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# Gelatin-immobilized pectinmethylesterase for production of low methoxyl pectin

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**Abstract**

The optimum conditions for the production of low methoxyl pectin using pectinmethylesterase (PME) from acerola (*Malpighia glabra* L.), immobilized in gelatin, have been established by factorial design and response surface methodology. In the case of the free enzyme, the optimum conditions for activity, within ranges adequate for food processing, are low NaCl concentrations (0.10 M), relatively high temperatures (55 °C) and slightly basic pH values (pH = 9). The temperature and pH seem to have strong influence on the observed activity. In the immobilized enzyme, optimum NaCl concentration was 0.15 M, while the optimum pH remained at 9.0.


*Keywords:* Pectinmethylesterase; Acerola; Activity optimum; Purification; Immobilization

## 1. Introduction

The enzyme pectinmethylesterase (PME, EC: 3.1.1.11) has been found in plants, as well as in pathogenic fungi and bacteria (Giovane et al., 1994), and catalyses the hydrolysis of the methyl ester groups of pectin. After this treatment, the pectin can be further hydrolysed through the action of polygalacturonase. Plant PME has been used for the preparation of low methoxyl pectin (LM-pectin) and for the destabilization of cloud in fruit juices (Dinnella, Stagni, & Lanzarini, 1997; Versteeg, Rombouts, Spaansen, & Pilnik, 1980).

The use of soluble enzymes presents several disadvantages for a continuous process (Lozano, Manjón, Romojaro, & Iborra, 1988). These include the impossibility of reusing enzymes or the alteration of the organoleptic properties of the processed product. These drawbacks can be mitigated or even avoided by enzyme immobilisation for continuous fruit juice processing (Lozano et al., 1988). With the new trends in industrial application of PME, there is an enhanced interest in the immobilisation of this enzyme on insoluble carriers (Nighojkar, Srivastava, & Kumar, 1995).

Pectolytic enzymes play a crucial role in food processing industries, e.g., in the production of fruit juices, soft drinks, and liquors (Brawman, 1981; Naidu & Panda, 1999). These enzymes are also used in the maceration, liquefaction, and extraction of vegetable tissues (Anastasakis, Lindamoo, Chism, & Hansen, 1981; Burns & Pressey, 1988; Joslyn, Mist, & Lambert, 1952; Naidu & Panda, 1999). They also help to reduce the viscosity of fruit pulp which, in turn, improves filtration

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and clarification of fruit juices and wood aging (Fogarty & Kelly, 1983; Kilara, 1982; Naidu & Panda, 1999). As a result, pectinases are one of the upcoming enzymes of the commercial sector. In 1998, worldwide enzyme sales amounted to over $1.5 billion, with a predicted annual growth rate ranging from 2% in the leather industry to 15% in paper production and 25% in feed enzymes and various sources. Enzymes produced for in-house use amount to approximately 10% of the total enzyme market (Beilen & Li, 2002).

PME from acerola fruit has been partially purified and characterised (Assis, Martins, Quaglianoni, & Faria Oliveira, 2002) and has shown significant thermal stability (Assis, Lima, & Faria Oliveira, 2000; Assis et al., 2002). The goal of this work is to establish the optimum conditions for demethoxylation of pectin, using a factorial design and response surface methodology as a preliminary basis for production of low methoxylated pectin with PME immobilized in gelatin.

## 2. Materials and methods

### 2.1. Chemicals

Citrus pectin (P9135, methoxy content 9.4%), and bovine serum albumin were purchased from Sigma Chemical Co. Gelatin was purchased from Merck. Sodium arsenite was purchased from Fluka. All the other chemicals used were of high quality analytical grade.

### 2.2. Preparation of pulp

The acerola fruits were collected from several trees from the culture in the Faculty of Science Agrary and Veterinary of Jaboticabal, SP – Brazil. In each tree, the fruits were collected at different points to assure a representative sample. The fruits selected were in the mature stage of development. The sample was obtained by mixing the fruits collected. The pulp was obtained by crushing the fruits with a marine propeller.

