

# Determination of pectin methylesterase activity in commercial pectinases and study of the inactivation kinetics through two potentiometric procedures

*Determinação da atividade da pectina metilesterase de pectinases comerciais e estudo da inativação por dois procedimentos potenciométricos*

Samantha Lemke GONZALEZ<sup>1</sup>, Neiva Deliberali ROSSO<sup>1\*</sup>

## Abstract

Pectinases are enzymes that degrade pectic substances and are widely used in juice and fruit beverages to improve the quality of the process. The objective of this study was to determine the optimum pH and temperature of two samples of commercial pectinases and propose an alternative procedure to determine the residual activity comparing the data with those of the traditional procedure. The pectin methylesterase (PME) activity in Pectinex 100 L Plus and Panzyn Clears was determined by potentiometry. The reaction consisted of 5.00 mg.mL<sup>-1</sup> apple pectin, 0.100 mol.L<sup>-1</sup> NaCl, and 50 µL enzyme to a total volume of 30 mL. The pectin reaction in the presence of PME in all experiments revealed a first order kinetics. The PME in the two enzyme preparations showed higher activity at pH 4.0 to 4.5 and temperature of 45 °C. From the results of both procedures  $\Delta V_{\text{NaOH}}/\Delta t$  and  $\Delta \text{pH}/\Delta t$ , it was concluded that the inactivation of PME occurred at 75 °C. The results obtained from the ratio  $\Delta \text{pH}/\Delta t$  showed good correlation with those obtained from the ratio  $\Delta V_{\text{NaOH}}/\Delta t$ . In the reaction accompanied by the ratio  $\Delta \text{pH}/\Delta t$ , the release of H<sub>3</sub>O<sup>+</sup> occurred in the real time reaction.

**Keywords:** pectin; pectin methylesterase; potentiometric procedure; residual activity.

## Resumo

As pectinases são enzimas que degradam substâncias pécicas, são amplamente utilizadas pelas indústrias de sucos e bebidas para melhorar a qualidade do processo. O objetivo deste trabalho consistiu em determinar o pH e a temperatura ótima de duas amostras de pectinases industriais, propor um procedimento alternativo para determinar a atividade residual e comparar os resultados com aqueles do procedimento tradicional. A atividade da PME na Pectinex 100 L Plus e Panzyn Clears foi determinada por potencimetria. A reação consistiu de 5,00 mg.mL<sup>-1</sup> de pectina de maçã, 0,100 mol.L<sup>-1</sup> de cloreto de sódio e 50 µL de enzima pectinolítica para um volume total de 30 mL. A reação da pectina em presença da PME em todos os experimentos mostrou uma cinética de primeira ordem. A PME nas duas preparações enzimáticas apresentou maior atividade em pH 4,0 a 4,5 a 45 °C. A partir dos resultados de ambos os procedimentos  $V_{\text{NaOH}}/\Delta t_{\text{tempo}}$  e  $\Delta \text{pH}/\Delta t_{\text{tempo}}$ , concluiu-se que a inativação da PME ocorreu na temperatura de 75 °C. Os resultados obtidos por  $\Delta \text{pH}/\Delta t_{\text{tempo}}$  apresentaram boa correlação com aqueles por  $\Delta V_{\text{NaOH}}/\Delta t_{\text{tempo}}$ , na reação acompanhada por  $\Delta \text{pH}/\Delta t_{\text{tempo}}$  tem-se a liberação de H<sub>3</sub>O<sup>+</sup> no tempo real da reação.

**Palavras-chave:** pectina; pectina metilesterase; procedimentos potenciométricos; atividade residual.

## 1 Introduction

Pectinolytic enzymes or pectinases can be divided into three large groups, protopectinases, depolymerases, and esterases. Protopectinases degrade the insoluble protopectin and give rise to highly polymerized soluble pectin. The depolymerases catalyze hydrolysis of  $\alpha$  (1 → 4) glycosidic linkages in pectic acid. The esterases catalyze the pectin de-esterification by the removal of methoxyl ester group. In the esterases group, the pectin methylesterase (PME; EC: 3.1.1.11) is found. Pectinases are found in plants, bacteria, and fungi. The PME present in plants and bacteria has optimum pH that ranges between 6 and 8, whereas the optimum pH of that produced by fungi is around 4 and 6, (BENEN et al., 2003; JAYANI; SAXENA; GUPTA, 2005). Commercial pectinases are produced by microbiologically safe strains from *Aspergillus sp.* They are widely used by the juice and fruit beverage industry because they favor clarification and

for making filtration easier resulting in a better performance of the process (ALKORTA et al., 1998).

