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Experimental Design Applied to the Optimization and Partial Characterization of Pectin Liase from a Newly Isolated *Penicillium brasilianum*

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ABSTRACT

Penicillium brasilianum was previously isolated from tea and identified by molecular biology technique. A Plackett-Burman design, followed by a complete second order design was used for the screening of most important factors and to maximize the pectin liase (PMGL) activity, respectively. The maximum PMGL activity by P. brasilianum achieved was 9.0 U/mL after 48 h of cultivation in a medium containing pectin (33.0 g/L), yeast extract (30.0 g/L) and potassium phosphate (2.0 g/L) at 30ºC, with a stirring rate of 180 rpm, initial pH 5.5 and $5x10^6$ spores/mL inoculum size. The kinetic evaluation in terms of substrate consumption demonstrated that the maximum production of PMGL was at 72 h, and 40% of the total organic carbon, 25% of the nitrogen, 88% of the magnesium, 13% of the potassium and 66% of the iron were consumed. The pH remained almost stable during the whole period of production (5.33 to 4.9). The partial characterization of the crude PMGL enzyme extract showed optimal pH and temperature of 5.5 and 37°C, respectively.

Key words: *Penicillium brasilianum*, pectin liase, experimental design technique

INTRODUCTION

Pectic polymers (chain of 1,4-linked-α-D-galacturonic acid and methoxylated derivatives) are the major structural constituents of middle lamellae in plant cell wall. They play an important role in adhesion of adjacent cells together and have a great impact on the viscosity and turbidity of fruit juices (Lozano et al. 1990; Sandri et al. 2013). Pectinases are the group of enzymes involved in depolymerisation of the pectic polymers. The group of enzymes, which are involved in the degradation of ‘‘smooth region’’ (homogalacturonan) include deesterifying enzymes, i.e., pectin methyl esterases (PME, E.C. 3.1.1.11) and pectin acetyl esterase (PAE, E.C. 3.1.1.6) which remove methoxyl and acetyl residues of pectin producing polygalacturonic acid. The other subclass of homogalacturonan degrading group are broadly termed as depolymerases, which break the α-1,4-linkages either by hydrolysis, i.e., polygalacturonases (PG, E.C. 3.2.1.15) or via transelimination mechanism, namely pectate liases (PL, E.C. 4.2.2.2) and pectin liases (PMGL, E.C. 4.2.2.10) (Whitaker et al. 1990).

Pectin liases are the only known pectinases capable of degrading highly esterified pectins (like those found in fruits) into small molecules via β-elimination mechanism without producing methanol, in contrast with the combination of PG and pectinesterases (PE, E.C. 3.1.1.1), which are normally found in the commercial products (Whitaker et al. 1990). This is important because

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methanol is toxic and may present health hazards. Methanol may be lost in vapour during juice concentration, but a different condition occurs in viscous materials (purees, baby foods, etc) or non-concentrated juices where methanol is only partially released during pasteurization. In addition, the presence of undesirable enzymatic activity in commercial pectinases may be detrimental to aroma because they are responsible for producing unpleasant volatile off flavor (Targano et al. 1994). There are many reports of fruit juices clarification by pectin liases (Mantovani et al. 2005; Yadav et al. 2008; Kant et al. 2013; Wang et al. 2013). The alkaline pectinase are inappropriate to be used in the food industries due to acidic pH of fruit juices. However, they have a very high demand in the textile industries. They are used for retting of plant fibers such as ramie, sunn hemp, jute, flax and hemp (Cao et al. 1992; Kapoor et al. 2001).

Pectinases are biotechnologically important because they have potential applications in the clarification of fruit juices, retting of natural fibers (ramie, hemp, flax, bast), treatment of pectic waste water, coffee and tea leaf fermentation, oil extraction, virus purification, etc (Alkorta et al. 1998; Hoondal et al. 2002). Pectin liases are the only enzymes capable of depolymerising highly esterified pectin into small molecules without prior action of other enzymes (Delgado et al. 1992). PMGLs cleave pectin by β-elimination mechanism that results in the formation of 4,5-unsaturated oligogalacturonates without affecting the ester content of the polymer chain, which is responsible for specific aroma of fruits (Targano et al. 1994). Since new applications for pectin liases are emerging, the demands for production of these enzymes are increasing (Satyanarayana et al. 2005).

