



Synthesis and potential application of polygalacturonase from a *Penicillium brasilianum* isolate

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ABSTRACT. The aim of this study was to evaluate the synthesis of pectinase from *Penicillium brasilianum* in shake flasks and address their potential for industrial applications. A Plackett-Burman design followed by a complete second order design were used for the screening of most important factors and to maximize the polygalacturonase activity, respectively. Maximum polygalacturonase activity was 52.8 U mL⁻¹ at 48 hours of bioproduction. The kinetic evaluation for substrate consumption showed that 42% total organic carbon, 52 nitrogen, 23 magnesium, and 60% potassium were consumed. The crude enzyme complex was used on commercial mango juice clarification, and, at a 0.5% concentration (v v⁻¹) reduced viscosity by 10%, turbidity by 12% and clarification by 23%. Therefore, the results presented in this study could provide valuable and beneficial information for the food and enzyme industries (juice) as well as being a new landmark to microbiology by providing essential knowledge on *P. brasilianum* growing needs.

Keywords: microorganism, pectinases, industrial application.

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Introduction

Microbial pectinases have tremendous potential to offer mankind. Fungal pectinases are among the most important industrial enzymes and are of great significance with a wide range of application such as textile processing, plant blast fibers degumming, pectic wastewater treatment, papermaking, coffee and tea fermentations, juice and wine processing, improving liquefaction, clarification and filterability and greater color and flavor compounds release entrapped in the grape skin, thereby contributing to wine bouquet and easing phenolic compounds release (Esawy, Gamal, Kamel, Ismail, & Abdel-Fattah, 2013; Sandri, Piemolini-Barreto, Fontana, & Silveira, 2014; Rehman, Aman, Nawaz, & Qader, 2015; Pitol, Biz, Mallmann, Krieger, & Mitchell, 2016).

Usually, commercial pectinase preparations contain one or more types of microbial pectinolytic enzymes (depending on the specific use), as well as cellulases, hemicellulases, proteases, and amylases (Esawy et al., 2013). Molds such as *Aspergillus niger*, *Coniotryrium diplodiela*, *Penicillium* and *Rhizopus* species are preferred for industrial purposes since as much as 90% of the enzyme may be excreted into the culture medium (Souza, Silva, Maia, & Teixeira, 2003; Gomes et al., 2011; Pili et al., 2018).

Pectinase production by filamentous fungi varies according to the type of strain, cultivation conditions (pH, temperature, aeration, stirring rate, and incubation time), and the growth medium composition (especially carbon and nitrogen sources). Thus, they have to be specified for each and every single strain of interest (Martinez-Trujillo, Arreguin-Rangel, Garcia-Rivero, & Aguilar-Osorio, 2011; Meneghel, Reis, Reginatto, Malvessi, & Silveira, 2014).

A number of authors have studied extracellular pectinases production from *Aspergillus* sp. using pectic substrates, however, just a few studies using *Penicillium* sp. by submerged fermentation are to be found in the literature (Jayani, Saxena, & Gupta, 2005; Sandri, Fontana, & Silveira, 2015). In previous work by our research group (Stirling, 2003), a number of microorganisms capable of producing polygalacturonase were isolated from different sources. The relevance of this study lays in establishing the optimum conditions for maximum polygalacturonase production followed by kinetic evaluation of substrate consumption, pH and biomass progress from a newly isolated *Penicillium brasilianum* strain by submerged fermentation in a

synthetic medium. Therefore, this study aimed at providing valuable and beneficial information for the food and enzyme industries, as well as being a new landmark for microbiology providing essential knowledge on *P. brasilianum* growing needs, which has been currently lacking in the literature.

Material and methods

Microorganism

The microorganism used in the present study was isolated from tea and previously identified by microcultivation technique as belonging to the *Penicillium* genera (Zeni et al., 2011).

The newly isolated microorganism was identified following the molecular biology method. Fungi genomic DNA extraction was performed using liquid nitrogen for cell disruption (Tanner & Brunner, 1985) following DNA quantification using a NanoDrop 1000 model spectrophotometer (NanoDrop Technologies).

The regions ITS1, 5.8S and ITS2 of fungal rDNA were amplified using primers ITS1 and ITS4 (White, Bruns, Lee, & Taylor, 1990). The reactions were performed using a GeneAmp PCR System 9700 model thermocycler (Applied Biosystems) using the following conditions; 94° C for 5 min., followed by 33 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s; and a final extension of 72°C for 10 min. The product was purified with GFX™ PCR DNA Kit and Gel Band Purification (GE Healthcare) and sequenced in an automatic ABI PRISM 3100 Genetic Analyzer sequencer (Applied Biosystems).

