academicJournals

Vol. 12(40), pp. 5886-5896, 2 October, 2013
DOI: 10.5897/AJB12.970
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Experimental design applied to the optimization and partial characterization of pectin-methyl-esterase from a newly isolated *Penicillium brasilianum*

Jamile Zeni, Jonaína Gomes, Éllin Ambroszini,Geciane Toniazzo, Débora de Oliveira* and Eunice Valduga

Department of Food Engineering - URI - Campus de ErechimAv. Sete de Setembro, 1621 - Erechim - RS, 99700-000, Brazil.

Accepted 1 August, 2012

The objective of this work was to optimize the medium composition for maximum pectin-methyl-esterase (PME) production from a newly isolated strain of *Penicillium brasilianum* by submerged fermentation. A Plackett-Burman design was first used for the screening of most important factors, followed by a 2^3 full experimental design, to maximize the enzyme production. The maximum pectin-methyl-esterase activity was 4.0 U/mL at 24 h of bioproduction using a pectin concentration of 32.0 g/L, yeast extract of 30.0 g/L, potassium phosphate of 8.0 g/L, iron (II) sulfate of 0.02 g/L, at 30°C, stirring rate of 180 rpm and initial pH of 5.5. The kinetic evaluation showed that after 27 h of fermentation, a consumption of 15% of total organic carbon and 10% of nitrogen was observed. The crude enzymatic extract kept about 80% of its initial activity after 1848 h under low temperatures. An increase of PME activity was observed after incubation at high temperatures. The residual activity of the extracts after 1728 h of incubation was about 95% for all tested pH values (5, 7, 9 and 11). The application of 0.5% (v/v) of PME crude extract for clarification of peach juice showed a reduction on the viscosity (7.20%) and turbidity (14.11%).

Key words: *Penicillium brasilianum*, pectin-methyl-esterase, experimental design, pectin-methyl-esterase (PME).

INTRODUCTION

Pectin is a major plant cell wall polysaccharide contributing to tissue integrity and rigidity. The main structural element of pectin, governing most of its functional properties, is a linear homopolymer of α-(1-4) linked D-galacturonic acids, which can be methyl-esterified (Ridley et al., 2001). Changes in the pectin structure take place during fruit ripening, processing and storage, and can be both chemical (for example, alkaline demethoxylation, β-eliminative depolymerization) and enzymatic (Bordenave, 1996; Rexova-Benkova and Markovic, 1976).

Many of the structural polysaccharides in the plant-cell wall undergo enzymatic hydrolysis, transglycosylation reactions, solubilization and depolymerization, which are thought to contribute to wall loosening (Brummell, 2006; Fischer and Bernnett, 1991; Wakabayashi et al., 2001). The disassembly of the cell wall structure occurs through the action of hydrolases including pectinases like polygalacturonase (PG), pectate lyase and pectin methylesterase (PME) (Brummell and Haspster, 2001).

Pectin methylesterase (PME, EC 3.1.1.11) is an enzyme of either plant or microbial origin that catalyses

*Corresponding author. E-mail: odebora@uricer.edu.br. Tel: +55 54 35209000. Fax: +55 54 35209090.

the demethoxylation of pectin, thus altering the degree and pattern of methyl-esterification and releasing methanol (Voragem et al 2001). PMEs are thought to play an important role in determining the extent to which demethylated polygalacturonans are accessible to degradation by PGs (5), by releasing galacturonic acid (exo-PG) or oligogalacturonate (endo-PG). Their action also influences the availability of homogalacturonan carboxylic groups for Ca^{2+} binding. Increasing the number of Ca^{2+} bridges between homogalacturonan chains leads to a decrease in their accessibility to cell wall hydrolases (Micheli, 2001).

In spite of its industrial importance, just a few works are presented in the current literature about the microbial PMEs. Acidic microbial PMEs (*Aspergillus japonicus*, *Aspergillus niger*, *Aspergillus foetidus*) catalyze a random cleavage of esterified carboxyl groups (Jayani et al*.,* 2005; Ishii et al., 1979) (multiple chain mechanism). Alkaline PMEs from higher plants (tomato, orange, alfalfa) and from fungal origin (*Trichoderma reesei*) produce blocks of free carboxyls groups, that is, they catalyze demethoxylation of pectin linearly along the chain (single chain mechanism). Pectinases synthesized by filamentous fungi have some advantages, since the microorganisms have the ability of adaptation to a wide type of substrates, being excellent decomposers of organic material. Moreover, the fungi pectinases present important characteristics for application in bioprocesses, as stability to pH and temperature (Martins et al., 2004).

In this context, the objective of this study was to evaluate the PME production by a newly isolated strain of *Penicillium brasilianum* using submerged fermentation of a conventional media. After the media optimization, a kinetic evaluation was carried out and the enzymatic extract was partially characterized and tested for juice clarification.

MATERIALS AND METHODS

Microorganism

The microorganism used in the present study was previously isolated from tea. Newly isolated microorganism was identified by molecular biology technique, following the methodology described here. Firstly, after incubation in potato dextrose (PD) medium at 28°C for three to four days, we used a protocol for extraction of yeast genomic DNA using liquid nitrogen for cell disruption (Fernandes-Salomão et al., 1996) following DNA quantification using a spectrophotometer model NanoDrop, ND-1000 (NanoDrop Technologies). The newly isolated microorganism was identified as *P. brasilianum.*

The propagation of this culture was done on potato dextrose agar (PDA) slant medium containing malt extract (10 g/L), yeast extract (4 g/L), glucose (4 g/L) and agar (20 g/L) and incubated at 30°C until sporulation (1 week). Stock cultures of these strains were prepared with 20 weight% glycerol water and stored at −80°C (MDF - U3086S - Sanyo). The harvesting of the spores from the slants was done using 5 ml of Tween 80-water (0.02%). For spores counting, 1 ml of the suspension, aseptically withdraw, was diluted 10 to 10^3 times in sterile aqueous solution of Tween 80 (0.1 % v/v). The resulting suspension was transferred to Neubauer chamber for spores counting. To determine the best spore concentration (spores/mL) for the production of PME, a previous study was carried out (data not shown) using different spores concentration: 5.10^3, 5.10^4, 5.10^5, 5.10^6, 5.10^7 and 5.10^8. In 100 ml of bioproduction medium (10 g/L pectin, 10 g/L yeast extract, pH 5.5) 1 ml of spores was added, in the desired concentration. The cultivation conditions were 30°C, 180 rpm for 24, 48, 72 and 96 h. Higher PME activity (3.0 U/mL in 24 h) was obtained using a spore concentration of 5.10^6 spores/mL.

Pectin-methyl-esterase bioproduction

A Plackett-Burman experimental design was employed to evaluate the effects of the culture medium and the operational conditions on the bioproduction of pectin-methyl-esterase (PME). The independent variables were: concentrations of pectin (2 to 22 g/L), L-asparagine (0 to 4 g/L), yeast extract (0 to 20 g/L) and magnesium sulfate (0 to 1 g/L). The variables temperature (30°C), agitation (180 rpm), pH (5.5) and cell concentration (5.10^6 spores/mL) were kept fixed in all experiments.