Ceci and Lozano (1998) determined the PME activity in the commercial enzyme samples Rohapect D5S and Pectinol. They observed that the optimum pH for the enzyme was 4.6. Mutlu et al. (1999) studied the Ultra SP-L Pectinex kinetic parameters, monitored the viscosity change of the pectin solution by enzyme action, and determined the PME activity at pH 3.5 at 35 °C. Commercial pectinases are also used for the extraction of orange juice (KASHYAP et al., 2001) because they promote an increase in sugar extraction as well as soluble solutions, increase productivity, and decrease juice viscosity. Duvetter et al. (2005) studied the inactivation kinetics of purified PME from *Aspergillus aculeatus*. The enzyme inactivation occurred at the temperature range of 46 to 56 °C

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<sup>1</sup> Laboratório L11 de Pesquisa Química Analítica e Inorgânica, Departamento de Química, Mestrado em Ciência e Tecnologia de Alimentos, Universidade Estadual de Ponta Grossa – UEPG, Av. Carlos Cavalcanti, 4748, CEP 84030-900, Ponta Grossa – PR, Brasil, E-mail: ndrosso@uepg.br

\*Corresponding author

and followed a first order kinetics. They also observed that there was no decrease in the activity after pressurization at 700 MPa for an hour at 25 °C. Grassin et al. (2005) studied the commercial pectinases composition used in apple maceration. They verified that the sample composition depends on the *Aspergillus sp.* employed in the enzymatic preparation, fermentation conditions, and methods used in recovering and purifying the enzyme. The authors found that there is enzymatic diversity in the products called pectinases, in which the PME concentration and polygalacturonase predominates. Nikolic' and Mojovic (2007) studied the *Aspergillus niger* (FPG) polygalacturonase, *Aspergillus niger* pectin methyltransferase (FPE), and the tomato pectin methyltransferase (PPE) action in the Budimka (*Pyrus malus L.*) apple pectin hydrolysis. The reaction was conducted by the individual and combined action of FPG, FPE, and PPE enzymes, the optimum pH values verified, 4.5, 3.5, and 6.5, and the optimum temperature of 40, 45, and in the 40-45 °C range, respectively. It was also observed that the FPE enzyme presented higher efficiency in the pectin hydrolysis when compared to PPE.

Fraeye et al. (2007) extracted and purified strawberry pectin, characterized and compared its molecular weight, the esterification, and the composition of sugar with those of apple pectin. Both pectins were treated with *Aspergillus Aculeatus* PME in order to determine the speed of de-esterification at 0.1 to 500 Mpa pressure and temperatures of 20 to 60 °C. The *A. aculeatus* PME optimum activity observed was at 200 to 300 Mpa pressure at 45-55 °C.

Sampedro, Rodrigo and Hendrickx (2008) determined the PME inactivation kinetics via treatment in combined conditions of high pressure and temperature in an orange juice and milk beverage. They observed that for complete inactivation, a treatment at 90 °C for one minute or one at 700 MPa at 55 °C for two minutes was necessary. Wilinska et al. (2008) studied the exogenous *Aspergillus niger* PME stability and inactivation kinetics in fruit juice, apple and blueberries juice, at the temperature range 52-66 °C. They observed that the enzyme activity decreased with the increase in temperature, and that the juice composition caused a considerable difference in the enzymatic susceptibility to the thermal treatment. They also verified that the exogenous PME inactivation reaction followed a first order kinetics. Plaza et al. (2008) studied the influence of pH, ionic strength and also the presence of salt and polyols in the recombinant *Aspergillus aculeatus* PME thermal stability. The authors observed that the PME was thermostable at 50 °C in the presence of NaCl at pH 5.0, and that the polyols presented higher protection effect for the enzyme than the sugars. They also found that pH and ionic strength were important parameters for the enzymatic stability.