For the sequence consensus construction, the Phred/Phrap and Consed softwares (Altschu, Gish, Miller, Myers, & Lipman, 1990) were used and the sequence was compared with data from GenBank National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (Blast) software. The global alignment of the sequences and the phylogenetic analysis were performed using MEGA version 4.0 software. Cladistic analyses were constructed by the neighbor joining method using Jukes-Cantor for distance measurement. The confidence levels for individual branches of the resulting tree were assessed by bootstrap analysis, in which 1000 bootstrapped trees were generated from the re-sampled data.

Pectinase bio-production

The effects of culture medium composition were assessed by a 3-central-point Plackett-Burman Design (Screening Design). The independent variables (factors) studied were pectin (2.0-22.0 g L⁻¹), L-Asparagine (0-4.0 g L⁻¹), yeast extract (0-20.0 g L⁻¹), magnesium sulphate (0-1.0 g L⁻¹), potassium phosphate (0-4.0 g L⁻¹), and iron sulphate (0-0.02 g L⁻¹). Temperature (30°C), stirring (180 rpm), pH (5.5) and time (24 hours) were set at fixed levels.

A 2³ central composite rotatable design (CCRD) was performed based on the results obtained in the Plackett-Burman design. The independent variables studied were pectin (15.2-48.8 g L⁻¹), yeast extract (1.6-18.4 g L⁻¹), and magnesium sulphate (0-1.0 g L⁻¹).

Kinetic evaluation

The substrate consumption kinetics (total nitrogen, potassium, magnesium, and total organic carbon - TOC), cell mass, pH evolution, and PG production were followed by periodic sampling (3 - 48 hours) using the maximization condition established after using the experimental design method.

Crude enzymatic extracts partial characterization

The temperature stability of enzymatic extract was determined by enzyme incubation at a fixed pH_{initial} (5.5) and different temperatures: 25, 35, 45, and 55°C. The pH stability was achieved by incubating the extract obtained at 40°C at pHs 4.0, 5.0, 7.0, and 9.0. The samples were withdrawn at regular time intervals.

Crude enzymatic extract application in juice clarification

A commercial mango juice (Del Valle brand) was treated by the crude enzymatic extract obtained at, previously established, maximized bioproduction conditions. 0.01, 0.05, 0.1, and 0.5% enzyme concentrations (v v⁻¹) at 40°C, 100 rpm for 60 min. were used by evaluating viscosity, turbidity, and juice clarification percentage.

Analytical methodology

Polygalacturonase (PG) was determined by measuring the reducing groups release using the acid dinitrosalisilic (DNS) method, initially mentioned by Miller (1959). One PG unit was defined as the amount

of enzyme that releases 1 μmol D-galacturonic acid per minute of reaction ($U = \mu\text{mol min}^{-1}$). Pectin methylesterase (PME) was determined following a method described by Hultin, Sun, & Bulger (1966), with modifications. One PME unit was established as the amount of enzyme capable to catalyze the demethylation of pectin corresponding to the consumption of 1 μmol NaOH $\text{min}^{-1} \text{mL}^{-1}$ under assay conditions. Pectin lyase (PMGL) was determined using method described by Pitt (1988), with modifications. One enzyme activity unit was established as the amount of enzyme that changes 0.01 absorbance at 550 nm under assay conditions.

Initial and final pH values of the culture medium were determined using a digital pH meter (Digimed DMPH-2). Cell mass was quantified by drying at 105°C (Fanem SE-320) until reaching a constant mass.

Total organic carbon (TOC) was determined by oxidation using catalytic combustion at 680°C and infrared detection (Shimadzu TOC-VCSH model).

The total nitrogen content in the medium was determined by the Kjeldahl method (VELP DK-20 and UDK-126 D) according to the procedure described by Association of Official Analytical Chemists (AOAC, 2000). Macronutrients (Mg and K) were determined by flame atomic absorption spectrometry - FAAS (Varian Spectra AA-55), according to method described by AOAC (2000).

