17] reported an almost coincident molecular weight (30 kDa) for a *A. niger* pectinase, while other authors found polygalacturonase molecular weights close to that of this study, i.e. 34 kDa [49], 37 kDa [45], 42 kDa [50], and 45 kDa [51].

3.5 Pectin hydrolysis in hog plum juice

EndoPGs are pectinases that hydrolyze glycosidic bonds randomly, releasing compounds with short molecular structures; therefore, they are of special interest for food industries. Owing to the consequent reduction in viscosity, less energy is required for juice transport through the pipes, thus reducing the process costs, shortening the filtration time and increasing the yield of free juices [15].

Aiming these benefits, pectin hydrolysis in hog plum juice was performed according to a 2^2-factorial design where the hydrolysis time and temperature were selected as the independent variables and the viscosity reduction (*VR*), levels of reducing sugars (RS) and total soluble solids (TSS), pH and yield of free juice (*Y*$_J$) as the responses (Table 3). Whereas variation of RS, pH and TSS were not statistically significant ($p > 0.05$), those of *VR* and *Y*$_J$ were ($p < 0.05$), with the highest values of these responses [96.4% and 35.3% (v/v), respectively] being observed at 40°C after 60-min hydrolysis time.

Figs. 5A and C illustrate the simultaneous effects of temperature and hydrolysis time on viscosity reduction and juice yield, while the Pareto Charts of Figs. 5B and D allow identifying the statistically significant effects. It is noteworthy that the hydrolysis time exerted a strong positive effect on *VR*, while that of temperature was not statistically significant. On the other hand, their interaction led to an antagonist effect, i.e. *VR* may be enhanced by simultaneously decreasing the less influencing variable (temperature) and increasing the most influencing one (time). Despite these variables showed the same effect values, a similar trend was observed for statistical analysis of juice yield (%) (Fig 5D). The time exerted the strongest positive effect on the yield, followed by its negative interaction with temperature, which was confirmed by the negative effect of temperature.

Pectin hydrolysis was then optimized through a set of runs carried out according to an additional CCD where the conditions of independent variables previously ensured the highest *RV* and *Y* values (40°C and 60 min) were selected as the central point. The results of pectin hydrolysis in hog plum juice listed in Table 4 show that the run performed at 40°C for 88 min ensured be the best performance, corresponding to the optimum region in the response surfaces (Figs. 6A and C). Even though under these conditions *VR* achieved a maximum value of 96.8%, it has been reported that long-term exposition of

fruit juices to high temperatures may confer undesirable cooked taste and cause degradation of several thermolabile bioactive compounds such as vitamins, sugars, amino acids, ascorbic acid, carotenoids, phenolic acids, flavonoids and tannins [52]. Therefore, we selected the central point conditions as the optimal ones, for they ensured almost the same mean *VR* (96.2%) and Y_J (35.5% v/v) values in shorter time (60 min). The modeling of hog plum juice processing showed high adjustment for *VR* ($R^2 = 0.96$) and Y_J ($R^2 = 0.88$), and these responses were described by the following second order polynomial equations:

$$VR = 3.528 + 0.398 \; x - 0.00223 \; x^2 + 3.668 \; y - 0.0409 \; y^2 - 0.00181 \; xy \qquad (10)$$

$$Y_J = -211.746 + 1.174 \; x - 0.0124 \; x^2 + 10.274 \; y - 0.135*y^2 + 0.0104 \; xy \qquad (11)$$

where *x* and *y* are the coded levels of time and temperature, respectively.

The statistical analysis of linear, quadratic and interaction terms is depicted in the Pareto Charts of Figs. 6B and D. Temperature was the variable that more significantly influenced *VR* exerting a positive linear effect up to 40°C (Table 4). However, beyond this temperature, this response was not favored even for short exposure times due to the prevalence of the strong negative quadratic effect. Such a behavior can be related to polygalacturonase thermosensibility that reduced the endoPG activity [53]. The time exerted a positive linear effect on *VR*, which, however, was overcome by the its strong negative quadratic effect for hydrolysis time longer than 60 min, while the interaction term was not statistically significant (Fig. 6B).

Even though the hydrolysis time linearly enhanced Y_J (Fig 4D), its quadratic negative effect was stronger, suggesting that the prolonged exposure to high temperature

led to endoPG denaturation and, as a result, the increase in viscosity induced by gelatinous structure hindered juice formation by pressing and reduced the yield [54].