### 2.3. Extraction of the PME

The enzyme was extracted from the pulp at 4 °C, by using 50 mM borate–acetate buffer, pH 8.3 (buffer A), containing 0.60 M NaCl. The ratio of pulp to solvent was 1:3 ($g\,ml^{-1}$). The homogenate was squeezed through two layers of gauze and the extract was centrifuged at 10000*g* for 10 min to remove the solid particles (Körner, Zimmerman, & Berk, 1980). The supernatant was brought to 70% saturation by addition of solid ammonium sulphate, and was centrifuged at 10000*g* for 10 min after standing for 1 h (5 °C). The precipitate was resuspended in borate–acetate buffer in the ratio of 1:3 (w/v). The PME extract thus obtained was lyophilised.

### 2.4. Determination of PME activity

PME activity was measured titrimetrically by determining the free carboxyl groups formed as a result of enzyme action in pectin. The standard reaction mixture was composed of 30 ml of the 0.125% citrus pectin solution, containing 0.15 mol/l NaCl and 0.5 ml of enzyme. It has been shown in previous works (Assis, 2000) that conversion rate varies linearly with enzyme concentration in the range used in the present work. The amount of 0.1 M NaOH required to maintain the reaction mixture at pH 8.3 ($40 \pm 2$ °C) was measured by the method of Kertesz (1955). One unit of PME was defined as the amount of enzyme which released 1 μmol of carboxyl groups per minute. PME activity was calculated according to Assis et al. (2000).

### 2.5. Protein determination

Protein concentration was determined according to the method of Hartree (1972), using bovine serum albumin (Sigma Chemical Co.) as standard.

### 2.6. Degree of methoxylation of pectin

The spectrophotometric method of Wood and Siddiqui (1971) was used to determine the degree of methoxylation of pectin.

### 2.7. PME immobilization in gelatin

A modification of the procedure given by Nighojkar et al. (1995) was used. 500 mg gelatin was dissolved in 4.5 ml of distilled water at 50 °C and thereafter allowed to cool to room temperature. 0.5 ml of the enzyme preparation (150 mg protein), in borate acetate buffer, pH 8.3, 50 mM, was then added, with constant stirring. After 1 h at 5 °C, when the suspension jellified, 10 ml of a 10% glutaraldehyde solution, in buffer phosphate pH 7.4, 50 mM were added. After 30 min, the gelatin was cut into small pieces (mean diameter = 2.53 mm). The gelatin was finally washed with distilled water and maintained in 50 mM borate acetate buffer, pH 8.3, at 5 °C.

## 3. Experimental design

The influences of temperature, NaCl concentration and pH on the specific activity of the free enzyme were investigated. A factorial design, using four levels of each factor, was used (Table 1). Each factor combination was performed in triplicate, yielding 192 experiments.

In order to detect the operational conditions that optimise the specific activity, the results were fitted to a polynomial model:

Table 1
Response of three variables in determining the conditions that maximize the activity of free PME