The viscosity reduction of mango juice after enzymatic treatment with crude extract was evaluated using a Falling-ball viscometer (Abbas, Abdulkarim, Saleh, & Ebrahimian, 2010). Mango juice clarification after enzymatic treatment with crude extract was determined based on color intensity (Chatterjee, Chatterjee, Chatterjee, & Guha, 2004), and it was expressed as clarification (%), taking into account the control juice color intensity (without enzymatic treatment) and the enzyme-treated juice. Turbidity reduction (%) was calculated based on the control juice absorbance (Chatterjee et al., 2004).

Statistical analysis

The results were treated using the Statistic 5.0 software (Statsoft, Tulsa, OK, USA). All analyses were performed considering a 95% confidence level ($p < 0.05$).

Results and discussion

Microorganism identification

According to the method described in the previous section, the newly isolated microorganism was identified as *Penicillium brasilianum* with a 100% identification rate as a result of BLAST. This result was enhanced by a dendrogram (not shown) generated by the *neighbor joining* method, using the Jukes-Cantor model as distance measurement and 1,000 *bootstrap* replicates, where the fungal isolate was compared to NCBI sequences.

Pectinases bio-production

The Plackett-Burman design results are seen in Table 1. It was noted that the pectin concentration presented a positive significant effect ($p < 0.05$) under the polygalacturonase activity, within the studied range (Figure 1). L-asparagine, potassium phosphate, and iron (II) variables presented a significant negative effect ($p < 0.05$). Therefore, these variables on level -1 were excluded from the fermentation process as the concentration was null. However, the magnesium sulphate and yeast extract variables showed a significant negative effect ($p < 0.05$) on level -1, the concentration used was zero and these variables were excluded from the process.

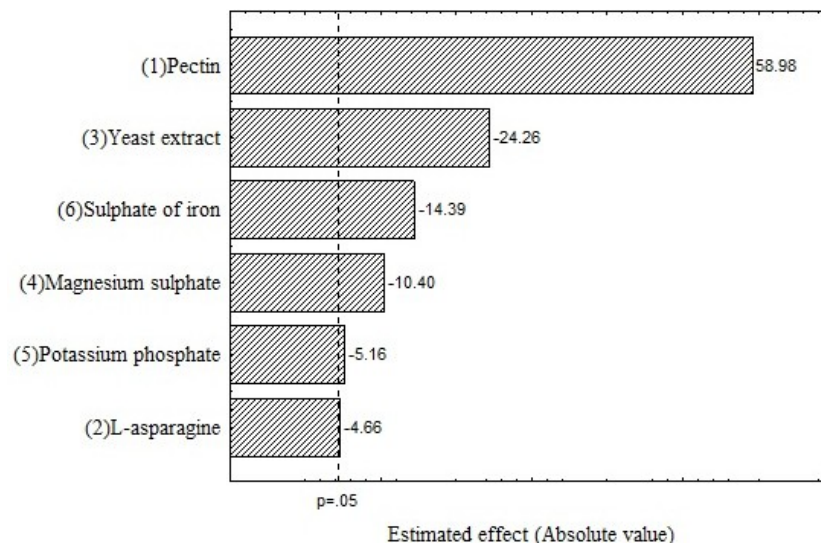
After analysing the first design, a second one was performed. Table 2 presents the coded and real values for the complete 2^3 factorial design and the responses in terms of polygalacturonase activity and pH. The maximum enzyme activity was obtained from assay 1.

The statistical analysis of these results allowed building Equation 1. It presents a second order coded model, which describes the exo-polygalacturonase activity as a function of the analysed independent variables (pectin, yeast extract, and magnesium sulfate), within the studied range. The model was validated by the analysis of variance with a 0.87 correlation coefficient and the F calculated was 2.19 times higher than the value listed in statistical tables, allowing for the construction of the contour curve presented in Figure 2.

Table 1. Plackett-Burman design matrix and the responses in terms of PG and pH_{final}.