4. Conclusions

Aspergillus aculeatus URM4953 was chosen among thirty-seven *Aspergillus* strains to produce polygalacturonases in submerged fermentation using passion fruit peel as carbon source and yeast extract as the nitrogen one. Optimum conditions to produce total PG and endoPG were concentrations of these ingredients of 3.0 and 0.1%, respectively. Fermentation carried out for 112 h under these conditions showed the highest total PG and endoPG activities after 96 h. EndoPG, which was characterized biochemically, showed hyperactivity under acidic conditions and high stability after 24 h. Citrus pectin was shown to be the proper substrate to determine total PG activity. A novel zymogram method allowed correlating the activity band with the protein one revealed by SDS-PAGE. Pectin hydrolysis was optimized and reduced the viscosity of hog plum juice in almost 100%. The results of this work give an idea of the great potential of *A. aculeatus* URM4953 PGs for possible industrial applications such as pectin degradation in fruit juices. Once such pectinases promoted high reduction of the viscosity and yield without change the physical-chemical parameters of the juice.

5. Acknowledgements

6. References

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Figures Captions

Fig 1. Statistical analysis of optimized productions total polygalacturonase (PG) and endo-polygalacturonase (endo-PG) from *Aspergillus aculeatus* URM4953 in 72-h submerged fermentations using passion fruit peel as a substrate. Fitted Surfaces of total PG (A) and endo-PG (C) activities. Pareto Charts of the effects of the independent variables on total PG (B) and endo-PG (D) activities.
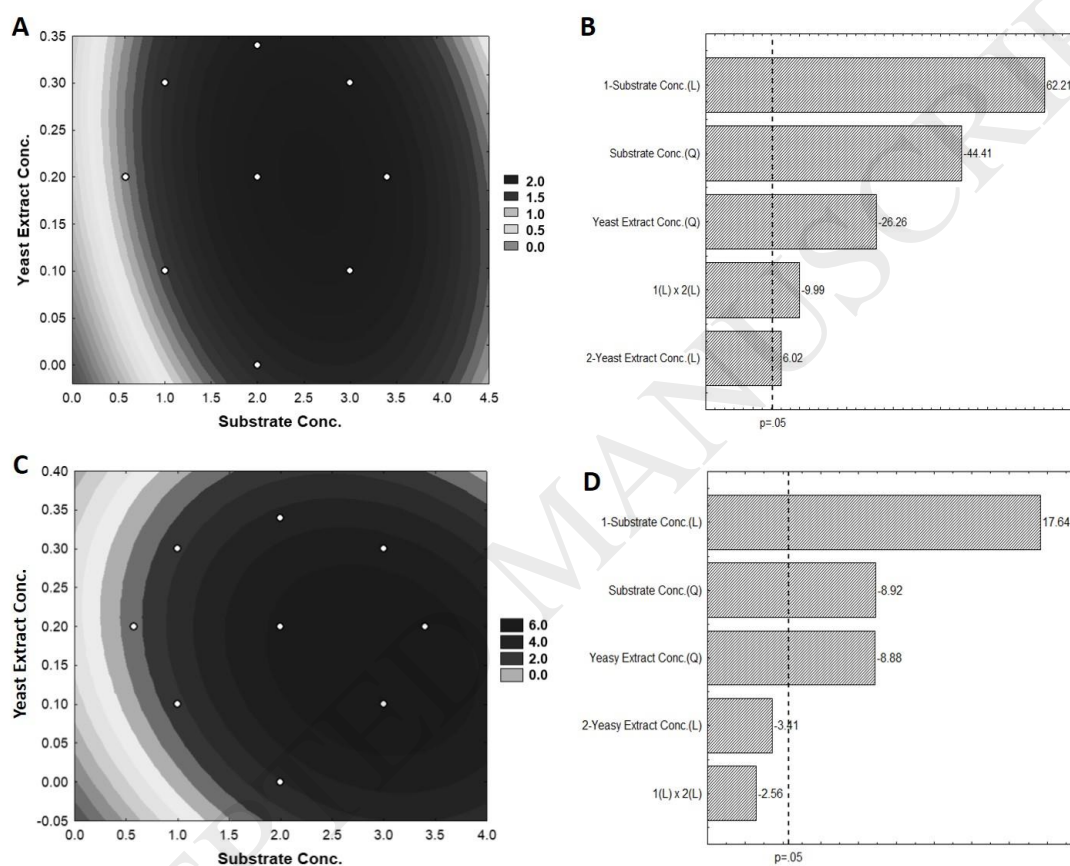
Fig 2. Time behaviors of biomass concentration (♦), pectin (substrate) concentration (●), total polygalacturonase activity (■) and endo-polygalacturonase activity (▲) during submerged fermentation by *Aspergillus aculeatus* URM4953 at 30°C and pH 4.5 using 3.0% (w/v) passion fruit peel as substrate and 0.1% (w/v) yeast extract as nitrogen source.
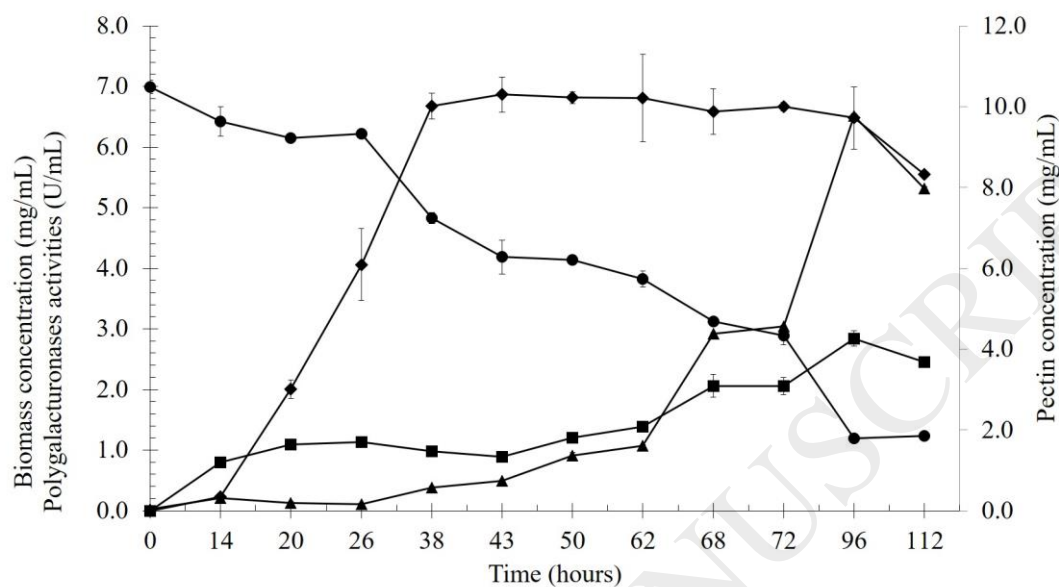