Pectic substances constitute a complex of heteropolysaccharides of high molecular weight, 25 to 360 kDa. These are present in most vegetable tissues, mainly in ripe fruit. They are the main component in the middle lamella among cells such as calcium and magnesium pectate (JAYANI; SAXENA; GUPTA, 2005; SCHELLER et al., 2007). The degree of esterification of pectin influences the gelling properties, gel pectins with a high degree of esterification at pH around 3.0, and in the presence

of sugar (ROLIN, 1993; FU; RAO, 1999, 2001). Pectin with low esterification degree forms gel in the presence of calcium ions in a wide pH range with or without sugar. Pectin with high methoxylation content can be treated with alkaline reagents or enzymes such as PME. The role of PME is to demethoxylate the galacturonic chain to produce low methoxylation content pectin.

PME acts in the pectin catalyzing the C-O bond hydrolysis of the methoxyl-ester groups forming methanol and converting pectin into pectate (Figure 1). During the pectate formation, there is a decrease in pH indicating  $H_3O^+$  release. For the hydrolysis of each mmol of the ester group, the release of one  $H_3O^+$  mmol occurs. Thus, the reaction can be followed by a potentiometric system accurately calibrated according to Martell and Motekaitis (1992). Studies aiming at elucidating the PME action and inactivation conditions are frequent issues in the research in this area.

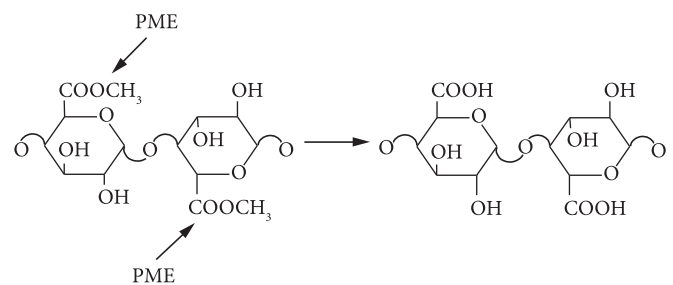
The objective of this work consisted of determining the optimum pH and temperature of two commercial pectinases samples by proposing an alternative procedure to determine the residual activity and comparing data with those obtained with the traditional procedure.

## 2 Materials and methods

Industrial enzymatic preparations of Pectinex 100 L Plus and Panzym Clears were supplied by Novozymes. These enzymatic preparations consisted of a mixture of pectinolytic enzymes with the predominance of polygalacturonase, pectinase and PME produced by *Aspergillus sp.* submerse fermentation. The samples were within the shelf life.

### 2.1 PME activity through NaOH volume variation

The PME activity was determined by titrimetry, which consists of quantifying free carboxyl groups formed by the enzyme action. The experimental system consisted of a double walled glass cell connected to a thermostated bath (Microquimica, MQBTC 99-20) with  $\pm 0.1$  °C precision and a B474 Micronal pHmeter equipped with a combined electrode, which was calibrated daily with buffer solutions, pH 4.0 and 7.0 (Merck). The experiments were carried out with controlled addition of  $CO_2$  free sodium hydroxide,  $0.05 \text{ mol.L}^{-1}$  (Merck) using a digital micro-burette (Denver instrument-Titrator 280), 0.01 mL precision. The reaction consisted of 0.150 g apple



**Figure 1.** Site in which the PME acts in the pectic substances. Source: Jayani, Saxena and Gupta (2005).

pectin, 5.00 mg.mL<sup>-1</sup> (Herbstreith and Fox, high methoxylation degree), 0.100 mol.L<sup>-1</sup> sodium chloride (Merck), and the volume completed with ultra-pure water up to 30 mL. After dissolving the components, 50 µL of commercial pectinolytic enzyme was added. Temperature and pH were kept constant, and the reaction was carefully controlled in terms of NaOH consumption to keep the pH constant. According to Fachin et al. (2002), the PME activity is proportional to the rate of NaOH ( $\Delta V_{\text{NaOH}}/\Delta t$ ) and can be expressed in units, U, defined as micromoles of acid produced per minute. In this study, the unit used to express the PME activity was mmol acid produced per second.