Assays	Independent variables* (factors)						Responses	
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	PG (U mL ⁻¹)	pH _{final}
1	1 (22)	-1 (0)	1 (20)	-1 (0)	-1 (0)	-1 (0)	42.52	4.70
2	1 (22)	1 (4)	-1 (0)	1 (1)	-1 (0)	-1 (0)	44.92	4.71
3	-1 (2)	1 (4)	1 (20)	-1 (0)	1 (4)	-1 (0)	5.67	5.57
4	1 (22)	-1 (0)	1 (20)	1 (1)	-1 (0)	1 (0.02)	13.51	5.25
5	1 (22)	1 (4)	-1 (0)	1 (1)	1 (4)	-1 (0)	31.78	4.66
6	1 (22)	1 (4)	1 (20)	-1 (0)	1 (4)	1 (0.02)	18.25	5.25
7	-1 (2)	1 (4)	1 (20)	1 (1)	-1 (0)	1 (0.02)	8.16	5.55
8	-1 (2)	-1 (0)	1 (20)	1 (1)	1 (4)	-1 (0)	6.89	5.50
9	-1 (2)	-1 (0)	-1 (0)	1 (1)	1 (4)	1 (0.02)	7.26	5.12
10	1 (22)	-1 (0)	-1 (0)	-1 (0)	1 (4)	1 (0.02)	49.30	4.25
11	-1 (2)	1 (4)	-1 (0)	-1 (0)	-1 (0)	1 (0.02)	11.01	5.25
12	-1 (2)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	12.09	4.52
13	0 (12)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	19.27	5.28
14	0 (12)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	19.27	5.26
15	0 (12)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	18.01	5.32

*X₁ = Pectin (g L⁻¹), X₂ = L-Asparagine (g L⁻¹), X₃ = Yeast extract (g L⁻¹), X₄ = Magnesium sulphate (g L⁻¹), X₅ = Potassium phosphate (g L⁻¹), X₆ = Iron sulphate (g L⁻¹).

**Figure 1.** Pareto chart of effects (absolute values) for the polygalacturonase (PG) activity response after the Plackett-Burman design.**Table 2.** 2⁵DCCR matrix (real and coded values) with polygalacturonase (PG) activity and pH_{final} responses.

Assays	Independent variables*			Responses	
	X ₁	X ₃	X ₄	PG (U mL ⁻¹)	pH _{final}
1	-1 (22.0)	-1 (5.0)	-1 (0.2)	42.65	4.86
2	1 (42.0)	-1 (5.0)	-1 (0.2)	36.06	4.77
3	-1 (22.0)	1 (15.0)	-1 (0.2)	25.94	5.17
4	1 (42.0)	1 (15.0)	-1 (0.2)	29.08	5.02
5	-1 (22.0)	-1 (5.0)	1 (0.8)	40.61	4.87
6	1 (42.0)	-1 (5.0)	1 (0.8)	38.51	4.74
7	-1 (22.0)	1 (15.0)	1 (0.8)	29.84	5.18
8	1 (42.0)	1 (15.0)	1 (0.8)	24.14	5.03
9	-1.68 (15.2)	0 (10.0)	0 (0.5)	29.20	5.14
10	1.68 (48.8)	0 (10.0)	0 (0.5)	16.97	4.88
11	0 (32.0)	-1.68 (1.6)	0 (0.5)	41.73	4.51
12	0 (32.0)	1.68 (18.4)	0 (0.5)	14.87	5.11
13	0 (32.0)	0 (10.0)	-1.68 (0)	26.27	4.95
14	0 (32.0)	0 (10.0)	1.68 (1.0)	26.89	4.87
15	0 (32.0)	0 (10.0)	0 (0.5)	37.73	4.93
16	0 (32.0)	0 (10.0)	0 (0.5)	37.22	4.91
17	0 (32.0)	0 (10.0)	0 (0.5)	36.48	4.93

*X₁ = Pectin (g L⁻¹), X₃ = Yeast extract (g L⁻¹), X₄ = Magnesium sulphate (g L⁻¹).

$$PG = 36.31 - 2.33.X_1 - 3.18.X_1^2 - 6.89.X_3 - 1.33.X_3^2 + 0.33.X_4 - 1.94.X_4^2 + 0.76.X_1.X_2 - 0.54.X_1.X_3 - 0.18.X_2.X_3 \quad (1)$$

where:

PG = Polygalacturonase (U mL^{-1});

X_1 = Pectin;

X_3 = Yeast extract, and;

X_4 = Magnesium sulphate.

Kinetic evaluation

Figure 3a and b present the kinetic evaluation in terms of substrate consumption pH evolution, biomass and polygalacturonase production in the maximized condition. The maximum polygalacturonase activity (52.8 U mL^{-1}) was reached at 48 hours of fermentation.

The fermentation medium pH (Figure 3a) presented a slight decrease in the first 36 hours (from 5.1 to 4.6) and it was higher at 48 hours. Such behavior could be due to the excretion of galacturonic acid in the first 48 hours of fermentation by pectinolytic enzymes action. After this period, higher pH values were observed due to the nitrogen consumption (about 52%).