| [NaCl] (M) | $T$ (°C) | pH | | | |
|---|---|---|---|---|---|
| | | 7.5 | 8.0 | 8.5 | 9.0 |
| 0.100 | 40 | 1.66 | 1.92 | 2.55 | 2.75 |
| | | 1.66 | 1.92 | 2.55 | 2.55 |
| | | 1.66 | 1.92 | 2.55 | 2.64 |
| | 45 | 2.13 | 2.87 | 3.30 | 3.41 |
| | | 2.24 | 2.87 | 3.30 | 3.62 |
| | | 2.18 | 2.87 | 3.30 | 3.51 |
| | 50 | 2.34 | 3.19 | 4.47 | 3.73 |
| | | 2.45 | 3.30 | 4.47 | 3.62 |
| | | 2.36 | 3.30 | 4.47 | 3.66 |
| | 55 | 3.19 | 3.76 | 3.56 | 4.15 |
| | | 3.19 | 3.83 | 3.62 | 4.15 |
| | | 3.19 | 3.89 | 3.62 | 4.15 |
| 0.125 | 40 | 1.66 | 2.13 | 2.55 | 2.64 |
| | | 1.66 | 2.13 | 2.45 | 2.75 |
| | | 1.66 | 2.13 | 2.55 | 2.75 |
| | 45 | 2.77 | 0.24 | 2.87 | 3.19 |
| | | 2.87 | 2.98 | 2.87 | 3.30 |
| | | 2.87 | 2.98 | 2.87 | 3.24 |
| | 50 | 2.54 | 2.98 | 3.19 | 3.03 |
| | | 2.45 | 3.09 | 3.30 | 3.12 |
| | | 2.45 | 3.09 | 3.30 | 3.06 |
| | 55 | 2.66 | 3.83 | 4.05 | 4.15 |
| | | 2.77 | 3.72 | 4.05 | 4.04 |
| | | 2.77 | 3.77 | 4.05 | 4.15 |
| 0.150 | 40 | 1.70 | 1.81 | 2.13 | 1.26 |
| | | 1.60 | 1.92 | 2.24 | 1.26 |
| | | 1.65 | 1.91 | 2.18 | 1.26 |
| | 45 | 1.92 | 2.34 | 2.34 | 2.98 |
| | | 1.92 | 2.34 | 2.23 | 2.98 |
| | | 1.92 | 2.34 | 2.28 | 2.98 |
| | 50 | 2.51 | 2.87 | 4.47 | 4.05 |
| | | 2.51 | 2.77 | 4.47 | 4.36 |
| | | 2.51 | 2.82 | 4.47 | 4.22 |
| | 55 | 2.66 | 3.09 | 2.66 | 3.30 |
| | | 2.66 | 3.41 | 2.66 | 3.30 |
| | | 2.66 | 3.30 | 2.66 | 3.30 |
| 0.175 | 40 | 1.49 | 2.13 | 2.66 | 2.96 |
| | | 1.49 | 2.13 | 2.77 | 3.06 |
| | | 1.49 | 2.02 | 2.77 | 3.06 |
| | 45 | 2.24 | 2.66 | 2.87 | 3.19 |
| | | 2.24 | 2.77 | 2.98 | 2.98 |
| | | 2.24 | 2.77 | 3.10 | 3.09 |
| | 50 | 2.02 | 2.34 | 2.66 | 2.77 |
| | | 1.92 | 2.34 | 2.77 | 2.66 |
| | | 1.97 | 2.34 | 2.71 | 2.72 |
| | 55 | 2.04 | 3.38 | 3.83 | 3.13 |
| | | 2.17 | 3.32 | 3.83 | 3.26 |
| | | 2.11 | 3.35 | 3.83 | 3.19 |

Table 2
Response of two variables in determining the conditions that maximize the activity of PME immobilized in gelatin, at 40 °C

| [NaCl] (M) | pH | | | |
|---|---|---|---|---|
| | 7.5 | 8.0 | 8.5 | 9.0 |
| 0.00 | 0.71 | 0.59 | 0.40 | 0.32 |
| | 0.71 | 0.59 | 0.40 | 0.32 |
| | 0.71 | 0.59 | 0.40 | 0.32 |
| 0.05 | 0.16 | 0.26 | 0.16 | 0.24 |
| | 0.16 | 0.2 | 0.16 | 0.24 |
| | 0.16 | 0.26 | 0.16 | 0.24 |
| 0.10 | 0.16 | 0.26 | 0.24 | 0.35 |
| | 0.16 | 0.2 | 0.24 | 0.26 |
| | 0.16 | 0.26 | 0.24 | 0.35 |
| 0.15 | 0.71 | 0.59 | 0.55 | 0.79 |
| | 0.63 | 0.59 | 0.71 | 0.63 |
| | 0.71 | 0.59 | 0.55 | 0.51 |

$$Y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} \sum_{j=i}^{n} a_{ij} x_i x_j + a_{123} x_1 x_2 x_3, \qquad (1)$$

where $Y$ is the response variable (specific activity), $x_i$ the $i$th independent variable, $a_0$ the constant of the model, $a_i$ the first order model coefficients, and $a_{ij}$ the second order coefficients. When $i \neq j$, then $a_{ij}$ is the coefficient for the interaction between factors $i$ and $j$.