The influence of pH and temperature in the Pectinex 100 L Plus and Panzym Clears commercial pectinases activity was verified. The pH effect on the enzyme activity was determined with the values 3.0; 3.5; 4.0; 4.25; 4.5; 5.0, and 5.5, keeping the reaction temperature constant at 45 ± 0.1 °C. The effect of temperature on the demethoxylation reaction was verified with the values of 25 – 35 – 45 – 55 – 65 – 75 °C, keeping the pH constant at 4.0. All experiments were performed in duplicate, and the activity obtained from the average values was represented on a graph.

To determine the residual activity of PME in the Pectinex 100 L Plus and Panzym Clears, samples of these preparations were placed in polyethylene bottles properly sealed and subjected to temperatures of 45, 50, 55, 60, 65, 70, and 75 °C for 10, 20, and 30 minutes at each temperature. Next, 50 µL of the enzyme were added to the reaction system, 5 mg.mL<sup>-1</sup> pectin, 0.100 mol.L<sup>-1</sup> NaCl and the volume was completed with ultra-pure water up to 30 mL. The reaction was then conducted at 45 °C and pH 4.0.

#### Determination of PME activity through pH variation

The PME activity for the commercial pectinase samples was also determined following the pH variation as a function of the time  $\Delta\text{pH}/\Delta t$  since the PME action on the pectin substrate produces H<sub>3</sub>O<sup>+</sup> ions and methanol. The H<sub>3</sub>O<sup>+</sup> ions can be directly quantified with a system containing a combined electrode, previously calibrated, connected to a pHmeter. The system and the conditions described above in section 2.1, were used; however, this procedure made possible to monitor the reaction without NaOH addition, which reduces the occurrence of errors.

Through the pH variation, the Pectinex 100 L Plus and Panzym Clears pectinases' activity was determined at 25 °C and pH 4.5. The residual activity was also determined with this procedure for both pectinase samples at 45, 50, 55, 60, 65, 70, and 75 °C for 10, 20, and 30 minutes of exposure at each temperature, as previously reported.