Like all pectinases, PMGL is also highly inducible enzymes even though there are reports of its considerable production in the absence of its natural inducer. PMGLs are mainly produced by fungi such as *Aspergillus*, *Penicillium* and *Fusarium* but there are a few reports on bacterial and yeast PMGL (Whitaker 1990; Fawole and Odunfa 2003; Gonçalves et al. 2012). In this context, the objective of this study was to evaluate the PMGL production by a newly isolated strain of *P. brasilianum* using submerged fermentation. After the media optimization, a kinetic evaluation was carried out and the enzyme was characterized in terms of optimum pH.

MATERIALS AND METHODS

Microorganism and growth conditions

The microorganism used in the present study was previously isolated from tea, which was identified by molecular biology technique. After incubation in Potato Dextrose (PD) medium at 28°C for 3-4 days, a protocol was used for the extraction of yeast genomic DNA using liquid nitrogen for cell disruption (Fernandes-salomão et al. 1996), followed by DNA quantification using a spectrophotometer model NanoDrop, ND-1000 (NanoDrop Technologies). The microorganism was identified as *Penicillium brasilianum.* The culture was on Potato Dextrose Agar (PDA) slant medium containing (g/L) malt extract 10.0, yeast extract 4.0, glucose 4.0 and agar 20 and incubated at 30ºC for one week. Stock cultures were prepared with 20 wt% glycerol water and stored at −80ºC (MDF – U3086S - Sanyo). The harvesting of the spores from the slants was done using 5.0 mL of Tween 80–water (0.02%). The spore suspension was collected in sterile falcon tube and stored at 4ºC until the use. The concentration of the suspension was adjusted to 5.10^6 spores per mL.

Pectin liase bioproduction

For studying the effects of the composition of the culture medium and operating conditions of the bioproduction pectin liase Plackett-Burman Design was used; the independent variables studied were: pectin (2.0 – 22.0 g/L), L-asparagine (0 – 4.0 g/L), yeast extract (0 – 20.0 g/L), iron sulphate II (0 – 0.02 g/L), potassium phosphate (0 – 4.0 g/L) and magnesium sulfate (0 – 1.0 g/L). Fixed variables were 30ºC, 180 rpm, pH 5.5 and $5x10^6$ spores/mL. This was followed by a 2^3 factorial design study comprising the variables: pectin (11.0 – 33.0 g/L), yeast extract (10.0 – 30.0 g/L), potassium phosphate (2.0 – 4.0 g/L), while maintaining the fixed temperature (30°C), agitation (180 rpm), pH (5.5) and time (48 h).

Kinetic evaluation

The kinetics of substrate consumption (total nitrogen, potassium, magnesium and total organic carbon - TOC), cell mass, pH evolution and PMGL production were followed by periodic sampling of the in the maximization condition (33.0 g/L of pectin, 30.0 g/L yeast extract, 2.0 g/L of potassium phosphate, 180 rpm, 30°C, pH 5.5 and $5x10^6$ spores/mL).

Partial characterization of crude enzymatic extracts

To determine the optimum values of temperature and pH in terms of enzyme activity for *P. brasilianum*, a central composite rotatable design (CCRD) 2^2 was accomplished using the enzymatic extract. The studied range for pH was from 3.0 – 6.0 and temperature was from 30 - 80°C. The temperature stability of the enzymatic extract was determined by enzyme incubation at a fixed pH (5.5) and different temperatures: 30 - 70°C. The pH stability was determined by incubating the enzyme extract obtained at 40ºC at the following pH: 3.0 - 8.0. The samples were withdrawn at regular time intervals.

Analytical determinations

Pectin liase (PMGL) activity: The pectin liase activity was determined using the method described by Pitt (1988). One unit of enzyme activity was defined as the amount of enzyme, which resulted a change in absorbance of 0.01 at 550 nm under the assay conditions.

pH: The pH was monitored using a potentiometer (DMPH-2, Digimed), after calibration with standard solutions pH 4.0 and 7.0.