A full 2^3 experimental design was carried out, taking into account the results of the first planning, evaluating the effects of concentrations of pectin (22 to 32 g/L), yeast extract (20 to 30 g/L), potassium phosphate (4 to 8 g/L), keeping fixed the temperature (30°C), agitation (180 rpm), pH (5.5), fermentation time (24 h) and iron sulfate (0.02 g/L).

Kinetic evaluation

The knowledge of the kinetics of a fermentative process is of extreme importance when transposing a laboratory experiment to an industrial scale, as well as when a quantitative comparison between different culture conditions is required.

The kinetics of substrate consumption (total nitrogen, potassium, magnesium and total organic carbon - TOC), cell mass, pH evolution and PME production was evaluated in the experimental condition previously maximized in the experimental design step (32 g/L pectin, 30 g/L yeast extract, 8 g/L potassium phosphate and 0.02 g/L iron sulfate, at 180 rpm, 30°C, pH 5.5 and initial cell concentration of 5.10^6 spores/mL) by periodic sampling of the fermentation medium.

Effect of salts and pectin concentration on the measurement of the pectin-methyl-esterase (PME) activity

From the maximized experimental condition defined previously, the effect of NaCl and $CaCl_2$ concentrations on the measurement of PME activity was evaluated. The salts concentration varied from 0.02 to 1 M, keeping fixed the citric pectin concentration at 1% (wt/v). Keeping the $CaCl_2$ concentration fixed at 0.5 M, the effect of pectin concentration on the enzyme activity was studied in the range from 0.5 to 2% (wt/v).

Partial characterization of the crude enzymatic extract

The partial characterization of enzymatic extracts is of fundamental importance for establishing the conditions of application. Such characteristics are optima temperature and pH and stability of the extract to the temperature and pH.

To determine the optimum values of temperature and pH in terms of enzyme activity for the PME from *P. brasilianum*, a central composite rotatable design (CCRD) 2^2 was accomplished using the enzymatic extract. The studied range for pH was from 5 to 11 and

the temperature varied from 30 to 80°C. The temperature of stability of the enzymatic extract was determined by enzyme incubation at a fixed pH (5.5) and different temperatures: 30 to 80°C. The stability pH was determined by incubation of the extract obtained by *P. brasilianum* at 40°C at the pH values ranging from 5.0 to 11.0. The samples were withdrawn from the reaction medium at regular time intervals. The stability of the crude enzymatic extract to low temperature was evaluated by the storage of the extract at 4, -20 and -80°C and determining the enzyme activity periodically.

Application of the crude enzymatic extract for the clarification of peach juice

A commercial peach juice (Sarandi, Brazil) was treated by the crude enzymatic extract obtained at the maximized conditions of bioproduction, defined previously. Enzyme concentrations of 0.01, 0.05, 0.1 and 0.5% (v/v) at 40°C, 100 rpm and 60 min were tested by evaluation of the viscosity, turbidity and percent of clarification of the juice.

Analytical determinations

Pectin-methyl-esterase activity

For all determination of PME activity, the bioproduction medium was filtered to separate the biomass. The filtrate was denominated by crude enzymatic extract: the activity of the pectin-methyl-esterase (PME) was determined following the methodology described by Hultin et al. (1966), with some modifications. 1 ml of enzymatic extract was added to 30 ml of citric pectin and 1ml of NaCl 0.2 M. The pH of the solution was adjusted to 7.0 for 10 min using NaOH 0.01 N. One unit of PME was defined as the amount of enzyme able to catalyze the demethylation of pectin corresponding to the consumption of 1 µmol of NaOH/min/mL, 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, in equipment Shimadzu model TOC-VCSH (AOAC, 1995).

Mineral compounds

Macro magnesium (Mg) and manganese (Mn) and micronutrients iron (Fe) and potassium (K) were determined by flame atomic absorption spectrometry - FAAS (Varian Spectra AA-55), following the methodology described by Association of Analytical Communities (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 nitrous oxide/acetylene. The readings of Mg, Mn, Fe and K were performed by FAAS, in absorption mode. To eliminate 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% (wt/v). For the determination of the minerals contents on 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).

Viscosity

The reduction of the viscosity of the peach juice after the enzymatic treatment using the crude extract was evaluated in viscometer "Falling Ball" (Ustok et al*.,* 2007).

Clarification

The clarification of the peach juice after enzymatic treatment with the crude extract was determined based on the color intensity (CI), by readings in spectrophotometer at 440 nm and 520 nm, with optical path of 1 mm (Silva et al., 2005), expressed as % of clarification, calculated taking into account the color intensity of control juice (without enzymatic treatment) and the enzyme-treated juice.

Turbidity

The reduction of turbidity of the juice after enzymatic treatment using the crude extract was calculated based on the absorbance, measured at wave length of 540 nm, using optical path of 1 mm against water, in spectrophotometer (Silva et al*.,* 2005), expressed as % of reduction of turbidity, calculated based on the absorbance of the control juice.

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 followed by Tukey's test. All analysis was performed using the software Statistica version 6.0 (Statsoft Inc, USA).

RESULTS AND DISCUSSION

Pectin-methyl-esterase bioproduction

Table 1 presents the matrix of the Plackett-Burman experimental design with the real and coded values of independent variables and the response in terms of pectin-methyl-esterase (PME) activity. From this table, one can verify that the maximum PME activity (2.50 U/mL) was obtained in the experiment 6, corresponding to the highest levels of pectin (22 g/L), yeast extract (20 g/L), L-asparagine (4 g/L), potassium phosphate (4 g/L) and iron II sulfate (0.02 g/L) concentrations.

The data presented in Table 1 were statistically treated and Figure 1 presents the Pareto chart with the estimated values of independent variables. The variables potassium phosphate, yeast extract and pectin concentrations presented a positive significant effect ($p < 0.05$), demonstrating that the displacement of these variables to upper levels could lead to an increase of enzyme activity. On the other side, the variables magnesium sulfate, L-

Table 1. Matrix of the Plackett-Burman experimental design with the coded and real values of independent variables and the response as pectin-methyl-esterase (PME) activity and pH.