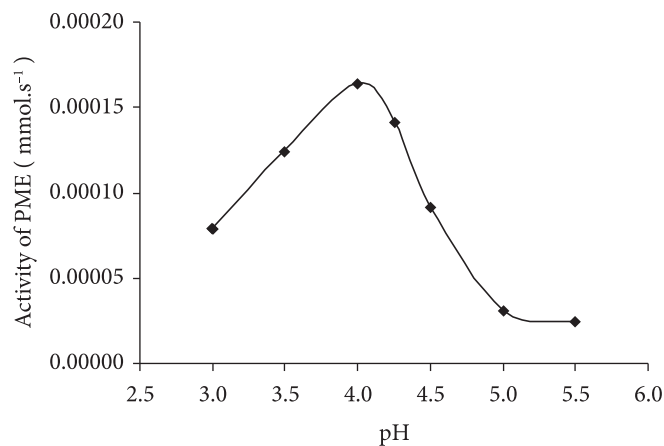
### 3 Results and discussion

#### 3.1 Determination of optimum pH, temperature, and residual activity of Pectinex 100 L plus and panzym clears

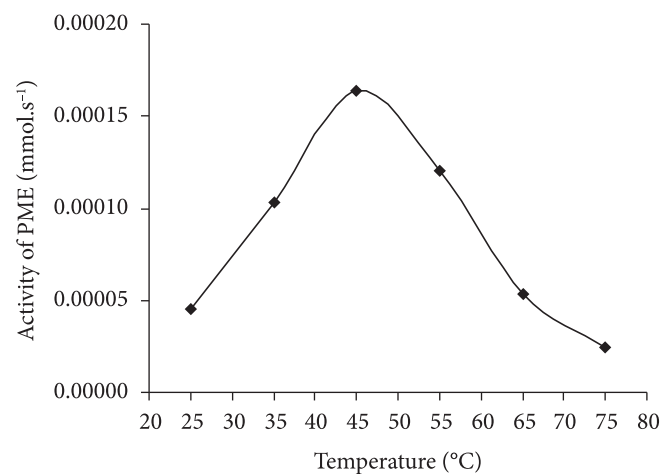
Figure 2 represents the influence of pH on the PME activity of a Pectinex 100 L Plus commercial pectinase sample keeping the temperature constant at 45 °C. The optimum pH observed

for Panzym Clears was of 4.0, value at which the PME maximum activity occurred. It was verified that the pH influenced the enzyme activity because for values above or below pH 4.0 there was a decrease in the enzyme activity. For the Panzym Clears commercial pectinase sample at constant temperature 45 °C, it was also observed that the maximum PME activity was at pH 4.0. These data is in agreement with those reported by Duvetter et al. (2005), Dinu et al. (2007) for commercial pectinase samples obtained from *Aspergillus aculeatus* and *niger*.

Figure 3 shows the temperature influence on the PME activity of Pectinex 100 L Plus keeping the pH constant at 4.0. The optimum temperature observed was 45 °C, and at this value the PME activity was maximum, which is in accordance with that determined by Fraeye et al. (2007). At temperatures above or below 45 °C, the PME activity decreased. It was also verified that the maximum PME activity of Panzym Clears commercial pectinase sample at pH 4.0 was at 45 °C. This optimum temperature is in accordance with that verified by Nikolic' and Mojovic (2007) for the PME of a commercial pectinase sample produced by *Aspergillus niger*.



**Figure 2.** pH influence on PME activity in the Pectinex 100 L Plus at 45 °C. The deviation range for the measurements was from  $1 \times 10^{-5}$  to  $5 \times 10^{-5}$ .



**Figure 3.** Temperature influence on the PME activity in the Pectinex 100 L Plus at pH 4.0. The deviation range for the measurements was from  $1 \times 10^{-5}$  to  $5 \times 10^{-5}$ .

Figure 4 shows the thermal stability of a Pectinex 100 L Plus commercial pectinase sample. It was observed that with the increase in temperature, the PME inactivation occurred. The maximum PME activity was observed with the treatment at 45 °C for 10, 20, and 30 minutes. However, for the treatment at 75 °C, the PME residual activity dropped to 2.49% for 10 minutes of exposure, and after 20 minutes, the complete inactivation occurred. For the Panzym Clears commercial pectinase sample in thermal treatment at 45 °C for 10, 20, and 30 minutes, the PME activity remained at the maximum value. The complete inactivation occurred at 75 °C during the first 10 minutes of PME exposure.

From the study of the two commercial samples, it was observed that the Panzym Clears pectinase activity was  $2.62 \times 10^{-4} \text{ mmol}\cdot\text{s}^{-1}$ , and the Pectinex 100 L Plus was  $1.64 \times 10^{-4} \text{ mmol}\cdot\text{s}^{-1}$  at the pH value of 4.0 and temperature 45 °C. The sample Panzym Clears showed higher activity under the same conditions; therefore, it contains a higher concentration of PME. The activation energy can be considered the amount of energy necessary so that the substrate molecule reaches the activated state in the reaction system. The substrate molecule firstly absorbs energy, becoming activated, and then it converts itself into a product. The enzymatic reaction activation energy can be calculated by the Arrhenius equation and through a graphic of the PME Ln activity as a function of the reciprocal of the absolute temperature in K degrees.

For the calculation of the PME activation energies of Panzym Clears and Pectinex 100 L Plus pectinases, sample graphs of the Ln activity were drawn as a function of the reciprocal temperature. The temperatures applied were those in which the PME activity increases until it reaches a maximum value at the optimum temperature. The Pectinex 100 L Plus and Panzym Clears activation energies obtained through the data were 54.73 and 57.35  $\text{kJ}\cdot\text{mol}^{-1}$  with a correlation coefficient of 0.9995 and 0.9997, respectively. A high value for the activation

energy means that the reaction speed increases rapidly with the increase in temperature. The values of activation energy for the samples studied are in accordance with those reported in the literature. Sarioglu et al. (2001) and Mutlu et al. (1999) determined the PME activation energy of a commercial sample, Pectinex Ultra SP-L, as  $39.00 \text{ kJ}\cdot\text{mol}^{-1}$ .