Total organic carbon (TOC): The total organic carbon (TOC) content was determined by the method of oxidation by catalytic combustion at 680°C and detection by infrared using a Shimadzu model TOC-VCSH analyzer (AOAC 1995).

Mineral compounds: Macro (Mg and Mn) and micronutrients (Fe and K) were determined by flame atomic absor ption spectrometry - FAAS (Varian Spectra AA-55), following the methodology described by AOAC (1995). Hollow cathode lamps of Mg and Fe were used as radiation source. The elements were measured in optimized operation conditions by FAAS in flame of air/acetylene or nitrous oxide/acetylene. The readings of Mg, Mn, Fe and K were performed by FAAS in absorption mode. To eliminate the possible interferences in the determination of Mg content, lanthanum chloride was added to the samples and to the standard solutions at a proportion of 1 % (w/v). For the determination of the minerals contents in the samples, calibration curves of standard solutions were used. The nitrogen content was determined by the Kjedahl method (VELP DK-20 e UDK-126D), following the methodology of AOAC (1995).

Statistical Analysis

The statistical analysis related to the estimated effects of each variable and process optimization was performed using the global error and the relative standard deviation between the experimental and predicted data. The other results were treated by analysis of variance (ANOVA), followed by Tukey's. All the analysis were performed using the software Statistica version 6.0 (Statsoft Inc, USA).

RESULTS AND DISCUSSION

Pectin liase (PMGL) bioproduction

Table 1 presents the matrix of a Plackett-Burman design with encoded values (actual) of the independent variables studied and the response in terms of pectin liase activity (PMGL). The maximum activity of pectin liase was obtained in run 5 at a central point with an activity of approximately 6.81 U/mL, when the cultivation medium contained 22.0 g/L pectin, 4.0 g/L L-asparagine, 1.0 g/L magnesium sulfate, and 4.0 g/L potassium phosphate, without the addition of yeast extract and iron sulphate (II).

Figure 1 shows a Pareto chart with the estimated effects of the variables studied in the Plackett-Burman design. Evidently pectin, yeast extract and potassium phosphate had significant ($p<0.05$) positive effects, which meant that when the concentration and/or range of this variable increased from level -1 to +1, which might increase enzyme activity. However, L-asparagine had a significant ($p<0.05$) negative effect; therefore, as at level -1, the concentration was zero, this variable was excluded from the process.

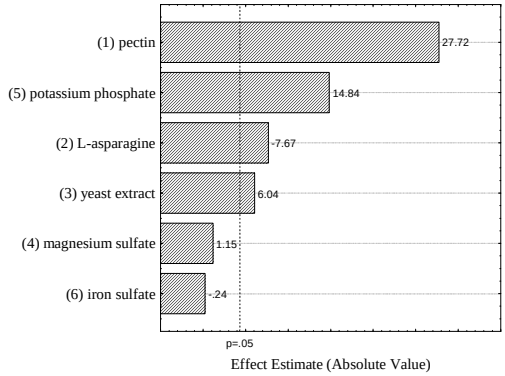

Figure 1 - Pareto Chart with the estimated effect (absolute value) of the variables tested in the experimental design of type Plackett-Burman, for the production of pectin liase.

Table 1 - Matrix of planning type Plackett-Burman (real and coded values) with the response in activity of pectin liase (PMGL) and pH.