Assay	Independent variable						Response	
	X_1	X_2	X_3	X_4	X_5	X_6	PME (U/mL)	pH
1	1 (22)	-1 (0)	1 (20)	-1 (0)	-1 (0)	-1 (0)	1.00	5.15
2	1 (22)	1 (4)	-1 (0)	1 (1)	-1 (0)	-1 (0)	0.40	4.67
3	-1 (2)	1 (4)	1(20)	-1 (0)	1 (4)	-1 (0)	2.00	5.65
4	1 (22)	-1 (0)	1 (20)	1 (1)	-1 (0)	1 (0.02)	1.00	5.38
5	1 (22)	1 (4)	-1 (0)	1 (1)	1 (4)	-1 (0)	1.30	4.56
6	1 (22)	1 (4)	1 (20)	-1 (0)	1 (4)	1 (0.02)	2.50	5.68
7	-1 (2)	1 (4)	1 (20)	1 (1)	-1 (0)	1 (0.02)	0.60	5.37
8	-1 (2)	-1 (0)	1 (20)	1 (1)	1 (4)	-1 (0)	2.00	5.50
9	-1 (2)	-1 (0)	-1 (0)	1 (1)	1 (4)	1 (0.02)	0.80	5.20
10	1 (22)	-1 (0)	-1 (0)	-1 (0)	1 (4)	1 (0.02)	1.50	4.44
11	-1 (2)	1 (4)	-1 (0)	-1 (0)	-1 (0)	1 (0.02)	0.20	4.56
12	-1 (2)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	0.00	4.92
13	0 (11)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	1,20	5.14
14	0 (11)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	1,10	5.17
15	0 (11)	0 (2)	0 (10)	0 (0.5)	0 (2)	0 (0.01)	1,00	5.11

X_1= Pectin (g/L); X_2= L-asparagine (g/L); X_3= yeast extract (g/L); X_4= magnesium sulfate (g/L); X_5= potassium sulfate (g/L). X_6 = iron II sulfate (g/L); Fixed variables: 180 rpm, 30°C, 24 h, $pH_{initial}$ 5.5 and initial cell concentration of 5.10^6 spores/mL.

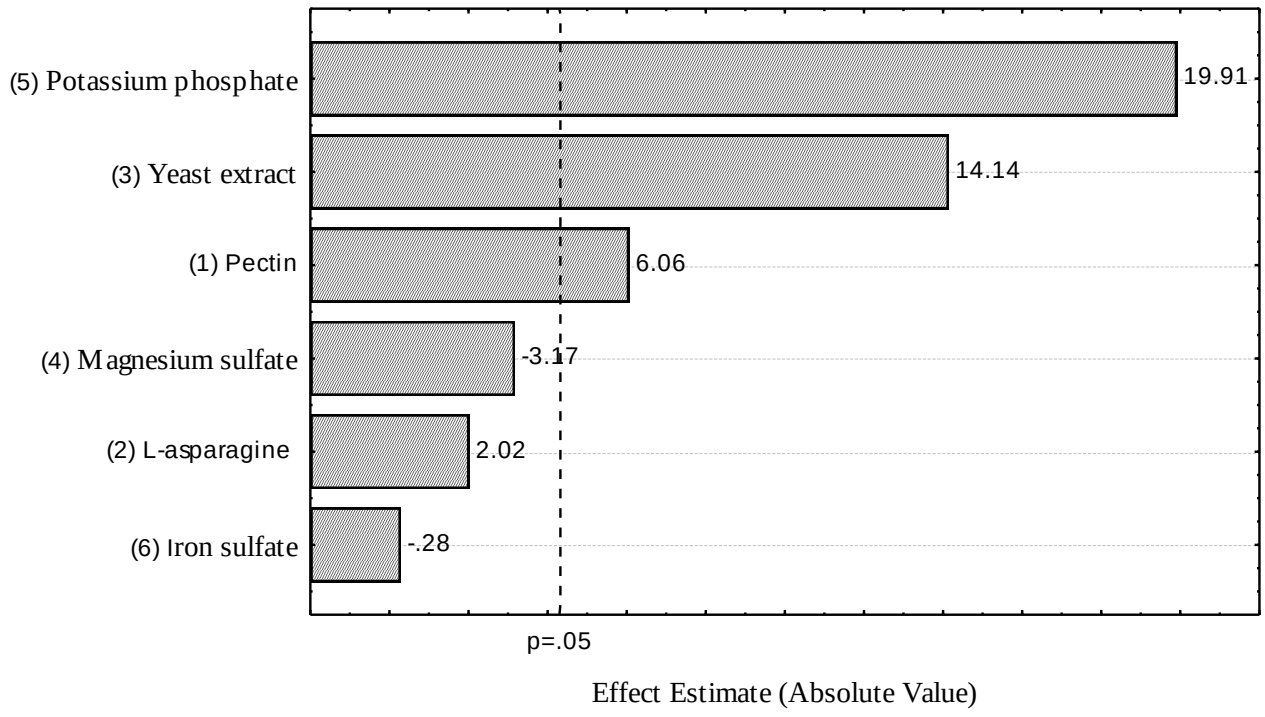

Figure 1. Pareto chart with the estimated effect (absolute value) of the studied variables on the Plackett-Burman experimental design for pectin-methyl-esterase (PME) production.

asparagine and iron (II) sulfate concentrations did not influence significantly ($p < 0.05$) the enzyme production, within the studied ranges. Based on this information, magnesium sulfate and L-asparagine were not added to

Table 2. Matrix of the 2^3 factorial design and response as pectin-methyl-esterase (PME) activity and pH.

Assay	Independent variable			Response	
	X_1	X_3	X_5	PME (U/mL)	pH
1	-1 (22)	-1 (20)	-1 (4)	1.70	5.20
2	1 (32)	-1 (20)	-1 (4)	2.10	4.92
3	-1 (22)	1 (30)	-1 (4)	2.50	5.16
4	1 (32)	1 (30)	-1 (4)	2.40	5.02
5	-1 (22)	-1 (20)	1 (8)	3.10	5.07
6	1 (32)	-1 (20)	1 (8)	3.50	4.96
7	-1 (22)	1 (30)	1 (8)	3.50	5.14
8	1 (32)	1 (30)	1 (8)	4.00	5.00
9	0 (27)	0 (25)	0 (6)	3.20	5.01
10	0 (27)	0 (25)	0 (6)	3.00	5.05
11	0 (27)	0 (25)	0 (6)	3.20	5.10

*X_1= Pectin (g/L), X_3= yeast extract (g/L), X_5= potassium phosphate (g/L). Fixed: iron (II) sulfate 0.02 g/L, 180rpm, 30°C, 24 h and $pH_{initial}$=5.5.

the production medium in the second experimental design. The iron (II) sulfate was fixed at 0.02 g/L, based on the highest PME activity achieved in experiment 6 of Table 1. A 2^3 full experimental design was carried out in order to maximize the enzyme production. Results obtained in this step are presented in Table 2.

From Table 2, one can verify that the maximum PME activity (4.0 U/mL) was obtained in experiment 8 (32 g/L pectin, 30 g/L yeast extract, 8 g/L potassium phosphate, 0.02 g/L iron (II) sulfate) at 180 rpm, 30°C, 24 h of bioproduction, initial pH of 5.5 and initial cell concentration of 5.10^6 spores/ml. The variables potassium phosphate and yeast extract presented a positive significant effect ($p < 0.05$) on the enzyme production and the pectin concentration influenced the pH reduction.

Equation 1 presents the first order coded model as a function of pectin, yeast extract and potassium phosphate concentrations. The model was validated by analysis of variance, with a correlation coefficient of 0.95 and a calculated F 7.48 times higher than the tabled one. The non significant parameters were added to the lack of fit for the analysis of variance (ANOVA) test.

$$PME = 2.93 + 0.25\ X_3 + 0.67\ X_5 \quad (1)$$

Where, PME = pectin-methyl-esterase activity (U/mL); X_3 = yeast extract (g/L); X_5= potassium phosphate (g/L).

