

Research Article

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Process Development to Recover Pectinases Produced by Solid-State Fermentation

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Abstract

Leaching, or solid-liquid extraction, is the first step that must be done in the recovery process of a metabolite produced by Solid State Fermentation (SSF). In this work, the leaching of a Polygalacturonase (PG) produced by a strain of *Aspergillus niger* by SSF of citrus dried pulp was performed. A fractionated factor design 2^{4-1} was developed to establish the influences of five factors: solid-liquid ratio, temperature, pH, agitation and surfactant addition (Tween 80). Agitation and surfactant addition effects were confounded by second order effects according to the fractionated factor design. Results showed that pH and surfactant addition did not influence the recovery process. The leaching process was characterized through the corresponding kinetic parameters for PG and total protein recovery, corresponding to first order kinetics. In the case of PG leaching, the theoretical C_s was 17993 U L^{-1} and the k was 0.107 min^{-1} , and in the case of total protein extraction, values were 13159 mg L^{-1} and 0.177 min^{-1} , respectively. The process was improved by using a system of six successive steps. The results showed that when a single step was performed the concentration obtained was 137 U g^{-1} (d.b.), while after successive extractions the PG extracted achieved a concentration of 537 U g^{-1} (d.b.), improving the process by 74%.

Keywords: Enzyme Recovery; Leaching kinetics; Solid state fermentation; Pectinase; *Aspergillus niger*

Abbreviations and Symbols: PG: Polymethylgalacturonase; SSF: Solid state fermentation; (d.b.): Dry basis; ANOVA: Variance analysis; p: Probability; C_{aPG} : PMG concentration at time t (IU/mL); C_p : Total protein concentration at time t (mg/mL); PG_{act} : IU PG/g (d.b.); X: Stage number; Y: mg protein /g (d.b.); r^2 : Regression coefficient

Introduction

Annually, about 34 million tons of citrus residues (peel, seeds) are produced from the processing of citrus fruits [1-3]. Although a part of these residues is applied in the diet of monogastric animals, its accumulation in the biosphere causes several environmental problems. At the same time, citrus peel can be an important and economical raw material to produce enzymes, mainly pectinase, by fermentative processes. This material contains almost all nutrients needed for microorganism growth, and several processes have been studied for the production of many important metabolites including enzymes [4].

Polygalacturonases, including endoPGases and exoPGases, comprise a family of enzymes named pectinases that degrade pectin and pectic acid. These enzymes are extensively used in the feed and drink industries, chiefly in juice clarification because they are capable of reducing the viscosity of liquors during the clarification process [5,6].

The production of PG by SSF employing agro-industrial residues and different microorganisms has been previously studied [7-13]. In SSF, it is necessary to consider that the produced metabolites remain in a solid matrix and, therefore, must be extracted by solid-liquid extraction or leaching. Therefore, this is the first step in any recovery and purification process that is intended for any desirable metabolite produced by SSF [14,15].

The study of leaching involves establishment of the factors and the corresponding parameters that characterise the process to reach acceptable levels in yield during the purification process. However, there are only a few reports regarding the leaching of metabolites from a solid state fermented matrix [16,17] and, in particular, from matrixes

containing PG. These reports mainly discuss how different factors affect enzyme extraction, but there is a lack of complete representation of the leaching process through the corresponding kinetic pattern. However, several mechanisms were postulated for their study from biological material [18].

The aim of this work was to analyse a leaching process to improve the recovery of PG produced by *Aspergillus niger* F3 growing on citrus wastes by SSF, as part a strategy to apply the enzyme PG in juice clarification within a global project to reuse the solid waste generated during citrus juice production. This study was done by statistically determining the significance of different factors that may influence the process. Once the significance of these factors was established, the kinetic patterns of the process were determined. The process was considered a proper procedure by using successive extraction steps.

Materials and Methods

Microorganism and inoculum preparation

The strain *A. niger* F3 with low sporulation was employed. This strain was obtained from the Federal University of Paraná (UFPR) and maintained on Potato Dextrose Agar (PDA) slants. Mycelial biomass was produced by growing the *A. niger* F3 on Czapek liquid medium for 72 hours at pH 5, 30°C and 120 rpm.