Runs	Independent variables *						Responses	
	X_1	X_2	X_3	X_4	X_5	X_6	PMGL (U/mL)	pH
1	1 (22)	-1 (0)	1 (20)	-1 (0)	-1 (0)	-1 (0)	5.97	5.07
2	1 (22)	1 (4)	-1 (0)	1 (1)	-1 (0)	-1 (0)	2.36	6.95
3	-1 (2)	1 (4)	1(20)	-1 (0)	1 (4)	-1 (0)	2.22	6.85
4	1 (22)	-1 (0)	1 (20)	1 (1)	-1 (0)	1 (0.02)	5.19	5.51
5	1 (22)	1 (4)	-1 (0)	1 (1)	1 (4)	-1 (0)	6.81	4.7
6	1 (22)	1 (4)	1 (20)	-1 (0)	1 (4)	1 (0.02)	5.70	5.63
7	-1 (2)	1 (4)	1 (20)	1 (1)	-1 (0)	1 (0.02)	1.31	7.36
8	-1 (2)	-1 (0)	1 (20)	1 (1)	1 (4)	-1 (0)	3.45	6.27
9	-1 (2)	-1 (0)	-1 (0)	1 (1)	1 (4)	1 (0.02)	2.80	4.34
10	1 (22)	-1 (0)	-1 (0)	-1 (0)	1 (4)	1 (0.02)	6.31	4.36
11	-1 (2)	1 (4)	-1 (0)	-1 (0)	-1 (0)	1 (0.02)	0.07	4.53
12	-1 (2)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	0.76	4.2
13	0 (11)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	6.86	6.26
14	0 (11)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	6.55	6.21
15	0 (11)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	6.99	6.54

X_1= Pectin (g/L), X_2= L-asparagine (g/L), X_3= yeast extract (g/L), X_4= magnesium sulphate (g/L), X_5= potassium phosphate (g/L), X_6 = iron sulphate II (g/L); independent variables fixed: 180 rpm, 30°C, 48 h, $pH_{inicial}$ 5.5 and $5x10^6$ spores/mL.

Based on the above results, a 2^3 factorial design was carried out. The results presented in Table 2 showed that the maximum activity of PMGL was 7.07 U/mL (run 4) in a culture medium composed 33.0 g/L of citrus pectin, 30.0 g/L of yeast extract and 2.0 g/L of potassium phosphate under conditions of 180 rpm, 30°C, 48 h and $pH_{initial}$ 5.5. Equation 1 shows the first order model that describes the activity of PMGL as a function of the variable pectin within the range studied. The model was validated by the analysis of variance, where a correlation coefficient of 0.95 was obtained. The calculated F value was 1.46 times higher than the value listed in statistical tables, which allowed the construction of the contour curve (Fig. 2), demonstrating that maximum production of PMGL was in a range of pectin concentration exceeding 22 g/L and in the range 20.0- 30.0 g/L of yeast extract.

$$PMGL = 5.862 + 1.195\ X_1 \quad (1)$$

Where PMGL is the activity of pectin liase (U/mL) and X_1 is the pectin concentration (g/L).

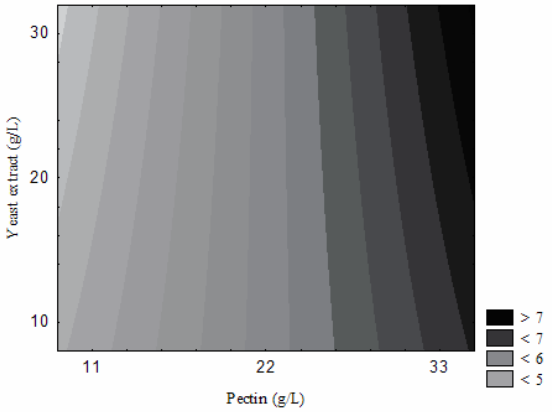

Figure 2 - Contour curve for the activity of PMGL as a function of pectin *versus* yeast extract.

Table 2 - Matrix of 2^3 factorial design and response in production of pectin liase (PMGL) and pH.

Runs	Independent variables *			Responses	
	X_1	X_3	X_5	PMGL (U/mL)	pH
1	-1 (11)	-1 (10)	-1 (2)	6.08	5.51
2	1 (33)	-1 (10)	-1 (2)	6.86	4.80
3	-1 (11)	1 (30)	-1 (2)	4.61	5.59
4	1 (33)	1 (30)	-1 (2)	7.07	4.89
5	-1 (11)	-1 (10)	1 (6)	2.75	5.02
6	1 (33)	-1 (10)	1 (6)	6.01	5.42
7	-1 (11)	1 (30)	1 (6)	3.48	5.47
8	1 (33)	1 (30)	1 (6)	6.53	4.92
9	0 (22)	0 (20)	0 (4)	6.36	4.92
10	0 (22)	0 (20)	0 (4)	5.54	4.89
11	0 (22)	0 (20)	0 (4)	6.28	4.91

*X_1= pectin (g/L), X_3= yeast extract (g/L), X_5= potassium phosphate (g/L). Independent variables fixed: 180 rpm, 30°C, 48 h and $pH_{inicial}$ 5.5.