Kinetic evaluation

The kinetic evaluation of the PME bioproduction, substrate consumption, biomass and pH was carried out taking into account the experimental conditions maximized (32 g/L pectin, 30 g/L yeast extract, 8 g/L potassium phosphate, 0.02 g/L iron (II) sulfate, 5.10^6 spores/mL, 180 rpm and initial pH 5.5) in the experimental design step performed in flasks (Figure 2).

From Figure 2a, one can observe that the maximum activity (4.80 U/mL) was reached after 27 h of bioproduction. The highest PME activity can be related to the beginning of the exponential phase for microorganism growth. The pH value was stable (approximately 5.5) during the first 36 h of bioproduction, with posterior decrease, reaching, after 48 h, values of about 4.5.

The analysis of the substrates assimilation permit us to observe a similar behavior compared to the nitrogen (Figure 2a), iron and potassium (Figure 2b), showing a slow and gradual decrease during the bioproduction, with a consumption of 13, 13 and 11%, respectively. From the mineral sources, the more expressive assimilation was observed for manganese and magnesium, corresponding to 26 and 82%, respectively.

The concentration of total organic carbon was practically constant until 24 and at 27 h period of maximum PME bioproduction (4.80 U/mL); it had the highest consumption of the carbon source (15%). After this period, the carbon concentration was constant (222 to 210 mg/L).

Studies about the production of PME by microorganisms are scarce in the current literature. The most cited works are referred to the enzymatic extract from papaya (Chatterjee et al., 2004; Lourenço and Catutani, 1984), apple (Johnston et al*.,* 2002), peach (Oliveira et al., 2005), raspberry (Lannetta et al*.,* 1999), carambola (Chin et al., 1999), pear (Brummell and Haspster*,* 2004), tomato (Hobson, 1963; Resende et al., 2004) banana (Hiltin and Levine, 1966; Sales et al*.,* 2004) and mango (Prasanna et al., 2003). Here, it is important to mention that the PME activity obtained by

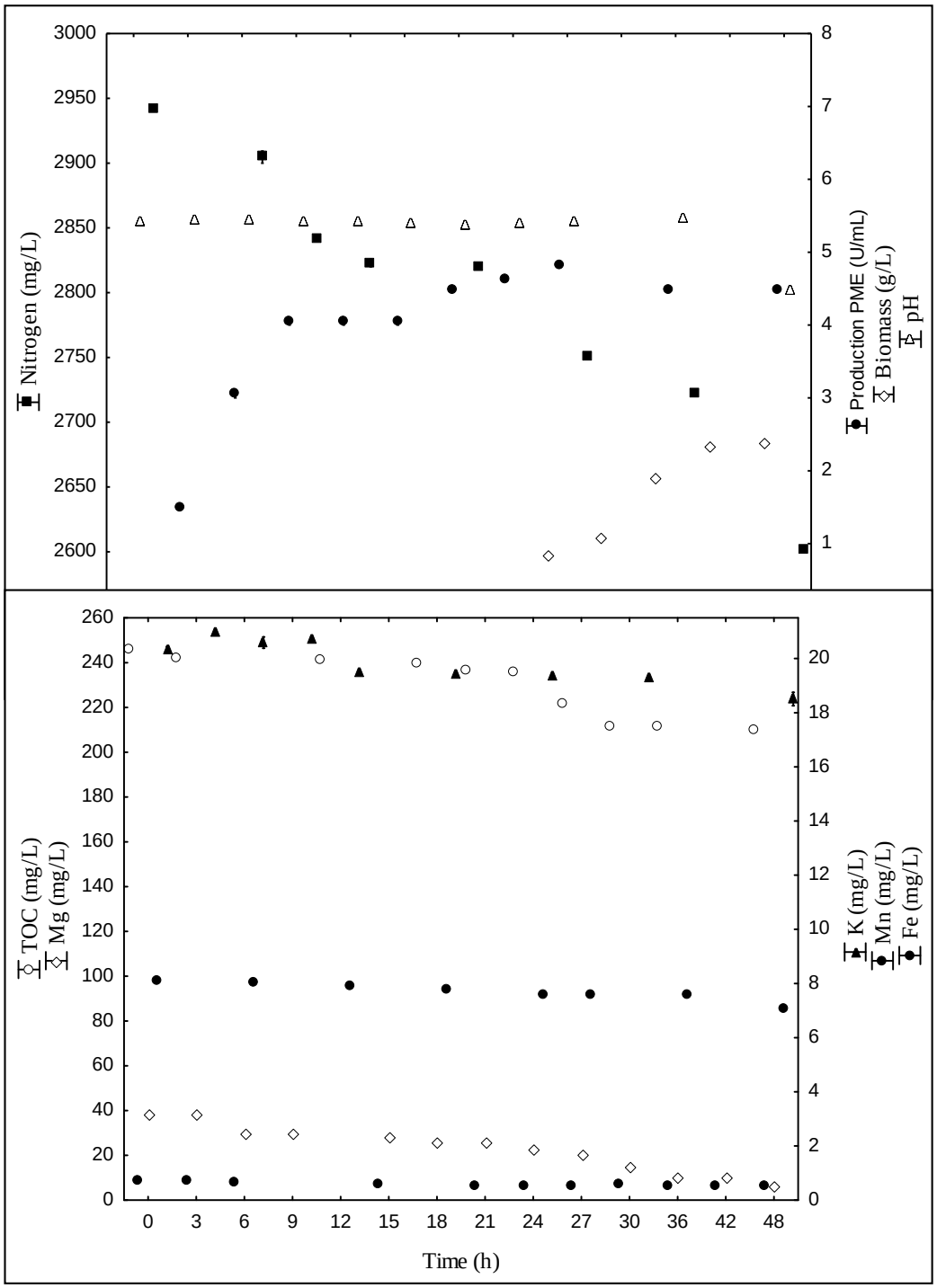

Figure 2. Kinetic curves for bioproduction of pectin-methyl-esterase (PME) as a function of enzyme activity (U/mL), biomass production (g/L), nitrogen and pH (a) and carbon consumption and minerals content (b).

the filamentous fungi *P. brasillianum* (4.80U/mL) was similar to that cited by Mantovani et al. (2005) for a commercial PME extract (Pectinex AR), which presented activity of 3.58 U/mL and higher than that obtained from industrial residues of pineapple pump (*Ananas comosus* L. Merr*)* (1.47 U/mL) (Kapoor et al., 2001).