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Solid state fermentation

Dried citrus peel (75% of the peel had a particle size ranging from 0.8 mm to 2 mm, and the other 25% had a particle size between 2 mm and 3 mm) was used and supplemented with the following substances in dry basis: NH_4NO_3 0.43%; Na_2SO_4 0.021%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.077%; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.042%; KCl 0.162%; and Ca(OH)_2 0.011%. Water was added to obtain 60% of initial humidity. Inoculation was made in a 1:10 (v/w) ratio in 1 kg of wet solid media. Fermentations were carried out in a cylindrical bioreactor (25 cm diameter and 50 cm height) loaded with 2 kg for 96 h at controlled room temperature (30°C). Aeration intensity was set at 1 VkgM (1 LAir $\text{kg}^{-1} \text{min}^{-1}$) [19].

Moisture content

Moisture content was determined in an infrared balance (Sartorius model MA-50) at 105°C.

Polygalacturonase (PG) activity

PG was assayed by measuring the release of reducing sugars by the di-nitro-salicylic (DNS) method [20]. In this case, galacturonic acid (Sigma) was considered as simple sugar formed after pectin degradation. In a test tube, 0.9 mL of 0.5% (w/v) pectin (Sigma) in 0.1 M citrate buffer (pH 4.0) was added to 0.1 mL of the diluted enzyme solution. After incubation for 15 min at 50°C, the reaction was stopped by the addition of 1 mL DNS, and the mixture was heated in boiling water for 5 min. Distilled water (5 mL) was then added to each sample. Absorbances of the samples were read at 540 nm. One unit of PG activity was defined as the amount of enzyme that liberates 1 μmol of D-galacturonic acid per minute at 50°C and pH 4.0 [19].

Protein concentration

Protein was determined with a Folin-phenol reagent [21] using Bovine Serum Albumin (BSA) as the protein standard. O

Enzyme leaching

Extractions were carried out in a stirred and jacketed tank (Heidolph RZR 2021; Germany) with 1 L of effective volume and a paddle agitator. Different solid-liquid relations were studied to determine the best condition to extract the enzymes. Enzymatic extracts were prepared by adding different masses of fermented solid matrix in 1L of pure water to obtain the desired solid-liquid ratio. Mass of solids was added considering the moisture content to express the solid concentration in dry basis. Mass of solids was calculated by the following equation:

$$M_s = \frac{\text{Sol} / \text{liq}_{(d.b.)} \cdot V}{\left(1 - \frac{\% \text{Moist}}{100}\right)}$$

Where:

M_s : Mass of solids (g).

$\text{Sol} / \text{liq}_{(d.b.)}$: Solid-liquid relation in dry basis (g dry solids mL^{-1}).

V: Total working volume (mL)

%Moist: Moisture content of the fermented solid matrix.

Experimental design

Four independent factors were analysed as follows: temperature, pH, agitation and addition of a non-anionic detergent as surfactant (Tween 80). After pH adjustment and temperature stabilisation, solids were agitated for 30 min to obtain enzymatic extracts. Extracts were centrifuged at 4,000 rpm (Sigma centrifuge; B. Braun Biotech International) for 15 min to analyse the enzymatic activity.

The selected factors considered in the experimental design that may influence the leaching study were the following:

Temperature (A): This factor influences both enzyme stability and enzyme diffusion into the solvent or liquid phase.

pH (B): This parameter may have an important effect on enzyme characteristics and enzyme-surface ratios but a lower impact on diffusion in the process.

Surfactant addition (C): This factor may avoid solute attachment to the solid matrix and increase cell permeability to increase extract yield. The surfactant selected was Tween 80 due to its non-anionic character.

Agitation (D): The most important influence of this factor is related to the gradients that may eventually form during the process. In addition, aeration influences the liquid film formed around the solid particles, which conducts the mass transfer from the solute to the solvent.

A two level fractionated factorial design (2^{4-1}) with three replicates at the central point was carried out to evaluate the influence of the selected factors and their possible interactions in the leaching process [22]. This procedure allowed the statistical analysis of these factors with a minimal number of experiments. Table 1 shows the levels and values for the independent factors analysed in the 2^{4-1} plan developed for the study.

The factorial plan was generated by using a full three factorial experimental design involving three factors (A, B and C) and then confounding the remaining factor (D) with interactions generated by the following plan generators: $D = A * B$. The defined relation allowed the establishment of an alias that determined which effects were confounded with each other through the coefficients of the predicted response equation as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD \quad (1)$$

The experimental design matrix is shown in Table 2. Runs were carried out in a random sequence with central point repetition.