In agreement with the results obtained in the 2^3 factorial design where only the variable pectin had a significant effect ($p<0.05$), an experiment was carried out in triplicate by varying the concentration of pectin between 33.0, 40.0 and 45.0 g/L and keeping the concentrations of yeast extract (30.0 g/L) and potassium phosphate (2.0 g/L) at 180 rpm, 30°C, 48 h and $pH_{initial}$ 5.5. The maximum production of PMGL was 6.69 U/mL at a citrus pectin concentration of 33.0 g/L. At 40.0 and 45.0 g/L citrus pectin, there was a significant reduction in the activity, indicating that an increase in the concentration of citrus pectin could inhibit the production of this enzyme due to excess substrate, which also might make the medium viscous and decreased transfer of oxygen.

Kinetic evaluation

Figures 3A and 3B present the kinetic evaluation in terms of substrate consumption (total nitrogen, potassium, magnesium and total organic carbon) and pH evolution, biomass and pectin liase production in the maximized condition (33.0 g/L citrus pectin, 30.0 g/L yeast extract, and 2.0 g/L potassium phosphate at 180 rpm, 30°C and $pH_{initial}$ 5.5) as a function of time, respectively.

Figure 3A showed that the maximum PMGL activity (9.0 U/mL) was obtained after 72 h of culture incubation; during the same period, the microorganism reached the end of its exponential growth phase.

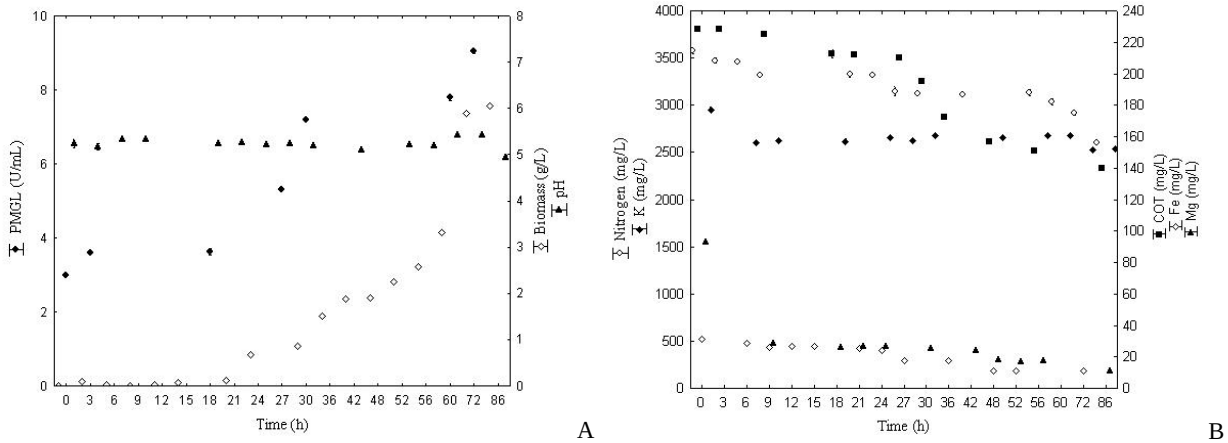

Figure 3 - kinetic Study of bioproduction of PMGL depending on the activity, production of biomass and pH by time (A) and on the basis of consumption of carbon, nitrogen and minerals by time (B), respectively.

The enzymatic activity of PMGL (9.0 U/mL) was similar to that reported by Santos et al. (2008), who studied the production and characterization of pectinolytic enzymes produced by *P. oxalicum* using agro-industrial waste and found that when the medium contained orange peel (1%), higher production of pectin liase (9.59 U/mL) occurred after 96 h of cultivation at 170 rpm and 28ºC. Camargo et al. (2005) studied the production of extracellular pectin liase from *Aspergillus* sp. isolated from the soil, half submerged in medium containing residue of orange and citrus pectin as sole source of carbon; after 68 h, the activity was 11.3 U/mL. The pH (Fig. 3A) was almost stable during the whole period of production (5.33 to 4.9).