For substrates consumption (Figure 3b) it was observed a TOC decrease in the first 6 hours of fermentation (from 152.6 to 134 mg L^{-1}). After this period the concentration remained constant until reaching 24 hours. A TOC concentration reduction after this time could be observed and it was noted that the polygalacturonase activity was at its maximum at 48 hours with the TOC concentration of 88 mg L^{-1} (42% TOC consumption).

The potassium content gradually decreased in the first 27 hours of fermentation (from 453 to 385 mg L^{-1}). After this period, a 78% reduction at 60 hours could be observed. Magnesium consumption presented similar behavior as it reduced by 10 in the first 30 hours. The highest polygalacturonase activity was achieved at 23% magnesium consumption after 48 hours evaluation.

In this work, pH values of all assays were monitored and it was noted that the behavior was not associated with the polygalacturonase production. A slight pH reduction could be observed (from 5.5 to 4.5) in all assays, as seen in Table 1 and 2. According to Cordeiro & Martins (2009), this pH reduction could be due to the glucuronic acid release into the medium caused by pectinolytic enzymes action. Such enzymes are produced by the microorganisms during the first hours of fermentation.

According to Chu, Lee, & Li (1992), pH changes in the culture medium are result of substrate consumption. When ammonium ions are used by the microorganisms, the medium is acidified and when the organic nitrogen (amino acids and peptides) are assimilated the medium is alkalinized. Considering this relationship between the polygalacturonase and the organic compounds utilization, the changes in pH values could explain certain behaviors in the polygalacturonase production.

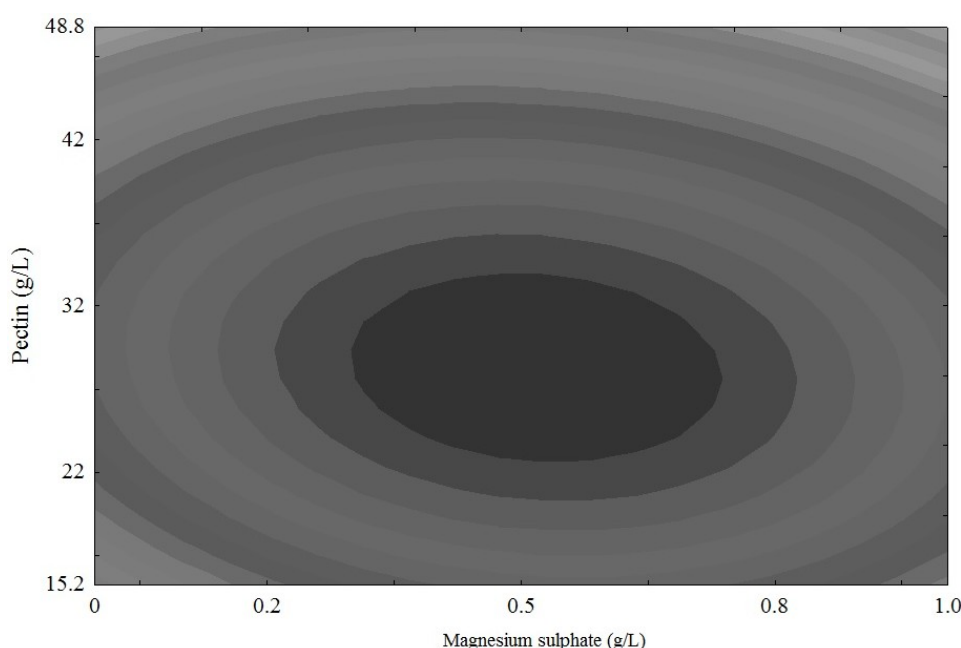


Figure 2. Contour curve for polygalacturonase activity (U mL^{-1}) in terms of pectin and magnesium sulphate concentration.