In order to optimize the extraction a central composite design

| Factors | Corresponding variable | Coded level of variable | | |
|---------|------------------------|-------------------------|------|-----|
| | | - 1 | 0 | 1 |
| A | Temperature (°C) | 30 | 40 | 50 |
| B | pH | 3 | 5 | 7 |
| C | Tween 80 (% v/v) | 0 | 0.75 | 1.5 |
| D | Agitation (rpm) | 100 | 300 | 500 |

Table 1: Experimental range and levels of independent variables studied using a two level fractionated factorial design (2^{4-1}) in terms of actual and coded factors.

| Run | A | B | C | D = A*B |
|---------------|----|----|----|---------|
| 1 | -1 | 1 | 1 | 1 |
| 2 | 1 | -1 | -1 | -1 |
| 3 | -1 | 1 | -1 | -1 |
| 4 | 1 | 1 | -1 | 1 |
| 5 | -1 | -1 | 1 | 1 |
| 6 | 1 | -1 | 1 | -1 |
| 7 | -1 | 1 | 1 | -1 |
| 8 | 1 | 1 | 1 | 1 |
| Central Point | 0 | 0 | 0 | 0 |
| Central Point | 0 | 0 | 0 | 0 |
| Central Point | 0 | 0 | 0 | 0 |

Table 2: Experimental design matrix for the 24-1 plan developed for independent factors analyzed in the PG extraction.

was developed after determining the significant factors for the PG extraction. In Table 3, the corresponding experimental design matrix is shown. All data were processed using the Design Expert Version 5.07 program.

Effect of ions on the recovery of PG

The effect of anions (Cl^- , SO_4^{2-} , NO_3^- , citrate anion, acetate anion and EDTA) and cations (Na^+ , K^+ , Ca^{+2} , Mg^{+2} , Mn^{+2} and Zn^{+2}) on enzyme activity was investigated by the addition of acid solutions containing these anions to the solvent for 30 min at room temperature (approximately 30°C). For all runs the ionic concentration was 30mM. The EDTA was added at a concentration of 50 mM. Distilled water was considered as the control.

Results and Discussion

PG from *Aspergillus niger* F3 was produced by SSF employing citrus peel during 96 hours. PG concentration at the end of the fermentation was 265 U g⁻¹(d.b.) and the final moisture was 61.23%. Based on this result the extraction of PG from the solid matrix for further downstream processing was analysed. The PG concentration obtained is higher than that reported in a review by Favela-Torres et al. [23] which is 71.2 - 81 U g⁻¹ for medium composed by orange bagasse.

Solid-liquid relation

Different quantities of fermented solid matrix were processed in 1L of distilled water in order to obtain the best solid-liquid relation. Table 4 shows the results of concentration, the activity expressed as U mL⁻¹. It is important to point out that when working with a 1:10 solid-liquid relation, all water was absorbed and retained by the solid and it was impossible to obtain an extract. According to the results showed in Table 3, the higher values of enzyme concentration were obtained at 1:20 g mL⁻¹ in dry basis, but it was also the condition with lower yield, whereas in the condition of 1:50 g mL⁻¹ enzyme was more diluted and the yield of the extraction was higher. There is an apparent contradiction, the more concentrated the activity was, the lower yield was obtained in the extraction. To explain this it is necessary to point out that when the yield was higher the amount of solid employed was lower and it was easier to extract the enzyme present in the solid matrix. But, at the same time, this condition represented more volume used to extract, so more steps required for concentration and more liquid residues generated, influencing negatively on the economy of the process. Applying an engineering criterion for extractive processes, “the best condition for recovery is where the product is more concentrated and not where more is extracted”, it is possible to economize time and energy during the

| Factor | Units | Coded level of variable | | | | |
|-------------|-------|-------------------------|-----|-----|-----|-------|
| | | - α | - 1 | 0 | 1 | α |
| Temperature | (°C) | 25.86 | 30 | 40 | 50 | 54.14 |
| Agitation | (rpm) | 88 | 150 | 300 | 450 | 512 |

Table 3: Experimental range and levels of independent variables studied using a central composite design in terms of actual and coded factors.

| Solid-liquid relation (g mL ⁻¹) (d.b.) | Mass of solids added (g) | PG concentration (U L ⁻¹) | Yield PG Extraction (%) |
|--|--------------------------|---------------------------------------|-------------------------|
| 1:10 | 257.9 | - | - |
| 1:20 | 129.0 | 11348 | 37.40 |
| 1:30 | 86.0 | 9276 | 42.14 |
| 1:50 | 64.5 | 6329 | 44.40 |
| 1:60 | 51.6 | 4983 | 47.58 |

Table 4: Concentration obtained for different solid-liquid relations considering dry basis.

concentration steps and in all recovery process. It was considered as the best condition where the PG was recovered more concentrated and it was decided to go on working in this research considering 1:20 g mL⁻¹ in dry basis as the solid liquid relation.