Figure 3B showed that there was a slow consumption of organic carbon up to 27 h, afterwards a gradual decrease until 86 h was observed with a total consumption of 40% (final TOC 136 mg/L). A similar behavior was observed for the iron content, with a total consumption of 66% (Fe 11.21 mg/L). However, the assimilation of nitrogen was slow and gradual throughout the production, with a total consumption of 25%. Regarding potassium, the decrease was higher in the first 6 h of the production (from 2,900 to 2,600 mg/L) and after this period, it remained almost constant (2,600 to 2,520 mg/L). The same behavior was observed for the magnesium, but in the first nine hours, the consumption was 60% and after 86 h, its assimilation was 88%.

Partial characterization of pectin liase (PMGL) crude extract

Effect of temperature and pH on crude enzymatic extracts activity

Table 3 shows the matrix of the full factorial design 2^2 (3 central points) as well as the results of the enzymatic activity of pectin liase. The highest activity was 11.97 U/mL in run 2 at pH 5.5 and 37°C.

Table 3 - Matrix of the full factorial design 2^2 (real and coded values) with the responses of and response in enzyme activity of pectin liase as a function of temperature and pH.

Runs	Independent variables*		Pectin liase
	pH	Temperature (°C)	(U/mL)
1	-1 (3.4)	-1 (37)	7.32
2	1 (5.5)	-1 (37)	11.97
3	-1 (3.4)	1 (73)	7.01
4	1 (5.5)	1 (73)	10.85
5	-1.41 (3)	0 (55)	7.20
6	1.41 (6)	0 (55)	9.14
7	0 (4.5)	-1.41 (30)	8.96
8	0 (4.5)	1.41 (80)	7.84
9	0 (4.5)	0 (55)	9.91
10	0 (4.5)	0 (55)	10.44
11	0 (4.5)	0 (55)	10.17

Equation 2 showed the second order model that described the concentration of PMGL as a function of the analyzed variables (temperature and pH), within the ranges studied. The model was validated by the analysis of variance, where a correlation coefficient of 0.88 was obtained and the calculated F value was 1.41 times higher than the value listed in the statistical tables, allowed the construction of the contour curve (Fig. 4).

$$PMGL = 10.17 + 1.41.pH - 0.63.(T)^2 \quad (2)$$

Where PMGL is the activity of pectin liase (U/mL) and T is the temperature (ºC).

The maximum enzymatic activity of the crude extract occurred in the range of pH 4.5 to 6.5 and 30 to 60°C (Fig. 4). Yadav et al. (2009) found that the pH and temperature optima for PMGL produced by several microorganisms varied from 5.0 to 10.5 and 32 to 60°C, respectively, depending on the microorganism. Silva et al. (2002) studied the production of PMGL by a strain of *P. viridicatum* RFC3, which showed maximum activity at pH 10.5 and 50ºC.

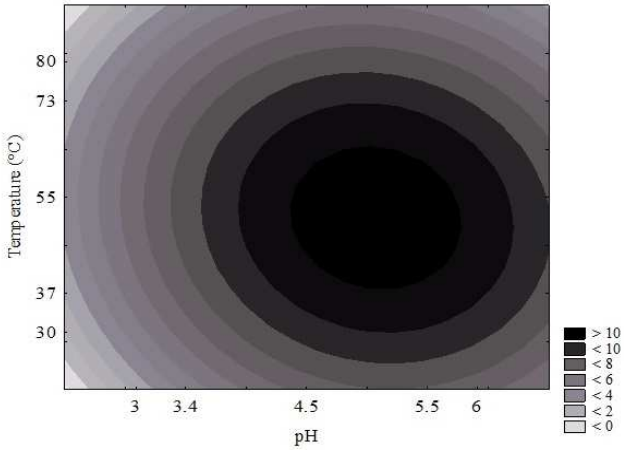

Figure 4 - Contour curve for the activity of PMGL as a function of temperature and pH.