The experimental data were first fitted to the model by multiple regressions. The statistical significance of each of the terms was evaluated by analysis of variance (ANOVA). After the non-statistically significant terms were removed from the model, any possible outliers were identified and withdrawn. The model-fitting cycle was then repeated with the remaining experimental data.

A similar procedure was performed to model the influence of NaCl concentration and pH on the specific activity of the enzyme immobilized in gelatin. Again, a factorial design, using four levels of each factor, was used and, as before, each factor combination was performed in triplicate, yielding 48 experiments (Table 2). The ranges of pH, temperature and NaCl concentration were chosen in order to be suitable for food processing. The pH values of the study were established according the conditions established in our previous work (Assis et al., 2000, 2002). The NaCl values tested were not higher due to the deleterious effects on the organoleptic properties of the final food products, such as jelly or juice.

## 4. Results and discussion

The effects of temperature, sodium chloride concentration and pH of the bioconversion medium on the specific activity of the free enzyme, $SA_{free}$, were evaluated. Multiple regression analysis was performed to correlate the effect of these parameters on specific activity, within a

temperature range of 40–55 °C, a pH range of 7.5–9.0 and a sodium chloride concentration range of 0.1–0.175 M. Best fit was obtained with a polynomial function (2)

$$SA_{free} = -29.3 - 58.3x_1 + 6.23x_3 + 1.17x_1x_2 + 11.0x_1x_3 + 0.0222x_2x_3 - 0.397x_3^2 - 0.241x_1x_2x_3, \quad (2)$$

where $x_1$ is the concentration of NaCl, $x_2$ is the temperature and $x_3$ the pH. The model fitted the experimental data with an $R^2$ value of 0.794 (Fig. 1).

Fig. 2 shows the effects of each parameter on the activity of free PME. The obtained regression coefficients show that a simple linear relationship between the response and the factors does not apply. Also, interactions between factors, especially between NaCl concentration and pH, are highly significant. The optimum conditions for activity, within the tested ranges, are low NaCl concentrations, high temperatures and slightly basic pH values.

The same procedure was undertaken to study the influence of pH and NaCl concentration on the activity of the enzyme immobilized in gelatin at a temperature of 40 °C. Although the activity at this temperature is not maximized, the gelatin support softened at temperatures above 45 °C (data not shown), not allowing them to be used in fixed bed reactors.

The specific activity of the immobilized enzyme, $SA_{im}$, was evaluated in bioconversion media with sodium chloride concentration ranging from 0 to 0.15 M and pH of 7.5–9.0. The following correlation (3) was obtained:

$$SA_{im} = 2.29 - 35.7x_1 - 0.216x_2 + 66.3x_1^2 + 3.20x_1x_2, \quad (3)$$

where $x_1$ is the concentration of NaCl and $x_2$ is the pH. The model fitted the experimental data with an $R^2$ value of 0.911.