Statistical analysis for fractionated factorial design 2⁴⁻¹

The statistical analysis was carried out by searching for possible factor interactions. It was considered that the confounded effects in the coefficients may be deduced from the defining relations of the plan, and confounded effects higher than second order were overlooked.

The response matrix (Y) obtained from the experiments developed in the fractionated factorial plan 2⁴⁻¹ is shown in Table 5. The highest activities obtained in the developed experiments corresponded to runs 5, 7 and 1. These runs coincided with the lowest values of the temperature, while for the rest of the independent factors analysed, the values appeared at higher or lower level in different cases. It is possible to think that temperature is the only factor with significant influence on the PG activity in the extract.

In order to explain this behaviour, analysis of variance (ANOVA) was performed to obtain the significant coefficients corresponding to Equation (1) in terms of coded factors and to validate the regression model. In Table 6, the results for the values and discrimination of coefficients ($\beta_0, \beta_1, \beta_2, \beta_3, \beta_{12} = \beta_4, \beta_{13} = \beta_5, \beta_{14} = \beta_6, \beta_{23} = \beta_7$) are shown, while independent factor (β_4) is associated with the interaction between temperature and pH (β_{12}).

In Table 6 coefficients obtained from ANOVA of the fractionated factorial design developed are shown. The β_1, β_{12} and β_{13} coefficients were statistically significant at $\alpha = 0.05$ confidence level and the rest of the coefficients had no significant influence on the process. Once the significant coefficients were established, it was important to analyse which effects represented each coefficient through the confounding effects determined by the alias pattern and the defining relations. The β_1

| Run | Temperature (°C) | pH | Tween 80 (%) | Agitation (rpm) | PG Concentration (U L ⁻¹) |
|-----|------------------|----|--------------|-----------------|---------------------------------------|
| 1 | 30 | 3 | 0 | 500 | 11982 |
| 2 | 50 | 3 | 0 | 100 | 7785 |
| 3 | 30 | 7 | 0 | 100 | 10457 |
| 4 | 50 | 7 | 0 | 500 | 8884 |
| 5 | 30 | 7 | 1 | 500 | 12007 |
| 6 | 50 | 3 | 1 | 100 | 7792 |
| 7 | 30 | 3 | 1 | 100 | 12162 |
| 8 | 50 | 7 | 1 | 500 | 7831 |
| PC1 | 40 | 5 | 0.75 | 300 | 10267 |
| PC2 | 40 | 5 | 0.75 | 300 | 10354 |
| PC1 | 40 | 5 | 0.75 | 300 | 10199 |

Table 5: Concentration of PG extracted from fermented solid matrix according to the fractionated factorial design 2⁴⁻¹.

| Coefficient (confounded effects) | Value | p |
|----------------------------------|----------|----------|
| β_0 (Block effect) | 9862.65 | - |
| β_1 (A) * | -1789.61 | 0.002165 |
| β_2 (B) | -29.03 | 0.006773 |
| β_3 (C) | 85.50 | 0.054505 |
| β_{12} (D; AB) * | 313.53 | 0.006769 |
| β_{13} (AC; BD) * | -346.81 | 0.006223 |
| β_{14} (AD; BC) | 77.45 | 0.105698 |
| Lack of fit* | - | 0.010336 |

Table 6: Factor coefficients discrimination corresponding to the fractionated factorial design 2⁴⁻¹.

coefficient showed the largest influence on the process, which means that the temperature is the most important independent factor analyzed. At the same time, second order interactions of temperature and pH, and temperature and Tween 80 are statistically significant. However second order interaction is confounded with the agitation and it is not possible to determine which of them has the real significant influence on the extraction. On the other hand, the fit of the model obtained was 0.86514 and its lack of fit was statically significant too. This behaviour suggested that a non-linear model may adjust better in the range analysed and that there may be a zone of optimal conditions.