Fig 3. Biochemical characterization of novel endo-polygalacturonase (Endo-PG) from *Aspergillus aculeatus* URM4953. (A) Activity profile and stability, (B) pH profile in different buffers, and pH stability as function of the time, (D) activity on different substrates.
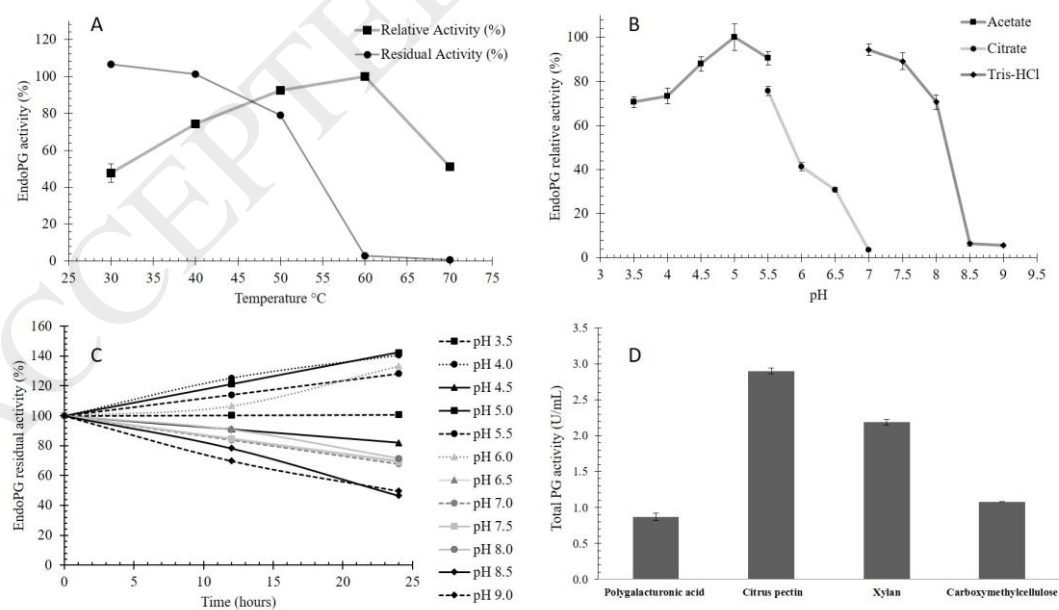
Fig 4. SDS-PAGE and polygalacturonic acid zymography analysis. MM - molecular weight markers, CE – crude extract and Z – polygalacturonase zymogram on electrophoresis.
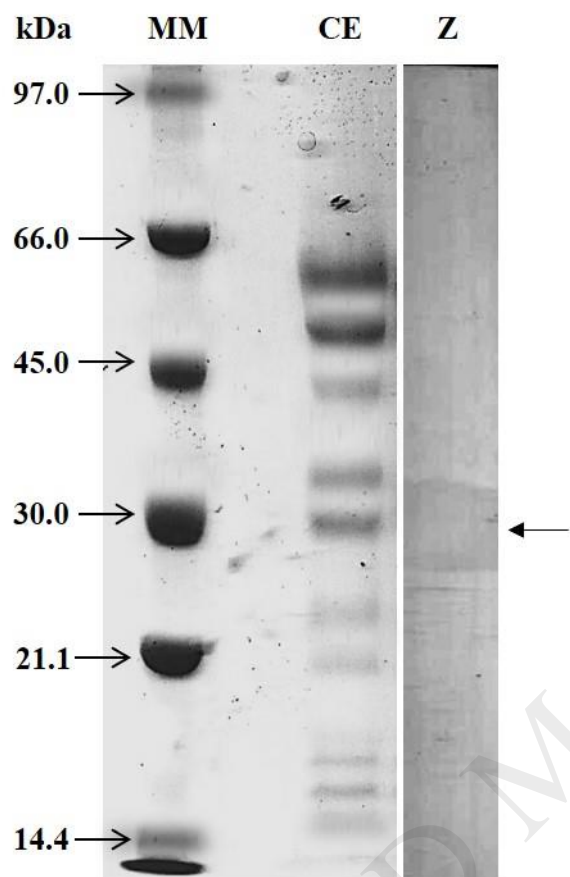
Fig 5. Statistical analysis of pectin hydrolysis in hog plum (*Spondias mombin* L) juice by polygalacturonases from *Aspergillus aculeatus* URM4953 performed according to the 2^2-factorial