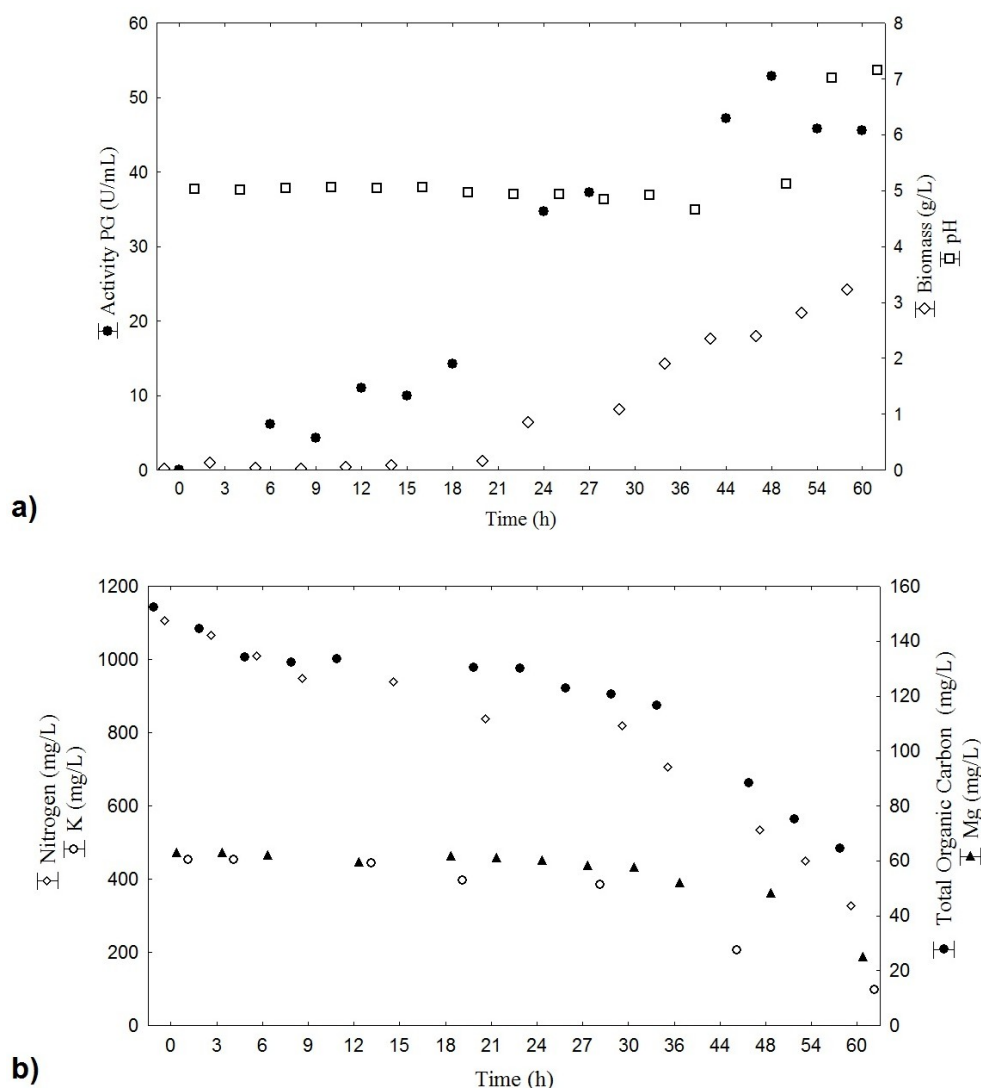


Figure 3. Kinetic in terms of polygalacturonase activity, biomass production, pH evolution (a) and substrates consumption: N₂, K, Mg and TOC (b) at the maximized condition (32.0 g L⁻¹ pectin, 10.0 g L⁻¹ yeast extract and 0.5 g L⁻¹ magnesium sulphate, at 30°C, 180 rpm and pH 5.5).

The current literature presents different polygalacturonase activities by fungi in submerged fermentation demonstrating the influence of the strain, the culture conditions, and substrates. Sandri et al. (2015) obtained a 42 U mL⁻¹ polygalacturonase activity from *A. fumigates* LB-01-AP using pectin (20 g L⁻¹), glucose (22 g L⁻¹), yeast extract (0.05 g L⁻¹) and supplemented with mineral sources [5 g L⁻¹ (NH₄)₂SO₄, 0.5 g L⁻¹ MgSO₄, 2.5 g L⁻¹ KH₂PO₄, 6.3x10⁻⁴ FeSO₄.7H₂O, 6.2x10⁻⁴ ZnSO₄, 1 x10⁻⁵ MnSO₄]. Gomes et al. (2011) obtained a 52 U L⁻¹ polygalacturonase activity from *A. niger* ATCC 9642 using pectin (32 g L⁻¹), L-asparagine (2 g L⁻¹), potassium phosphate (0.06 g L⁻¹), iron sulfate (1 g L⁻¹), 180 rpm, 25°C and 4.0 pH_{initial}. Pili et al. (2015) obtained a 18.5 U mL⁻¹ polygalacturonase activity (specific activity was 195.6 U mg⁻¹) from *A. niger* ATCC 9642 using orange peel (80 g L⁻¹), corn steep liquor (60 g L⁻¹), parboiled rice water (450 g L⁻¹), 180 rpm stirring rate, 30°C and 5.5 pH_{initial}.