Optimisation of the solid liquid conditions

A central composite design based on the results of the fractionated factorial design was carried out to optimise the conditions for lixiviation. The design and results are shown in Table 7. Analysis of variance was performed to validate the regression model. The regression coefficients and *p*-values are presented in Table 8. Linear and quadratic coefficients of temperature had significant influence in the extraction of PG, also the quadratic coefficient of the agitation formed a curve in the surface obtained, therefore it was possible to optimize the extraction of PG. Non-significance of the linear coefficient of agitation and interaction are in accordance with the results showed in the fractionated factorial design, and at the same time it is possible to affirm that interaction between temperature and pH had significance influence on the extraction and not the linear coefficient of agitation. The fit of the model was 0.9525, therefore, the statistical model accounted for 95.25% of the response. Statistical model was obtained considering only significant coefficients and is expressed by Equation 2:

$$Y = 10225.55 - 2893.9X_1 - 229.2X_1^2 - 140.68X_1^2 \quad (2)$$

Decoded values for temperature and agitation were 25°C and 300 rpm respectively.

| Run | Temperature (°C) | Agitation (rpm) | PG Extracted (U L ⁻¹) |
|-----|------------------|-----------------|-----------------------------------|
| 1 | 3.5 | 150 | 12194.08 |
| 2 | 6.5 | 150 | 7129.85 |
| 3 | 3.5 | 450 | 12252.49 |
| 4 | 6.5 | 450 | 7185.91 |
| PC1 | 5.0 | 300 | 10235.53 |
| PC2 | 5.0 | 300 | 10296.22 |
| PC3 | 5.0 | 300 | 10162.26 |
| PC4 | 5.0 | 300 | 10209.74 |
| Ax1 | 2.9 | 300 | 14790.63 |
| Ax2 | 7.1 | 300 | 5076.88 |
| Ax3 | 5.0 | 90 | 10295.16 |

Table 7: Response matrix (Y) of the Central Composite Design developed for optimizing PG extraction.

| Coefficient (linear and quadratic effects) | Value | p |
|--|----------|----------|
| β_0 (Block effect) | 10225.55 | - |
| β_1 (L) * | -2983.90 | 0.000001 |
| β_{11} (Q) * | -229.20 | 0.001910 |
| β_2 (L) | -51.16 | 0.081112 |
| β_{22} (Q) * | -140.68 | 0.007836 |
| β_{12} | -0.50 | 0.986844 |

Table 8: Factor coefficients discrimination corresponding to the Central Composite Design.

Effect of ions on the recovery of PG

The control sample had an activity of 13682 U mL⁻¹. Figures 1 and 2 show that none of the anions and cations used in the study had an activating effect when used at a concentration of 30 mM. It is possible to see that the inhibition effect was stronger for cations than for anions. Zn²⁺ and Mn²⁺ were the strongest inhibitors of PG activity with 46% and 51% of inhibition, respectively. For anions the strongest inhibitory effect was given by NO₃⁻, when 85% of the PG activity was achieved compared with the control.

EDTA addition improved the PG activity because it tends to chelate metal ions, which inhibit enzyme activity. Furthermore, the growth medium contained different ions that are considered to be inhibitors of enzyme activity. Therefore, the enzyme stability was tested in solution at room temperature (± 25°C) after 24 hours. The sample with EDTA conserved approximately 90% of the initial activity whereas the control sample lost nearly 33% of its initial activity.

Kinetic pattern of the PG leaching process

The leaching kinetic study was performed considering the optimal conditions determined for the extraction of PG from a solid state fermented dried citrus pulp by *A. niger* F3. These optimal conditions were 1 dried gram of solid fermented medium per 20 mL of solvent in agitated system at 300 rpm and 25°C of temperature. EDTA was added to get a concentration of 50 µM in the solvent employed.

Figure 3 illustrates the extraction kinetics. The highest concentra-

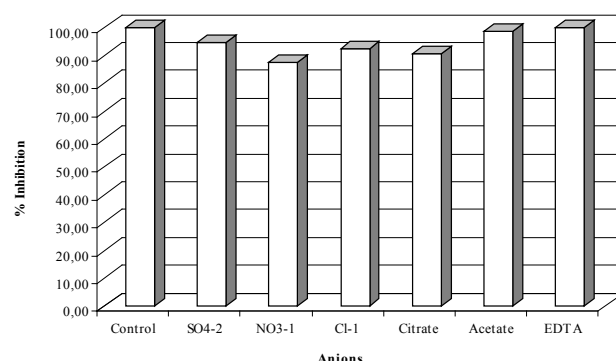


Figure 1: Effect of different anions on the PG activity considering the control to be 11.65 IU/mL leached from a solid fermented (by *A. niger* F3) dried citrus pulp.