design. Pareto Charts of the effects of the independent variables on viscosity reduction (A) and juice yield (B).
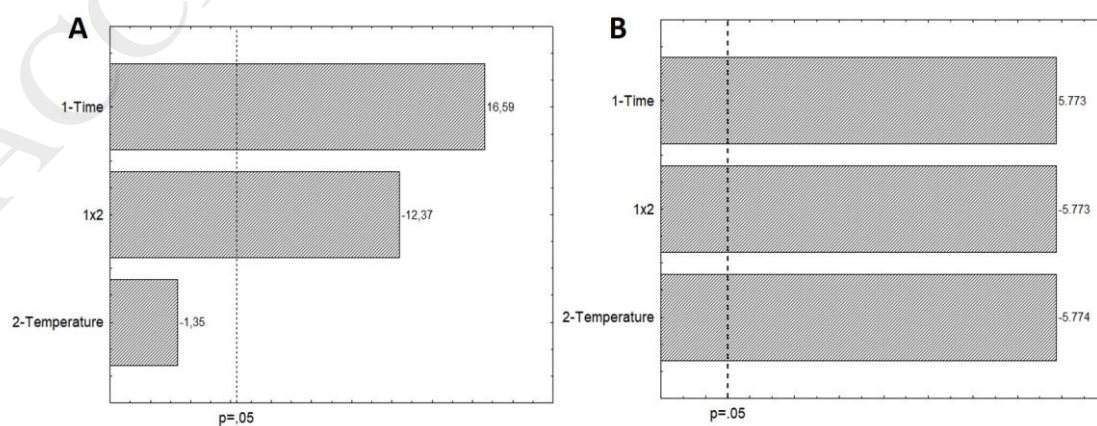
Fig 6. Statistical analysis of optimized pectin hydrolysis in hog plum juice performed according to the Central Composite Design. Fitted Surfaces of viscosity reduction (A) and juice yield (C) function of time and temperature. Pareto Charts of the effects of the independent variables (hydrolysis time and temperature) on viscosity reduction (B) and juice yield (D).
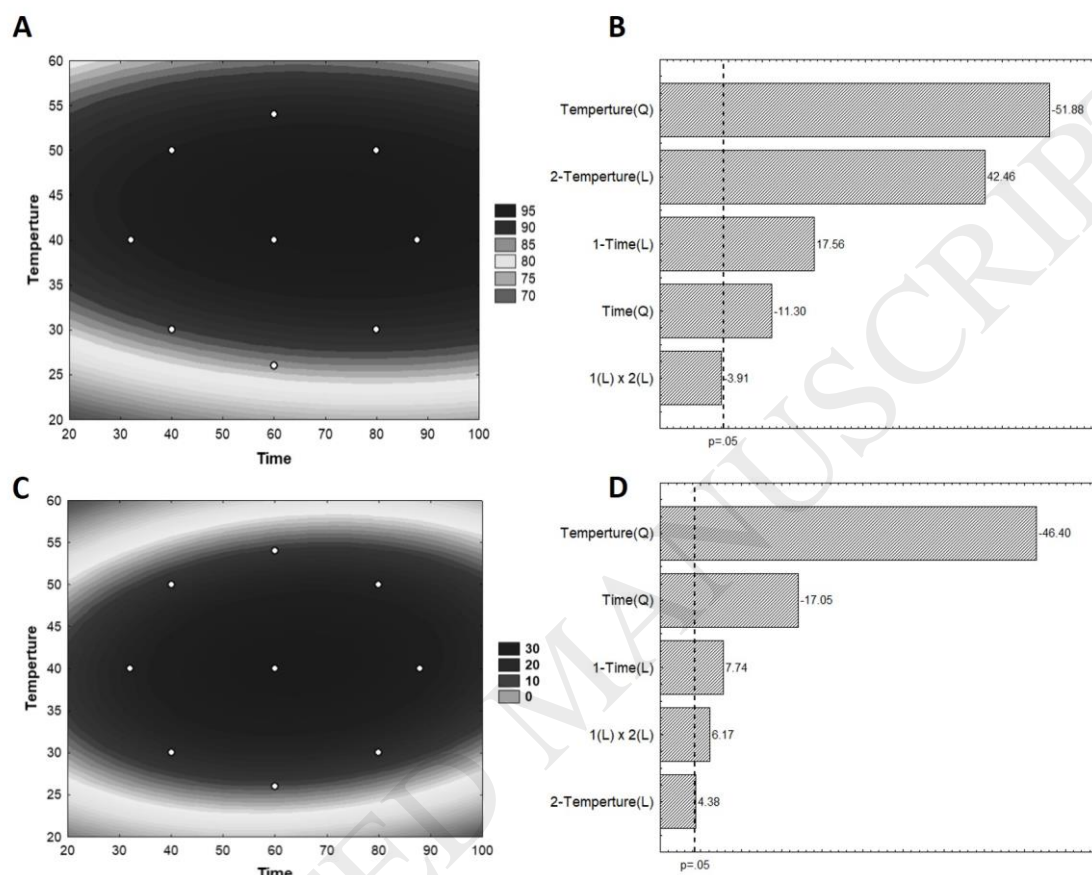
Table 1. Screening of *Aspergillus* strains with total polygalacturonase (PG) activity in submerged fermentation using passion fruit peel as a substrate.