### 3.2 Determination of PME activity through the pH variation procedure

In the PME reaction with pectin, the hydrolysis of the ester group with the production of one mmol methanol and one mmol ions  $\text{H}_3\text{O}^+$  occurs, which can be quantified using a potentiometric system thoroughly calibrated. The PME activity in both pectinase commercial samples was determined at 25 °C with initial pH of 4.5. The reaction was monitored for 10 minutes registering the pH value as a function of the time. A sharp pH decrease at the beginning of the reaction was observed, as the PME acted on the substrate. For the calculation of the activity, the pH variation in the first minutes of the reaction was used, Equation 1.

$$\Delta\text{pH} = \text{pH}_f - \text{pH}_0 \quad (1)$$

$\text{pH}_0$ : initial pH, enzyme free; and

$\text{pH}_f$ : final pH after enzyme addition at each interval of the reaction.

From the system pH the hydrogenionic concentration of the reaction is directly obtained, Equation 2.

$$\text{pH} = -\log [\text{H}^+] \quad (2)$$

Then, the  $\text{H}^+$  concentration is obtained, Equation 3.

$$[\text{H}^+] = 10^{(-\text{pH})} \quad (3)$$

For the PME activity calculation; the hydrogenionic concentration variation was used,  $\Delta[\text{H}^+]$ , Equation 4.

$$\Delta[\text{H}^+] = [\text{H}^+]_f - [\text{H}^+]_0 \quad (4)$$

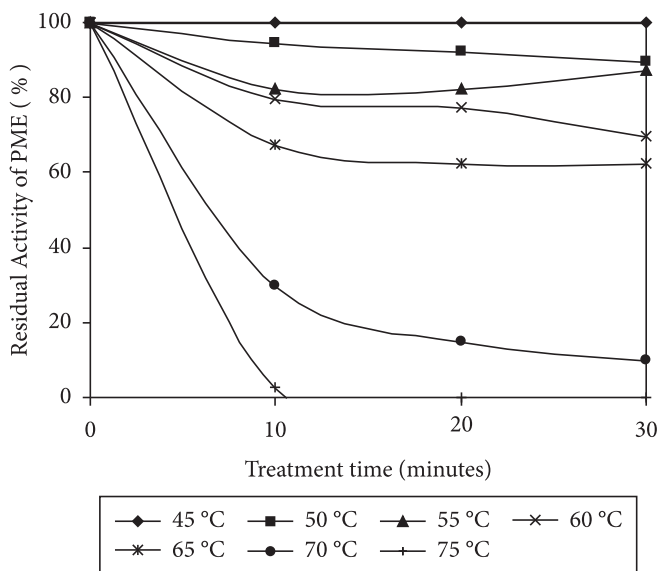
$[\text{H}^+]_0$ : Enzyme free hydrogenionic concentration; and

$[\text{H}^+]_f$ : Hydrogenionic concentration after enzyme action at each interval of the reaction.

The final and initial pH values were converted into hydrogenionic concentrations and substituted in Equation (4) in order to obtain a  $\Delta[\text{H}^+]$  at each different reaction interval, Table 1. The reaction follows a first order kinetics, and the PME activity is the straight line angular coefficient.

**Table 1.** pH conversion into hydrogenionic concentration  $[\text{H}^+]$  as a function of time and the hydrogenionic concentration variation,  $\Delta[\text{H}^+]$ , which corresponds to each time interval in the Pectinex 100 L Plus PME activity determination at 25 °C and pH 4.5.