Silva et al. (2007) producing endo-PG and exo-PG evaluated the solid-state fermentation of an orange bagasse and wheat bran mixture (1:1, at 28°C) with the *Penicillium viridicatum* RFC3. Exo-PG activity reached its maximum values at 336 hours of cultivation with 70 (5.8 U mL⁻¹) and 80% (8.9 U mL⁻¹) from initial moisture content.

Crude enzymatic extracts partial characterization

The stability of the crude enzymatic extracts obtained by *P. brasilianum* fermentations in relation to temperature (Figure 4) was verified in the 25 to 55°C range at a fixed 5.5 pH value.

The pH stability (Figure 5) was assessed in the 4.0 to 9 range using 100 µmol L⁻¹ sodium phosphate buffer at 37°C. The PG produced from *P. brasilianum* presented higher stability at pH 4.0 to 5.0 and

55°C. Tari, Dogan, & Gogus (2008), when investigating the effect of pH on stability, found that polygalacturonase from *A. soybean* was quite stable at pH 5.0 and retained 60 and 70% of its activity at pHs 3.0 and 7.0, respectively.

Kant, Vohra, & Gupta (2013) studied the stability of purified polygalacturonase from *A. niger* MTCC 3323 and found enzymatic stability from pH 4.0 to 5.5 for 1 hours. Gomes et al. (2011) evaluated the stability of PG produced from *A. niger* using a complex medium (32 g L⁻¹ pectin, 2 g L⁻¹ L-asparagine, 0.06 g L⁻¹ potassium phosphate and 1.0 g L⁻¹ iron sulfate) and observed that at pH 5.0 60% of the PG initial activity during 150 hours of storage was maintained. Silva et al. (2007) evaluated pectinolytic enzymes from *Penicillium viridicatum* RFC3 and obtained the maximum activity at pH 6.0 and 60°C.

Crude enzymatic extract application for juice clarification

Table 3 shows a turbidity reduction and a clarification increase of commercial mango juice treated with the crude enzymatic extract. It shows a 12% reduction in turbidity and a 23% increase in clarification, obtained using a 0.5% enzymatic concentration (v v⁻¹) at 40°C, 100 rpm for 60 min.

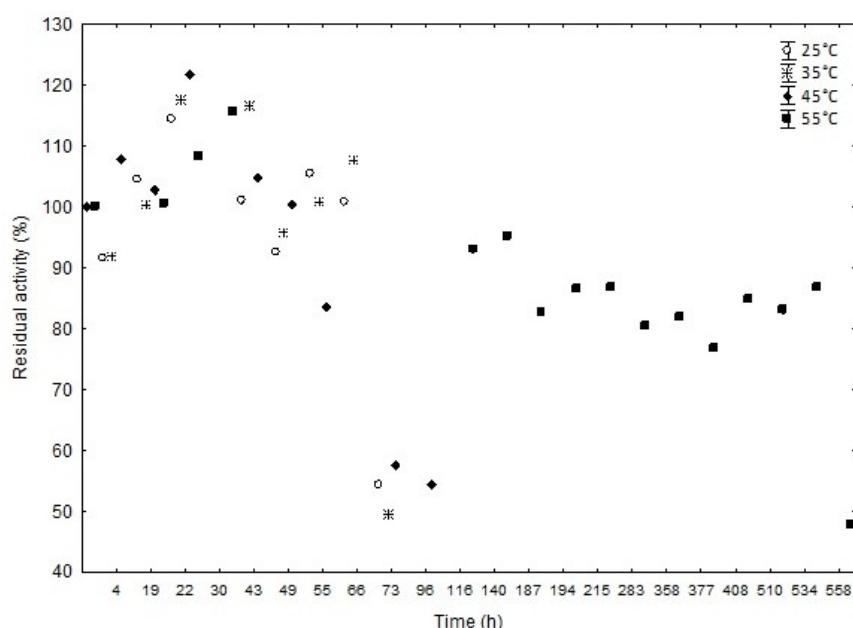


Figure 4. Stability of PG crude enzymatic extract at 25, 35, 45, and 55°C.