Temperature stability of the crude enzymatic extracts

Evaluation of the stability of the crude enzyme extract of PMGL was performed by incubating at different temperatures (30, 40, 50, 60 and 70°C), and monitoring its behavior as a function of time (Fig. 5). The crude enzyme extract presented the highest stability at 30 and 40°C, retaining 53 and 54% of its initial PGML activity after 2,300 and 2,100 h of storage, respectively. At 50, 60 and 70°C, the behavior was similar, maintaining residual activity of about 50% to 1,370 h. Busto et al. (2006) evaluated the stability of the commercial pectin liase, free and immobilized in support (sodium alginate) and found that at 40°C the free and immobilized enzyme retained 57 and 50% of the activity, respectively after 2.5 h incubation. The thermal stability was lower at higher temperatures, retaining only 7% activity after 2.5 h at 60°C. In spite of this, 40°C is most frequently used in juice processing Minussi et al. (1998), evaluating the stability of PMGL from *P. griseoroseum* observed that the enzyme was stable up to 40°C, but lost all its activity when maintained at 55°C during one hour. Silva et al. (2002) evaluated the stability of PMGL produced by *P. viridicatum* RFC3 and found higher thermal stability at 35ºC for one hour.

pH stability of the crude enzymatic extracts

The stability of the crude PMGL enzyme extract to different pH was performed by monitoring the behavior as a function of time. The extract was incubated at 40°C at an initial pH of 4.89 and then its activity was evaluated at different pH (3.0, 3.4,

4.5, 5.5, 6.0, 7.0 and 8.0). The results obtained are presented in Figure 6, which showed that the crude enzyme extract had maximum stability at pH 5.5, retaining 54% of its initial activity after 1,500 h of storage. Silva et al. (2002) reported higher stability of PMGL from *P. viridicatum* RFC3 at acidic pH.

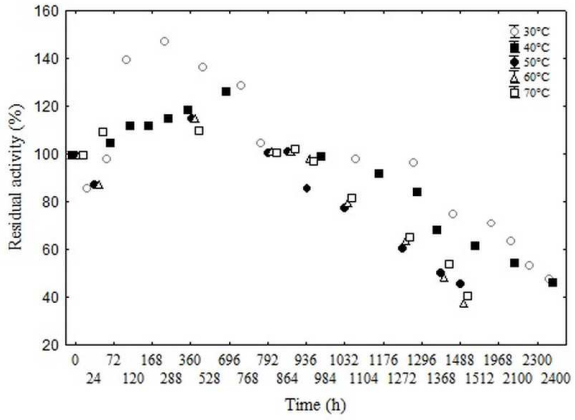

Figure 5 - Influence of temperatures of 30, 40, 50, 60 and 70°C on stability of the crude enzymatic extract obtained *Penicillium brasilianum*.

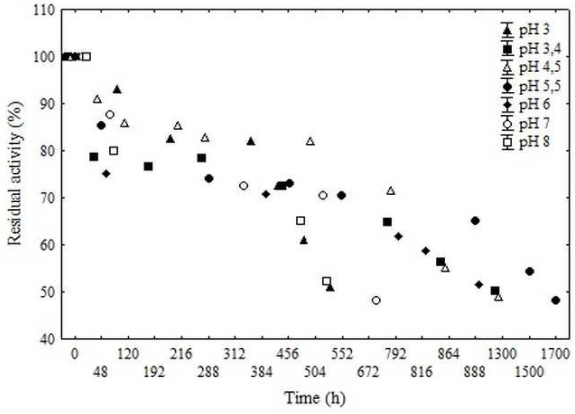

Figure 6 - Influence of pH on stability of the crude enzymatic extract obtained by *Penicillium brasilianum*.

CONCLUSIONS

The maximum production of pectin liase by *P. brasillianum* was 9.0 U/mL at 72 h with pectin concentration of 33.0 g/L, yeast extract 30.0 g/L and potassium phosphate 2.0 g/L at 30ºC, 180 rpm, initial pH 5.5 and $5x10^6$ spores/mL inoculum size. Partial characterization of the crude enzyme extract showed pH 5.5 and 37°C as optimal. The crude enzyme extract showed good stability at 30 and 40°C for 2,300 and 2,100 h, and at pH 5.5 for 1,500 h.

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