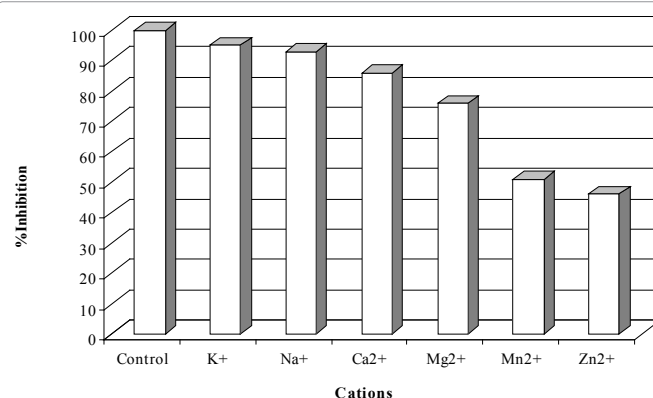
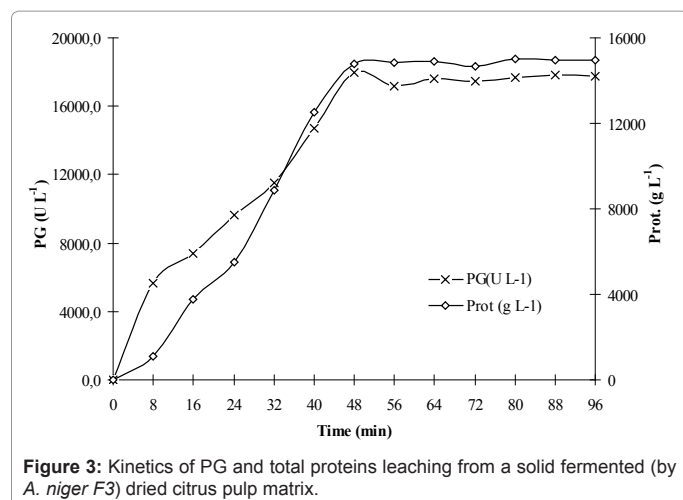


Figure 2: Effect of different anions on the PG activity considering the control to be 11.65 IU/mL leached from a solid fermented (by *A. niger* F3) dried citrus pulp.



tion was 16 IU/mL at 40 min of extraction. At this time, the extraction reached the equilibrium point and the activity remained constant for the rest of the time studied. The concentration obtained was similar to the concentration of PG normally produced in submerged fermentation by several species of *Aspergillus niger*.

The mechanism that controls the leaching process depends on the characteristics of the original matrix from which the product is extracted. If this mechanism depends on the following factors, then a basic equation for the leaching process is represented by a first order kinetic process: migration of the extracted solute from the particle surface into the bulk solution without interferences by the entry of the solvent into the particles; redistribution of the solvent in the cell compartments; expansion of the solid matrix; and the dissolution and/or degradation of solutes. The basic equation reported in scientific literature [24] is as follows:

$$C = C_s (1 - e^{-kt}) \quad (2)$$

Where:

k is the leaching specific rate constant (min^{-1}),

C is the enzyme concentration (IU/mL),

C_s is the saturated enzyme concentration (IU/mL)

t is the time (min).

From Equation (2), the following is deduced:

$$\ln\left(\frac{C_s - C}{C_s}\right) = -kt \quad (3)$$

Equation (3) represents a linear form of Equation (2). Therefore, k may be deduced from Equation (3) by linear correlation if the process is controlled only by the migration of the extracted solute from the particle surface into the bulk solution.

Because *Aspergillus spp.* have the general capacity to synthesise several enzymes, the leaching of other metabolites that may be present in the fermented solid matrix, mainly other undetermined enzymes, was considered in the leaching study. This assumption was made considering the total protein extracted from the solid matrix. Figure 4 shows the linear models obtained for the leaching of PG (IU/mL) and total protein (mg/mL) considering the points obtained until 40 min.

The following linear model is for PG leaching from the values reported in Figure 5:

$$\ln\left(\frac{C_s - C}{C_s}\right) = -0.1077t + 0.1548 \quad (4)$$

$$R^2 = 0.964$$

For the total protein extraction, the linear model is as follows:

$$\ln\left(\frac{C_s - C}{C_s}\right) = -0.177t + 0.1493 \quad (5)$$

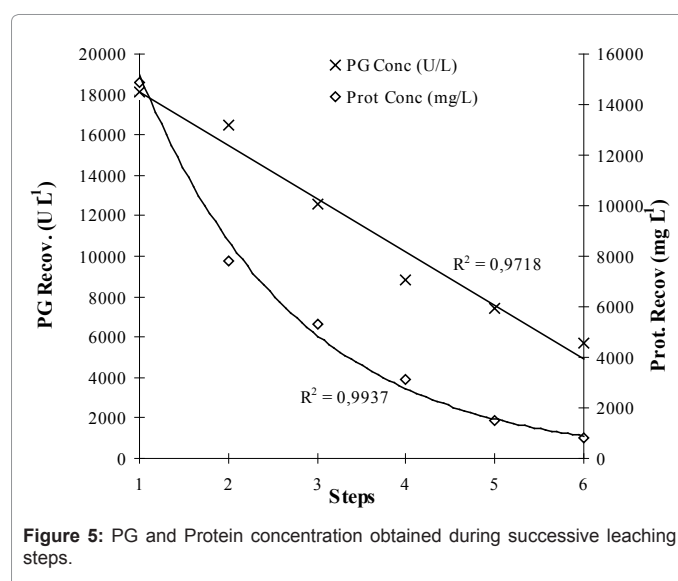
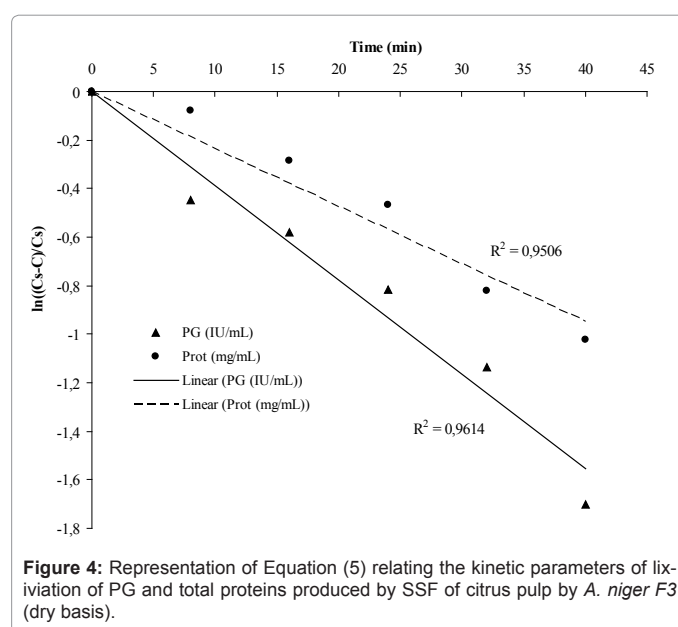
$$R^2 = 0.9506$$

From the former equations, the following can be deduced:

$$C_{PG} = 17.9(1 - e^{-0.107t}) \quad (6)$$

$$C_P = 13.1(1 - e^{-0.177t}) \quad (7)$$

Where C_{PG} is the PG concentration at time t (IU/mL), C_P is the total protein concentration at time t (mg/mL) and t is the time (min).



| Step | PG Conc. (U L ⁻¹) | PG Recov-ery (Ug ⁻¹ d. b.) | Protein (mg g ⁻¹ d. b.) | Specific Activity (Ug ⁻¹ prot.) | Yield Total PG Recov-ery (%) | Yield Total Protein Re-cov-ery (%) |
|------|-------------------------------|---------------------------------------|------------------------------------|--|------------------------------|------------------------------------|
| 1 | 17684 | 137.09 | 115.44 | 1.19 | 29.10 | 44.45 |
| 2 | 13491 | 104.58 | 60.78 | 1.72 | 22.20 | 23.40 |
| 3 | 10586 | 82.06 | 41.26 | 1.99 | 17.42 | 15.88 |
| 4 | 7653 | 59.33 | 24.03 | 2.47 | 12.59 | 9.25 |
| 5 | 6022 | 46.68 | 11.83 | 3.95 | 9.91 | 4.55 |
| 6 | 5329 | 41.31 | 6.38 | 6.48 | 8.77 | 2.46 |

Table 9: Results of PG leaching from citrus pulp fermented by *A. niger* F3 at six steps with a solid-liquid ratio of 1:25 in dry basis.

In the case of PG leaching, the C_s was 15.9 IU/mL and k was 0.107 min⁻¹. The corresponding values for total protein extraction were 13.1 mg/mL and 0.177 min⁻¹, respectively. These results demonstrated that the leaching process of PG was accompanied by the extraction of other proteins, and these proteins were easier to extract than PG according to their respective leaching specific rate constants. The kinetic constant for the total protein extraction was almost the double of the kinetic constant for the PG leaching, which has to be taken into account when the PG specific activity is considered in a further purification, because the total protein extracted does not only correspond to PG. Unfortunately, previous reports do not contain any values related to this topic.