Table 3 presents the PME activity (U/mL) in different concentrations of NaCl, $CaCl_2$ and mixture of NaCl/$CaCl_2$ for the measurement of the enzyme activity. From this table, one can observe that when $CaCl_2$ and 1.0% of citric

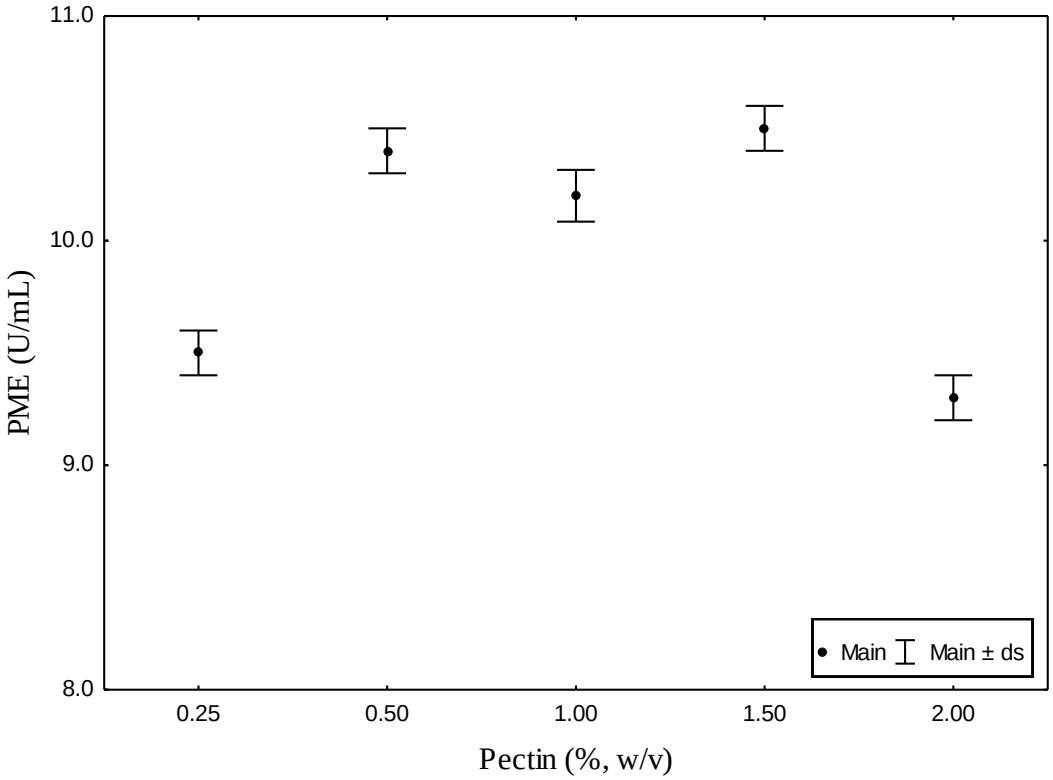

Figure 3. Pectin-methyl-esterase (PME) activity (U/mL) using different concentrations of citric pectin.

Table 3. Pectin-methyl-esterase (PME) activity (U/mL) in different concentrations of NaCl, $CaCl_2$ and mixture of NaCl/$CaCl_2$.

Salt concentration (M)	PME activity (U/mL)		
	NaCl	$CaCl_2$	NaCl/$CaCl_2$
0.02	5.10^{aA} (± 0.14)	4.87^{eA} (± 0.25)	3.30^{iB} (± 0.20)
0.04	5.25^{Aa} (± 0.16)	5.43^{eA} (± 0.12)	5.00^{hA} (± 0.28)
0.06	5.55^{aB} (± 0.29)	6.63^{dAB} (± 0.32)	7.27^{fA} (± 0.29)
0.08	5.90^{aB} (± 0.15)	6.70^{dA} (± 0.20)	6.9^{fA} (± 0.10)
0.1	5.05^{aC} (± 0.07)	7.27^{cdB} (± 0.23)	8.15^{eA} (± 0.07)
0.15	5.70^{aB} (± 0.30)	8.47^{cA} (± 0.25)	8.55^{deA} (± 0.35)
0.2	5.27^{aB} (± 0.35)	8.63^{bA} (± 0.23)	8.80^{dA} (± 0.10)
0.25	5.75^{aB} (± 0.35)	8.33^{cA} (± 0.21)	6.93^{fB} (± 0.32)
0.3	5.25^{aC} (± 0.07)	7.80^{cA} (± 0.30)	7.17^{fB} (± 0.31)
0.4	4.73^{aC} (± 0.15)	9.00^{bB} (± 0.15)	9.97^{bA} (± 0.15)
0.5	5.20^{aC} (± 0.12)	10.17 ± 0.25^{aA}	9.33 ± 0.15^{cB}
0.75	5.27 ± 0.35^{aB}	10.13 ± 0.38^{aA}	10.70 ± 0.10^{abA}
1	5.57 ± 0.45^{aB}	11.23 ± 0.23^{aA}	11.20 ± 0.20^{aA}

*PME activity (U/mL) ± standard deviation followed by equal lowercase/uppercase letters on the columns/lines indicates no significant difference at a confidence level of 5%.

pectin was used, the highest PME activities were obtained: 10.17, 10.13 and 11.23 U/mL, at salt concentration of 0.50, 0.75 and 1 M, respectively; these did not differ statistically ($p < 0.05$) from each other. This study was performed because the ions Na^+ and Ca^{+2} on the reaction medium can interact with the substrate (pectin)

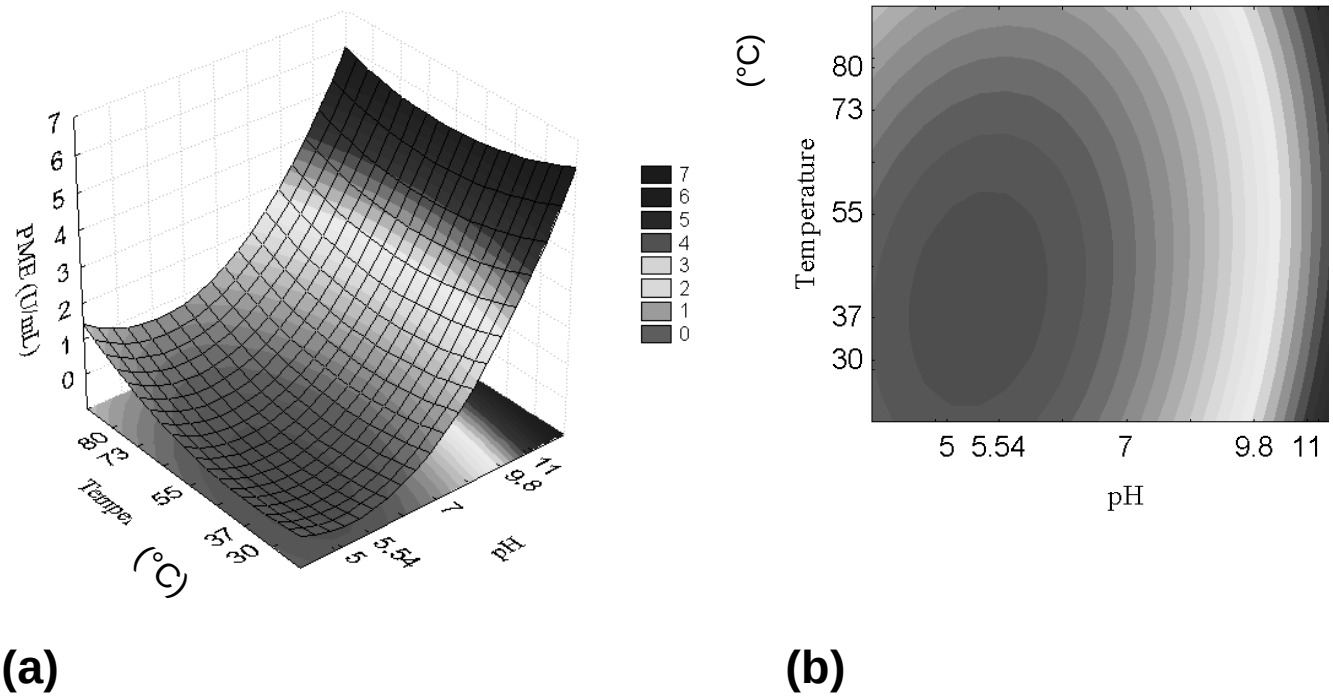

Figure 4. Response surface (a) and contour curve (b) for pectin-methyl-esterase (PME) activity (U/mL) as a function of temperature (°C) and pH.

catalyzing the removal of methoxyl groups. In this case, the PME activity is higher when the pectin is methylated.