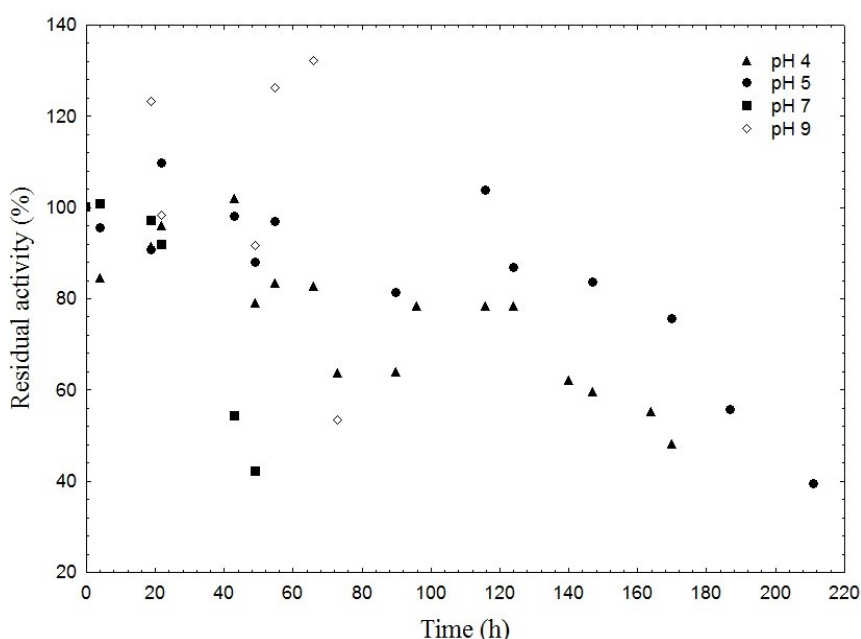


Figure 5. Stability of PG crude enzymatic extract at pHs 4, 5, 7, and 9.

Table 3. Viscosity, turbidity, and clarification reduction of commercial mango juice treated with pectinolytic crude enzymatic extract.

Determination	Enzymatic treatment*			
	0.01 % (v v ⁻¹)	0.05 % (v v ⁻¹)	0.1 % (v v ⁻¹)	0.5 % (v v ⁻¹)
Reduction of viscosity (%)	2.00 ± 0.03 ^c	4.40 ± 0.14 ^b	4.74 ± 0.20 ^b	10.06 ± 0.22 ^a
Reduction of turbidity (%)	-	7.91 ± 0.02 ^c	11.38 ± 0.28 ^b	12.01 ± 0.01 ^a
Clarification (%)	0.03 ± 0.01 ^d	2.10 ± 0.03 ^c	12.78 ± 0.44 ^b	23.02 ± 0.12 ^a

*Mean ± standard deviation followed by equal letters in the lines indicates no significant difference at a confidence level of 5%.

This effect was also observed by Chatterjee et al. (2004), and might be possibly related to the presence of other pectinases in crude enzymatic extracts, such as pectin methylesterase (PME) with 6.0 U mL⁻¹ activity and pectin lyase (PMGL) with 6.61 U mL⁻¹ activity. Thus, the results could have been influenced by these and other enzymes whose activities have not been quantified in the study. Clemente and Pastore (1998) and Vámos-Vigyázó (1981), using a commercial enzyme (Pectinex), which showed cellulases activity, verified a better performance of the clarification process for peach juice compared to the isolated enzymes. Poletto, Renosto, Baldasso, Zeni, and Silveira (2015), in the clarification of blackberry juices, verified a reduction in viscosity and turbidity of 40 and 50%, respectively. According to Echavarría, Torras, Pagán, and Ibarz (2011), the reduction of these parameters is paramount to ensure juice stability during storage.

Conclusion

The crude enzyme-complex as well as the pectinolytic activity of polygalacturonase (52.8 U mL⁻¹) showed pectin methylesterase (6 U mL⁻¹) and pectin lyase (6.6 U mL⁻¹) activities. The crude enzymatic complex with a 0.5% concentration (v v⁻¹) used for commercial mango juice clarification reduced the viscosity by 10%, turbidity by 12% and clarification by 23%. In this way, the results presented.

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