The C_s indicated that the water employed as solvent was saturated at a concentration equivalent to 15.9 IU/mL. This concentration constant also indicated that not all of the enzyme may be extracted in a specific volume of solvent, in one step. Therefore, the solid-liquid ratio needs to be greater, which may be undesired because it results in more liquid disposal to handle within the recovery process. This is contrary to the features of a solid state process in which the disposal problem should impact less when compared with submerged fermentations. Thus, successive extractions or a multistage process is required to determine the complete extraction of the initial enzyme.

Study of the leaching process by a multistage process

As it was observed in the study of solid-liquid relation, working at lowest levels implies in treating a high amount of solids and it is possible to achieve a high concentration of enzyme, but at the same time, part of the enzyme that is not extracted and remains in the solid matrix. In the present case it was decided to proceed with successive leaching steps at a low solid-liquid ratio (1/20) with the residual solid from the preceding step. Each step lasted for 45 min at 300 rpm and 25°C and there were 6 successive steps. Although a countercurrent process could be better at operation, our purpose was to establish the relationship between PG and total protein extracted relative to the quantity extracted and remaining, compared to a single stage. As in the previous kinetic study, the PG and the total protein extracted were considered. Table 9 reports the values obtained for each step.

From Table 9 it is observed that in the one step leaching process for PG at a solid-liquid ratio of 1/20, there was an extraction of 140.29 U g⁻¹ (d.b.) of PG with the residual PG activity as high as 395.98 IU g⁻¹ (d.b.), that is almost three times the value obtained in a one step extraction. It can also be observed that the concentration in each step diminished with successive steps, meaning that the kinetic pattern established former no longer remained and the transport of the solute to the exterior of the particles, or internal diffusion, started to control the process. The increase of the specific activity with the steps indicates that at the same time a purification of the PG took place. This is expected when considering the values for the specific kinetic constants obtained before, which established that proteins not related to PG are easier and faster

to extract. This increase in the specific activity demonstrates that in the preceding steps, protein not related to PG was not as strongly attached to the solid fermented matrix as the PG protein.

These results can be better observed by modeling the values reported in Table 9. The following models were determined from the data reported in Table 9, considering the step number as the independent variable:

$$PG_{Rec} = -2651(\#step) + 20808 \quad (6)$$

$$R^2 = 0.9718$$

and

$$Prot_{Rec} = 26881e^{-0.57(\#Step)} \quad (7)$$

$$R^2 = 0.9937$$

In the case of total protein extraction, an exponential pattern is observed, while in the case of PG it is linear. This fact shows that protein extraction is easier than PG extraction. It can be inferred that the PG protein was more attached to the porous surface of the fermented solid (citric pulp) than the other existing proteins. The high regression coefficients obtained in both models allow for this conclusion. The yields for each step were determined from the total amount of PG and total protein recovery in the 6 steps. In 3 steps 70% of the PG is recovered and also 83% of the total protein. It is remarkable that the yield for the leaching of total protein attached was 45% in the first step, which does not occur in the case of the PG extraction (26% only). After 3 stages it is noticeable that the total protein yields dropped, indicating that the remaining proteins are related mainly to residual PG so the specific activity increased. The increase of PG specific activity means that the system works as a purification procedure. The PG concentrations obtained in the 3 earlier steps are higher than 10 U mL⁻¹, values comparable with concentrations reported for submerged fermentations [9,24,25]. The former conclusion indicates that 3 liquid extractions from a solid state fermented substrate are equivalent to 3 submerged fermentations, emphasizing one of the advantages of this type of fermentation. Another point that is very important to emphasize is that the whole content of PG in the solid fermented substrate after six steps was calculated as 536.27 U g⁻¹ (d.b.), a value that is overlooked when considered at merely one stage. In the case of a single step, only 26% of the total enzyme is obtained. Comparing this value with those reported it can be concluded that the process for production of PG from citrus pulp employing the strain *Aspergillus niger* F3 is quite appropriate.

Conclusions

In this study, PG was recovered from a fermented solid matrix. The results indicated that the best conditions for extraction were room temperature and 1:20 solid-liquid ratio. None of the anions and cations assayed improved the enzyme activity when compared to the control, but addition of EDTA helped in the stabilization of PG, whose activity was measured 24 hours after extraction. Kinetic studies showed that the saturation concentrations for PG and protein were reached after 48 minutes. In three consecutive extraction steps it was possible to recover 70% of all PG produced by solid state fermentation.

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