Time (s)	pH	$[\text{H}^+]$ (mol)	$\Delta[\text{H}^+]$ (mol)
0	4.50	$3.162 \times 10^{-5}$	–
60	4.28	$5.248 \times 10^{-5}$	$2.086 \times 10^{-5}$
120	4.19	$6.456 \times 10^{-5}$	$1.208 \times 10^{-5}$
180	4.15	$7.079 \times 10^{-5}$	$0.623 \times 10^{-5}$



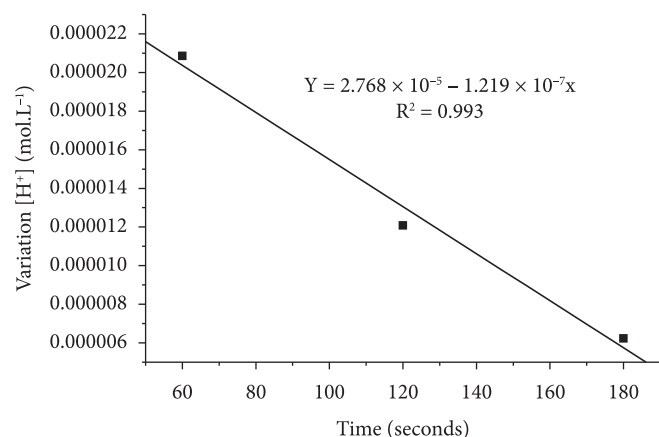
**Figure 4.** Pectinex 100 L Plus pectinase thermal stability in 5.00  $\text{mg}\cdot\text{mL}^{-1}$  of apple pectin, pH 4.0, at 45 °C and 0.100  $\text{mol}\cdot\text{L}^{-1}$  NaCl.

Figure 5 shows the reaction behavior as a function of  $\Delta[H^+]$  (mol), as a function of time (s), it was observed that the reaction follows a first order kinetics. As can be seen in Figure 5, it is possible to observe a linear response, Equation 5, between the hydrogenionic concentration variation in function of time. The activity of PME in the commercial Pectinex 100 L Plus sample was the angular coefficient, that is,  $1.219 \times 10^{-7} \text{ mol.s}^{-1}$ .

$$\Delta[H^+] = 2.768 \times 10^{-5} - 1.219 \times 10^{-7} \times t(s) \quad (5)$$

All experiments for the determination of PME activity were performed in duplicate: the activity value according to the second experiment was  $1.095 \times 10^{-7} \text{ mol.s}^{-1}$  and the average between the activities was  $1.157 \times 10^{-4} \text{ mmol.s}^{-1}$  for the Pectinex 100 L Plus PME sample at 25 °C and pH 4.5.

Table 2 shows the PME activity in both commercial samples through the  $\Delta\text{pH}$  and  $\Delta V_{\text{NaOH}}$  procedure at 25 °C and pH 4.5. Good agreement between the PME activity values determined by the  $\Delta V_{\text{NaOH}}$  and  $\Delta\text{pH}$  procedures was observed. The activity values determined in both procedures show that the Panzym Clears pectinase presented higher activity thus presenting higher PME concentration.



**Figure 5.** Pectinex 100 L Plus PME reaction kinetics with 5.00 mg.L<sup>-1</sup> pectin at 25 °C and pH 4.5.

**Table 2.** PME activity in both commercial samples through the  $\Delta\text{pH}$  and  $\Delta V_{\text{NaOH}}$  procedure at 25 °C and pH 4.5.