Figure 3 presents the PME activity using different concentrations of citric pectin. From this figure, it can be observed that the pectin concentrations of 0.5, 1.0 and 1.5% did not present significant difference (confidence level of 95%) among them, but differed from those that used 0.25 and 2% of pectin for the measurement of enzyme activity.

Cardello and Lourenço (1992) evaluated the use of NaCl and $CaCl_2$ and 0.125% of pectin in the reaction medium and verified that the best results were obtained using NaCl and $CaCl_2$ concentrations of 0.15 and 0.04 M, respectively, reaching activities of about 6 U/ml for both salts. When the same authors evaluated the effect of salts association (NaCl and $CaCl_2$) and 0.5% of citric pectin, varying the molarity, they verified that the activity was about 9U/mL, for all tested molarities (0.04 to 0.5 M).

Partial characterization of pectin-methyl-esterase (PME) crude extract

Optimum temperature and pH

Table 4 presents the matrix of the 2^2 full experimental design with the response as PME activity as a function of temperature and pH. The highest PME activity (40 U/mL) was obtained in experiment 6, at 55°C and pH 11. Similar results were observed in experiments 2 and 4, showing a range of optimum temperature (37 to 73°C) and pH (9 to 11).

Equation 2 presents the second order coded model for the PME activity as a function of temperature and pH, within the evaluated range. It is possible to observe that the variables influenced the enzyme activity. The model was validated by analysis of variance, with a correlation coefficient of 0.98 and a calculated F 4.9 times higher than the tabled one, making possible to build the response surface and contour curve, presented in Figure 4. The non significant factors were added to the lack of fit by analysis of variance (ANOVA).

$$PME\ (U/mL) = 11.744 + 13.754.pH + 6.314.(pH)^2 + 1.537.T + 1.761.(T)^2 - 1.150.(pH).(T) \quad (2)$$

Where, PME = pectin-methyl-esterase activity (U/mL); T = temperature (°C).

From Figure 4, it is possible to verify a wide range of temperature (30 to 80°C) and pH (9 to 11) that lead to higher PME activities. The literature points out the influence of pH and temperature on the measurement of the PME activity, mainly for pectinases extracted from fruits. Delgado et al., (1992) determined the optimal conditions for measure-ment of the activity of commercial PME (Pectinex 100 L Plus, Panzym Univers and Panzym

Table 4. Matrix of the full 2^2 experimental design with real and coded values and the response as pectin-methyl-esterase (PME) activity as a function of temperature and pH.

Assay	*Independent variable		PME activity (U/mL)
	pH	Temperature (°C)	
1	-1 (5.54)	-1 (37)	5.00
2	1 (9.8)	-1 (37)	36.00
3	-1 (5.54)	1 (73)	11.60
4	1 (9.8)	1 (73)	38.00
5	-1.41 (5)	0 (55)	2.90
6	1.41 (11)	0 (55)	40.00
7	0 (7)	-1.41 (30)	11.10
8	0 (7)	1.41 (80)	13.70
9	0 (7)	0 (55)	11.60
10	0 (7)	0 (55)	11.80
11	0 (7)	0 (55)	11.90

Clears) and observed that higher activities were obtained in pH values from 4.0 to 4.5 and temperature of 45°C. Assis et al. (2002) related higher activities for PME from acerola (*Malpighia glabra L.*) at pH of 9.0. Researches using other fruits as PME source presented higher activities at lower values of pH, as 8.0 for pectin-methyl-esterases from orange juice (Amaral et al., 2005), or 7.5 (Termote and Ilnik, 1977). Ly-Nguyen et al. (2002) found the optimal pH as 7.0 using crude PME extracts from strawberries. Targano et al. (1994), evaluating the activity of pectin-methyl-esterase (PME) and polygalacturonase (PG) of peaches, verified that the PG showed maximum activity at pH values around 5.0, and the PME showed higher activities at pH 7.0. The optimal temperature for both enzymes was 25°C.

Arbaisah et al. (1997) studied the purification of PME extracted from pulp of graviola (*Anona muricata*) and determined that the optimal range of pH was 7.0 to 8.0. Amaral et al. (2005) evaluated the effect of temperature on the activity of purified PME from orange and determined the optimal temperature as 50°C.

Thermal stability

The crude PME extract was stored at -80, -20 and 4°C and the enzyme activity was measured periodically. The extract kept 80% of its initial activity after storage at all three temperatures for, at least, 1848 h.

The stability of PME extract at high temperatures showed a differenced behavior. An increase of 15, 25, 10, 10 and 50% was observed for PME activity at 30, 40, 50, 60 and 70°C, after 1848 h, respectively. The PME activity at 100°C presented a decrease of 25% compared to its original value.

Results obtained here are similar to those of Kapoor et al., (2001) which evaluated the thermal stability of concentrated extract of PME from pineapple and verified an increase on the activity of 45.9% at 25°C and 100% after incubation at 40°C, both during 60 min. Leite et al. (2006) evaluated the thermal stability of concentrated PME extract from guava cv. Paluma and verified that samples submitted to 75°C presented an enhancement on enzyme activity of 64.77% compared to the initial activity.

Stability to pH

The stability of the crude PME was evaluated by incubation of the extract at different pH values (5, 7, 9 and 11) and monitoring the activity with time. The residual activity of the extracts after 1728 h of incubation was about 95%, for all tested pH values.

Cardello and Lourenço (1992) evaluated the stability of the partially purified PME extract from eggplant by incubation during 60 min and verified that the extract was stable in pH values in the range of 5 from 8.

Preliminary application of crude pectin-methyl-esterase (PME) extract to the clarification of peach juice

Table 5 presents the reduction of viscosity, turbidity, pH and soluble solids of the peach juice treated with pectin-methyl-esterase. From this table one can observe that the maximum reduction was 7.20 and 14.11% on the viscosity and turbidity, respectively, using an enzyme concentration of 0.5% (v/v) at 40°C, 100 rpm during 60 min. The pH of the juice was 3.69.

Results obtained here can be explained since pectinases hydrolyze pectin, leading to the flocculation of the complex pectin-protein (Lee et al., 2006) resulting in juice with reduced content of macromolecules and low turbidity (Sandri et al., 2011). The enzymatic treatment

Table 5. Reduction of viscosity, turbidity, pH and soluble solids of the peach juice treated with pectin-methyl-esterase.