Aspergillus strains	PG activity (U/mL)
A. niger URM5741	4.06±0.06[a]
A. niger URM5838	3.94±0.03[a]
A. aculeatus URM4953	3.87±0.17[a]
A. tamarii URM4634	3.45±0.07[b]
A. niger URM5756	3.44±0.01 [b]
A. carbonarius URM1546	3.44±0.00 [b]
A. parasiticus URM5778	3.40±0.02 [b]
A. terreus URM5864	3.13±0.00 [c]
A. niger URM3856	3.12±0.16 [c]
A. phoenicis URM4924	3.04±0.24 [c]
A. heteromorphus URM269	2.90±0.08 [c]
A. flavus URM5791	2.89±0.01 [c]
A. niger URM5863	2.74±0.16 [c]
A. japonicus URM3916	2.72±0.01 [c]
A. niger URM5218	2.65±0.01 [c]
A. flavus URM5794	2.60±0.09 [c]
A. japonicus URM5620	2.53±0.04 [d]
A. flavus URM5740	2.45±0.01 [d]
A. japonicus URM5242	2.29±0.01[e]
A. niveus URM5870	2.18±0.07[e]
A. niger URM5837	2.00±0.04[f]
A. carbonarius URM5182	1.99±0.03[f]
A. parasiticus URM5787	1.75±0.01[g]
A. sydowii URM5774	1.70±0.03[g]
A. carbonarius URM3818	1.48±0.05 [g]
A. tamarii URM3266	1.47±0.11 [g]
A. scherotiorum URM5792	1.45±0.07[h]