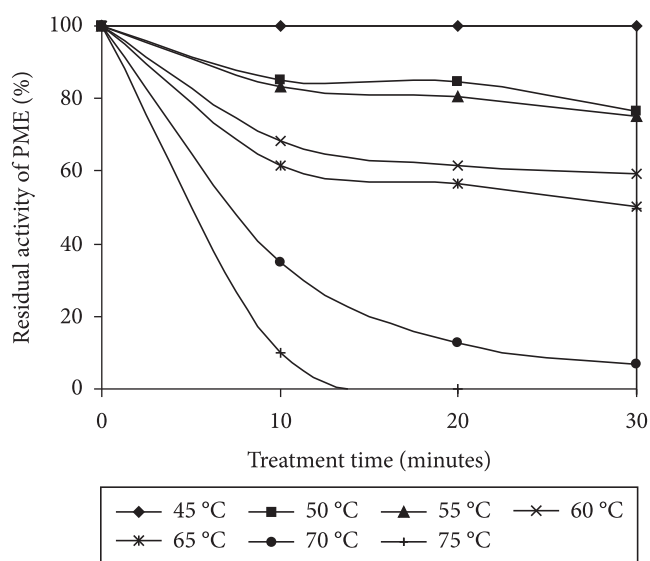
Pectinase commercial sample	PME activity (mmol.s <sup>-1</sup> )	
	$\Delta\text{pH}$	$\Delta V_{\text{NaOH}}$
Panzym Clears	$1.291 \times 10^{-4}$	$1.080 \times 10^{-4}$
Pectinex 100 L Plus	$1.157 \times 10^{-4}$	$0.941 \times 10^{-4}$

**Table 3.** Residual activity of Pectinex 100 L Plus, from both procedures in %.

Temperature (°C)	45		55		65		70		75	
	A	B	A	B	A	B	A	B	A	B
Procedimentos:										
A = $\Delta V_{\text{NaOH}}/\Delta t$										
B = $\Delta\text{pH}/\Delta t$										
Time (min)										
10	100	100	84	83.4	67.4	64.1	29.9	34.6	2.49	6.90
20	100	100	84	82.1	62.3	60.0	15.0	12.6	0.00	0.00
30	100	100	84	75.1	62.3	56.3	10.0	6.8	0.00	0.00

The Pectinex 100 L Plus and the Panzym Clears PME residual activity was also determined by the pH variation procedure. The Pectinex 100 L Plus PME residual activity data is shown in Figure 6. It can be seen that with the thermal treatment at 45 °C for 10, 20, and 30 minutes, the activity remained at the maximum value. This is due to the fact that the PME is at its optimum temperature. The PME complete inactivation occurred at 75 °C for 20 minutes. It was observed that temperature and time of exposure influence the PME residual activity. For the Panzym Clears pectinase, the PME activity remained at the maximum value in the treatment at 45 °C for 10, 20, and 30 minutes of exposure; the complete inactivation occurred at 75 °C for 10 minutes

The Pectinex 100 L Plus PME thermal treatment data obtained through the NaOH volume variation procedure (Figure 4) show good correlation with those obtained through pH variation procedure (Figure 6). Table 3 summarizes the percentage values of PME residual activity obtained through both procedures. It can be seen that in both methods the treatment at 45 °C for 10, 20, and 30 minutes of exposure the activity remained in the maximum value. Complete inactivation occurred at 75 °C for 20 minutes of exposure. Therefore, more reliable quantitative information was obtained through the  $\Delta\text{pH}/\Delta t$  procedure compared to the traditional procedure,  $\Delta V_{\text{NaOH}}/\Delta t$ .



**Figure 6.** Pectinex 100 L Plus PME residual activity in 5.00 mg.mL<sup>-1</sup> of apple pectin, pH 4.0, at 45 °C and 0.100 mol.L<sup>-1</sup> NaCl through  $\Delta\text{pH}$ .

## 4 Conclusions

The pectin reaction in the presence of PME in all experiments revealed a first order kinetics. It was also observed that the PME in both enzymatic preparations, Pectinex 100 L Plus and Panzym Clears, was the most active at pH range of 4.0 to 4.5. It was concluded that the enzyme in both pectinases presented the highest activity at 45 °C, and that the Panzym Clears sample contains the highest PME concentration. From the results of both procedures on the pectinase samples,  $\Delta V_{\text{NaOH}}/\Delta t$  and  $\Delta \text{pH}/\Delta t$ , it was concluded that inactivation occurred at 75 °C for 10 minutes of exposure. The results obtained through the pH variation procedure as a function of time presented good correlation with the traditional method. The reaction followed by pH variation as a function of time makes unnecessary the use of a number of reagents and relatively high cost materials. Through this procedure, there is the release of  $\text{H}_3\text{O}^+$  at the real reaction time thus reaching the most precise activity values.

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