Parameter	Enzymatic treatment**			
	0.01 % (v/v)	0.05 % (v/v)	0.1 % (v/v)	0.5 % (v/v)
Reduction of viscosity (%)	5.65^{c} (± 0.74)	4.42^{d} (± 0.20)	5.93^{b} (± 0.01)	7.20^{a} (± 0.20)
Reduction of turbidity (%)	0.08^{d} (± 0.14)	4.85^{c} (± 0.53)	11.96^{b} (± 0.08)	14.11^{a} (± 0.28)
pH				
Control*	3.67^{a} (± 0.02)	3.67^{a} (± 0.02)	3.69^{a} (± 0.01)	3.68^{a} (± 0.02)
Treated	3.66^{a} (± 0.03)	3.68 ^{a} (± 0.01)	3.68^{a} (± 0.01)	3.69^{a} (± 0.01)
Soluble solids (ºBrix)				
Control*	13.00^{a} (± 0.01)	13.00^{a} (± 0.01)	13.00^{a} (± 0.01)	13.00^{a} (± 0.01)
Treated	13.00^{a} (± 0.01)	13.00^{a} (± 0.01)	13.00^{a} (± 0.01)	13.00^{a} (± 0.01)

*juice without enzymatic treatment; ** Mean ±standard deviation followed by equal letters in the lines indicates no significant difference at a confidence level of 5%.

for degradation of pectin could cause reduction on the ability to retain water, reducing the viscosity of the juice by the liberation of water to the system (Sandri et al*.,* 2011). The pectin, in aqueous solution, can make the environment more viscous, even at low concentrations, depending on the degree of methoxylation (Lofgren and Hermansson, 2007).

The low efficacy of the PME extract on the reduction of turbidity and viscosity can be associated to the fact that the extract also presents pectin-lyase activity (2.96 U/mL) and possibly other non-measured activities. Clemente and Pastore, (1998) and Vámos-Vigyázó (1981) used a commercial enzyme (Pectinex), with PME and cellulase activities, and verified that the use of this enzyme improved performance of clarification process for peach juice, compared to the employment of isolated enzymes.

The enzymatic treatment and the time of processing (60 minutes) did not influence the values of pH and soluble solids. Similar values compared to the *in natura* juice were observed. This behavior was also verified by Ushikubo et al. (2007), Cao et al. (1992) and Barros et al. (2003), in the clarification process of different fruit juices. Santin et al*.* (2008) evaluated the viscosity of peach juice using a commercial pectinase, Pectinex AFPL-3 (Novozymes), at 45°C, 30 min of processing and enzyme concentration of 200 ppm. A reduction on viscosity of about 29% was observed.

Taking into account the results obtained for the clarification of peach juice evaluated here and also the partial characterization of crude PME extract, one can suggest other promising industrial applications for the extract, especially those that occur at high temperatures and pH near to the neutrality, such as production of processed fruit and/or lettuce food (purees, pulps, ketchup and pet food). Kashyap et al. (2001) showed that peels, seed residues and pulp ejected by the citrus fruit extractor can be used to make dried animal feed and the mixture is lime treated to increase the pH to 8.0 to take advantage of the citrus PME. Papaya puree is prepared by conversion of papaya-fresh into a semi-liquid product. As the puree is viscous even if the fruit contains endogenous PME and PG, it is necessary to depectinize it before concentration.

Conclusions

The experimental condition that conducted to the maximal production of PME (4.8 U/mL) in shaken flasks was: 32 g/L pectin, 30 g/L yeast extract, 8 g/L potassium phosphate and 0.02 g/L iron sulfate, 5.10^{6} spores/mL, 30°C, 27 h of fermentation, initial pH 5.5 and 180 rpm. The addition of $CaCl_2$ (0.5 M) and citric pectin (0.5%) led to an increase of 2.3 times in the PME activity.

The partial characterization of the crude PME extract indicated that higher activities (36 to 40 U/mL) were obtained at pH range from 9 to 11 in a wide interval of temperature (30 to 80°C). The extract kept about 80% of its activity after storage at 4°C during 1848 h. An increase on the PME activity was observed at high temperatures. The extract was stable during 1728 h at different pH values (5, 7, 9 and 11). The application of 0.5% (v/v) of PME crude extract for clarification of peach juice showed a reduction on the viscosity of 7.20% and turbidity of 14.11%.

ACKNOWLEDGEMENTS

The authors thank CNPq, CAPES, FAPERGS and URI-Campus de Erechim for the financial support and scholarships.

REFERENCES

Amaral SH, Assis SA, Oliveira OMMF (2005). Partial Purification and Characterization of Pectin Methylesterase From Orange (*Citrus Sinensis*) Cv. Pera- Rio. J. Food Biochem. 29:367-380.