Different superscript letters mean significant statistical difference between activities ($p <$ 0.05) according to the Tukey's test.

Table 2. Production of polygalacturonases by *Aspergillus aculeatus* URM4953 in submerged fermentations* carried out according to the Central Composite Design described in section 2.3, using passion fruit peel as a substrate.

Substrate concentration (% w/v)	Yeast extract concentration (% w/v)	Total PG Activity (U/mL)	Endo-PG Activity (U/mL)
1.0[b]	0.30	1.50±0.06	1.54±0.06
1.0 [b]	0.10	1.29±0.01	1.53±0.12
3.0[d]	0.30	2.06±0.06	4.97±0.03
3.0[d]	0.10	2.16±0.01	6.38±0.12
2.0[c]	0.20	2.12±0.01	4.90±0.05
2.0 [c]	0.20	2.14±0.04	5.42±0.09
2.0 [c]	0.20	2.15±0.04	5.32±0.05
2.0 [c]	0.34	1.94±0.01	3.26±0.13
2.0 [c]	0.00	1.58±0.00	2.84±0.24
3.4[e]	0.20	1.98±0.03	4.91±0.16
0.6[a]	0.20	1.01±0.01	1.00±0.01

*Fermentations medium with initial pH 4.56, shaken at 130 rpm, at 30°C for 72 h.

Pectin concentrations extracted from their respective substrate concentrations: [a] 2.0; [b] 3.5;

[c] 7.0; [d] 10.5 and [e] 12.0 mg/mL

Table 3. Pectin hydrolysis in hog plum juice by polygalacturonases from *Aspergillus aculeatus* URM495.

Time (min)	Temperature (ºC)	*VR* (%)[a]	Y_J (% v/v)[b]	RS (mg/mL)[c]	TSS (ºBrix)[d]	pH
20	40	77.8±0.1	23.5±0.2	18.3±0.1	9.0±0.1	3.7±0.2
60	40	96.4±0.3	35.3±0.2	19.9±0.1	10.1±0.2	3.9±0.1
20	60	84.9±0.2	23.5±0.2	19.2±0.2	9.4±0.1	3.7±0.2
60	60	87.6±0.4	23.5±0.2	19.4±0.3	9.4±0.1	3.6±0.2
40	50	92.9±0.1	29.4±0.2	19.4±0.3	9.9±0.2	3.8±0.1
40	50	94.2±0.1	28.4±0.2	18.4±0.2	9.4±0.1	3.8±0.1
40	50	93.8±0.2	29.4±0.2	19.3±0.2	9.4±0.1	3.8±0.1

[a]Viscosity reduction.

[b]Yield of free juice.

[c]Reducing sugar concentration.

[d]Total soluble solid level.

Table 4. Pectin hydrolysis in hog plum juice by polygalacturonases from *Aspergillus aculeatus* URM4953 carried out according to the Central Composite Design described in section 2.9.

Essay	Time Levels	Temperature Levels	Time (min)	Temperature (ºC)	VR (%)[a]	Y_J (% v/v)[b]
1	-1	-1	40	30	86.3±0.4	18.7±0.2
2	-1	1	40	50	94.0±0.1	18.7±0.2
3	1	-1	80	30	88.6±0.2	10.3±0.2
4	1	1	80	50	94.9±0.6	18.7±0.2
5	- α	0	32	40	92.5±0.1	18.7±0.2
6	+ α	0	88	40	96.8±0.1	35.3±0.4
7	0	- α	60	26	85.5±0.2	10.3±0.2
8	0	+ α	60	54	91.2±0.3	10.3±0.2
9	0	0	60	40	96.1±0.5	35.6±0.3
10	0	0	60	40	96.4±0.2	36.6±0.2
11	0	0	60	40	96.0±0.7	35.3±0.3

[a]Viscosity reduction.

[b]Yield of free juice.