The range used for testing the influence of NaCl on the activity of the immobilized enzyme was shifted to lower values than those used in the experiments with the free enzyme, as it was shown that the highest activity with the free enzyme was obtained at the lower limit of the tested NaCl concentration range. The first order coefficients have negative effects, while the second order coefficients have positive effects, confirming a significantly non-linear relationship between the factors and the response. The extreme NaCl concentration values within the tested range were those that resulted in higher immobilized enzyme activity, while the highest pH values were again those that maximized the immobilized

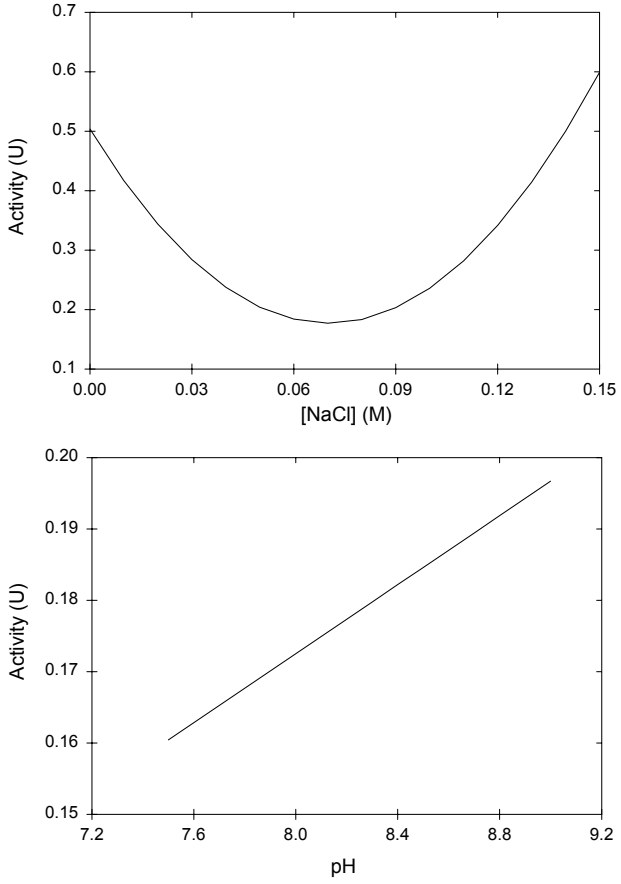


Fig. 2. Main effects of parameters on the activity of the enzyme. The influence of one parameter is shown at a time, while the remaining was set at the average value of the tested range.

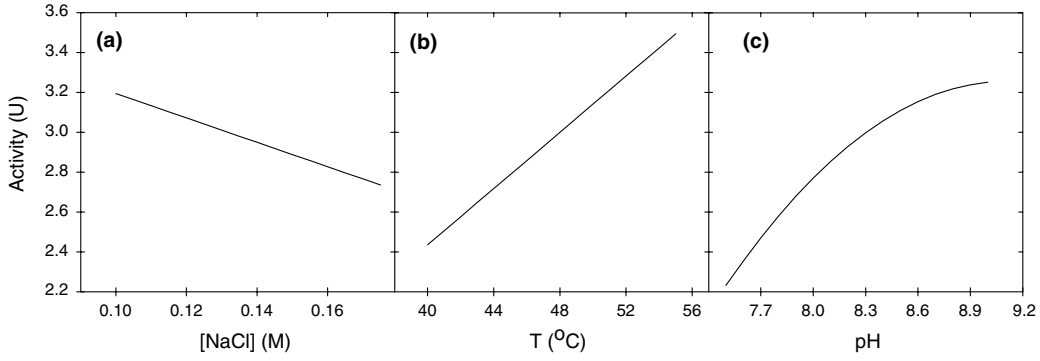


Fig. 1. Effect of sodium chloride concentration (a), temperature (b) and pH (c) on the specific activity of the free enzyme. The influence of each parameter is shown at a time, while the remaining were set at the average value of the tested range.

enzyme activity. NaCl concentration had a stronger effect on activity than pH, which is confirmed by the values of the coefficients in Eq. (3).

## 5. Conclusion

This study shows that a factorial design and response surface methodology can be used in determining the conditions that maximize the activity of acerola fruit. These conditions, determined within adequate ranges for food processing, are pH 9.0, 0.1 M NaCl and 55 °C for the free enzyme and pH 9.0 and 0.15 M NaCl for the enzyme immobilized in gelatin, at 40 °C.

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