Arbaisah SM, Asbi BA, Junainah AH, Jamilah B (1997). Purification and properties of pectinesterase from soursop (*Anona muricata*) pulp. Food Chem. 59(1):33-40.
Assis SA, Martins ABG, Quaglianoni DG, Oliveira OMMF (2002). Partial Purification and Characterization of Pectina Methylesterase from Acerola (*Malpighia glabra* L.). J. Agric. Food Chem. 50:4103-4107.
Association of Official Analytical Chemists - AOAC (1995). Official methods of analysis of the association of the analytical chemists, 16 ed. Washington.
Barros STD, Andrade CMG, Mendes ES, Peres L (2003). Study of fouling mechanism in pineapple juice clarification by ultrafiltration. J. Membrane Sci. 215(1-2):213-224.
Bordenave M (1996). Analysis of pectin methyl esterases. In H. F. Linskens (Ed.), Plant cell wall analysis. Berlin, New York: Springer. pp. 165-180.
Brummell DA (2006). Cell wall disassembly in ripening fruit, Funct. Plant Biol. 33:103-119.
Brummell DA, Harpster MH (2001). Cell wall metabolism in fruit softening and quality its manipulation in transgenic plant. Plant Mol. Biol. 47:311-340.
Brummell DA, Harpster MH (2004). Cell wall metabolism during maturation, ripening and senescence of peach fruit. J. Exp. Bot. 55(405):2029-2039.
Cao J, Zheng L, Chen S (1992) Screening of pectinase producer from alkalophilic bacteria and study on its potential application in degumming of ramie. Enzyme Microbial Technol. 14:1013-1016.
Cardello L, Lourenço EJ (1992). Partial purification and characterization of pectinmethylesterase from egg plant. Alimento Nutrição 4:111-123.
Chatterjee S, Chatterjee S, Chatterjee BP, Guha AP (2004). Clarification of fruit juice with chitosan. Process Biochem. 39:2229-2233.
Chin LH, Mohd Z, Lazan H (1999). Cell wall modification, degrading enzymes and softening of carambola fruit during ripening. J. Exp. Bot. 50(335):767-775.
Clemente E, Pastore GM (1998) Peroxidase and polyphenoloxidase, the importace for Food Technology. Boletim Sociedade Brasileira de Ciência e Tecnologia de Alimentos 32(2):167-171.
Delgado L, Blanca AT, Huitron C, Aguilar G (1992). Pectin lyase from *Aspergillus* sp. CH-Y-1043. Appl. Microbiol. Biotechnol. 39:515-519.
Fernandes-Salomão TM, Amorim ACR, Chaves-alves VM, Coelho JLC, Silva DO, Araujo EF (1996). Isolation of pectinase hyperproducing mutants of *Penicillium expansun*. Revista de Microbiologia 27:15-18.
Fischer RL, Bennett AB (1991). Role of cell wall hydrolases in fruit ripening. Annual Rev. Plant Physiol. 42:675-703.
Hiltin HO, Levine AS (1966). Pectin methylesterase in the ripening banana. J. Food Sci. 30:917-921.
Hobson GE (1963). Pectinesterase in normal and abnormal tomato fruit. Biochem. J. 86:358-365.
Hultin HO, Sun B, Bulger J (1966). Pectin methyl esterase of the banana. Purification and properties. J. Food Sci. 31(3):320-327.
Ishii K, Kiho S, Sugiyama S, Sugimoto H (1979). Low-methoxyl pectin prepared by pectinesterase from *Aspergillus japonicus*. J. Food Sci. 44:611-614.
Jayani RS, Saxena S, Gupta R (2005). Microbial pectinolytic enzymes:A Rev. Process Biochem. 40(9):2931-2944.
Johnston JW, Hewett EW, Hertog MLATM (2002). Postharvest softening of apple (*Malus domestica*) fruit: a review. New Zealand J. Crop Hort. Sci. 30:145-160.
Kapoor M, Beg QK, Bhushan B, Singh K, Dadhich KS, Hoondal GS (2001). Application of alkaline thermostable polygalacturonase from *Bacillus* sp. MG-ep-2 in degumming of ramie (*Boehemeria nivea*) and sunn hemp (*Crotalaria juncea*) bast fibers. Process Biochem. 6:803-807.
Kashyap DR, Vohra PK, Chopra S, Tewari R (2001). Applications of pectinases in the commercial sector: a review. Boiresour. Technol. 77:215-227.
Lannetta PPM, Van Den Berg J, Wheatley RE, McNicol RJ, Davies HV (1999). The role of ethylene and cell wall modifying enzymes in raspberry (*Rubus idaeus*) fruit ripening. Physiologia Plantarum 105:338-347.
Lee WC, Yusof S, Hamid NSA, Baharin BS (2006). Optimizing conditions for enzymatic clarification of banana juice response surface methodology (RSM). J. Food Eng. 73:55-63.
Leite KMSC, Tadiotti AC, Baldochi D, Oliveira OMMFO (2006). Partial purification, heat stability and kinetic characterization of the pectinmethylesterase from Brasilian guava, Paluma cultivars. Food Chemistry 94:565-572.
Lofgren C, Hermansson AM (2007). Synergistic rheological behaviour of mixed HM/LM pectin gels. Food Hydrocolloids 21:480-486.
Lourenço EJ, Catutani AT (1984). Purification and properties of pectinesterase from papaya. J. Sci. Food Agric. 35:1121-1127.
Ly-nguyen B, Loey AV, Fachin D, Verlent I, Duvetter T, Vu ST, Smout C, Hendrickx ME (2002). Strawberry Pectin Methylesterase (PME):Purification, Characterization, Thermal and High-Pressure Inactivation. Biotechnology Progress 18(6):1447-1450.
Mantovani CF, Geimb MP, Brandelli A (2005). Enzymatic clarification of fruit juices by fungal pectin lyase. Food Biotechnol. 19:73-81.
Martins ES, Silva D, Da Silva R, Gomes E (2004) Solid state production of thermostable pectinases from thermophilic *Thermoascus aurantiacus*. Process Biochem. 37:949-954.
Micheli F (2001). Pectin methylesterases:cell wall enzymes with important roles in plant physiology. Trends Plant Sci. 6:414-419.
Oliveira FER, De Abreu CMP, Asmar SA, Corrêa AD, Dos Santo CD (2005). Firmeza de pêssegos 'Diamante' tratados com 1-MCP. Revista Brasileira de Fruticultura 27(3):366-368.
Prasanna V, Yashoda HM, Prabha TN, Tharanathan RN (2003). Pectic polysaccharides during ripening of mango (*Mangifera indica* L.). J. Sci. Food Agric. 83:1182-1186.
Resende JM, Chitarra MIF, Maluf WR, Chitarra AB, Saggin Júnior OJ (2004) Atividade de enzimas pectinametilesterase e poligalacturonase durante o amadurecimento de tomates do grupo multilocular. Horticultura Brasileira 22(2):206-212.
Rexova-Benkova L, Markovic O (1976). Pectic enzymes. Advances in Carbohydrate Chem. Biochem. 33:323-385.
Ridley BL, O'Neill MA, Mohnen DA (2001). Pectins:Structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 57(6):929-967.
Sales AN, Botrel N, Coelho AHR (2004). Aplicação de 1-metilciclopropeno em banana 'prata-anã' e seus efeitos sobre as substâncias pécticas e enzimas pectinolíticas. Ciência e Agrotecnologia 28(3):479-487.
Sandri IG, Fontana RC, Barfknecht M, Moura da Silveira M (2011). Clarification of fruit juices by fungal pectinases. LWT - Food Sci. Technol. Doi:10.1016/ j.lwt. 2011.02.008.
Santin MM, Valduga E, Treichel H, Cabral L, Di Luccio M (2008). Evaluation of enzymatic treatment of peach juice using response surface methodology. J. Sci. Food Agric. 88:507-512.
Silva D, Martins ES, Silva R, Gomes E (2005). Pectinase production by *Penicillium viridicatum* RFC3 by solid state fermentation using agricultural wastes and agro-industrial by-products. J. Microbiol. 33(4):318-324.
Targano VM, Pelosof AMR (1994). Application of Doehlert design for water activity, pH and fermentation time, optimization for *Aspergillus niger* pectinolytic activity and production in solid state and submerged fermentations. Enzyme Microbiol. Technol. 25:411-419.
Termote F, Rombouts FM, Pilnik W (1977). Stabilization of cloud in pectinesterase active orange juice by pectic acid hydrolysates. J. Food Biochem. 1:15-34.
Ushikubo FY, Watanabe AP, Viotto LA (2007). Microfiltration of umbu (*Spondias tuberose* Arr. Cam.) juice. J. Membr. Sci. 288(1-2):61-66.
Ustok FI, Tari C, Gogus N (2007). Solid-state production of polygalacturonase by *Aspergillus sojae* ATCC 20235. J. Biotechnol.127:322-334.
Vámos-Vigyázó L (1981). Polyphenoloxidade and peroxidase in fruits and vegetables. Central Food Research Institute, semptember, Budapest, Hungary. pp. 49-127.
Voragem F, Beldman G, Schols H (2001). Chemistry and enzymology of pectins. In:McCleary, V.; Prosky, L., Edtors. Advanced dietary fibre technology, Blackwell Science, London. pp. 379-398.
Wakabayashi K, Chub JP, Huber JD (2001). Extensive solubilization and depolymerization of cell wall polysaccharides during avocado (*Persea americana*) ripening involves concerted action of poligalacturonase and pectinmethylesterase. Physiologia Plantarum 108:345-352.