

## Enzymatic Biocatalyst using enzymes from Pineapple (*Ananas comosus*) Peel Immobilized in Hydrogel Beads.

Biocatalisador enzimático usando enzimas da casca do abacaxi (*Ananas comosus*) imobilizado em esferas de hidrogel.

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### Abstract

The enzymatic extract from pineapple peels, considering the time factor, low cost and ease of preparation of the reaction system, makes the use of this plant material scrap an economical alternative for some reactions of synthetic interest. Therefore, this work aimed to prepare pineapple peel enzymatic extract containing bromelain, with and without mechanical grinding for a period of up to 9-days of extraction, and then immobilizing those extracts on hydrogel beads for their application as a biocatalyst to produce energy interest esters. The best protein content obtained was 1.95 mg mL<sup>-1</sup> for 6-days with mechanical grinding, as for the hydrolysis of p-NPP (p-nitrophenyl palmitate), 0.0125 U.mL<sup>-1</sup> for 1-day extraction with trituration. The best index esterification activity achieved for the lauric acid as a substrate was 1.8 U mL<sup>-1</sup> at 1-day extraction without grinding. For the hydrogel beads immobilization, a maximum protein yield of 28.8% was obtained with the 1-day extract and mechanical grinding, and in terms of p-NPP activity, a yield of 40.6% for the immobilized with 9-day extract without mechanical grinding. The immobilized and dehydrated beads with 1-day extract without grinding took the best esterification activity, 7.2 U g<sup>-1</sup> of biocatalyst. The best conversion performance in the biocatalysis of fatty esters was by esterification of the dodecanoate n-propyl, with 95.1% for a period of 48 hours of reaction. For the transesterification reaction, the methyl oleate yield reached 47.3% after 120 hours of reaction.

**Keywords:** Enzymatic extract, Bromelain, Pineapple, Fatty acids, Transesterification

### Resumo

O extrato enzimático das cascas de abacaxi, considerando o fator tempo, o baixo custo e a facilidade de preparo do sistema de reação faz com que o uso desse material da planta de abacaxi seja uma alternativa econômica para algumas reações de interesse sintético. Portanto, este trabalho teve como objetivo preparar o extrato enzimático de casca de abacaxi contendo bromelaína, com e sem trituração mecânica por um período de até 9 dias, e imobilizar esses extratos em esferas de hidrogel para sua aplicação como biocatalisador para produção de ésteres de interesse energético. O melhor teor de proteína obtido foi de 1,95 mg mL<sup>-1</sup> por 6 dias com moagem mecânica, quanto à hidrólise de p-NPP (p-nitrofenil palmitato), 0,0125 U mL<sup>-1</sup> para extração de 1 dia com trituração. A melhor atividade de esterificação do índice alcançada para o ácido láurico como substrato foi 1,8 U mL<sup>-1</sup> na extração de 1 dia sem trituração. Para a imobilização das esferas de hidrogel, obteve-se um rendimento proteico máximo de 28,8% com o extrato de 1 dia e moagem mecânica e, em termos de atividade de p-NPP, um rendimento de 40,6% para o imobilizado com extrato de 9 dias sem moagem mecânica. As esferas imobilizadas e desidratadas com extrato de 1 dia sem trituração apresentaram a melhor atividade de esterificação, 7,2 U g<sup>-1</sup> de biocatalisador. O melhor desempenho de conversão na biocatálise de ésteres graxos foi pela esterificação do dodecanoato n-propílico, com 95,1% por um período de 48 horas de reação. Para a reação de transesterificação, o rendimento de oleato de metila atingiu 47,3% após 120 horas de reação.

**Palavras-chave:** Extrato enzimático; Bromelina; Abacaxi; Ácidos graxos; Transesterificação

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## 1 Introduction

Enzymes are biodegradable and renewable resources of biocatalysis that play a valuable role in environmental-friendly transformations. Moreover, they are an excellent alternative for catalyzing reactions of industrial interest as the process has low energy requirements as well as mild temperature and pressure conditions (SILVA et al., 2016).

The use of enzymes is often convenient because these biocatalysts are very specific, selective and capable of exhibiting a very high activity compared to the conventional catalysts (JESIONOWSKI et al., 2014). Nevertheless, the use of enzymes for industrial biocatalysis has a high production cost because enzyme synthesis usually takes place in small concentrations and the extraction and purification process is expensive. In addition, enzymes have moderate stability under operational conditions, low activity versus non-physiological substrates and non-absolute specificity or selectivity on industrially relevant substrates.

Enzyme immobilization techniques may overcome some of these drawbacks by maintaining (or even increasing) enzyme activity, modulating enzyme selectivity or specificity, improving resistance to inhibitors (MATEO et al., 2007; GARCIA-GALAN et al., 2011; RODRIGUES et al., 2013) and allowing an easy recovery of the biocatalyst from the reaction medium, which reduces the overall operational costs (SHELDON; VAN PELT, 2013; DICOSIMO et al., 2013; LIESE; HILTERHAUS, 2013).

The literature reports the use of plants from the Brazilian diversity as biocatalysts in several organic reactions, such as *Manihot esculenta*, *Passiflora edulis*, *Vigna unguiculata*, *Glycine max*, sugar cane (*Saccharum officinarum*) and *Lens culinaris*, all of them presenting good conversions and very good enantiomeric excess (ee) at low cost (ASSUNÇÃO et al., 2008; MACHADO et al., 2009; BIZERRA et al., 2010).

Pineapple (*Ananas comosus*) is among the most produced fruits in Brazil (ROGÉRIO et al., 2007), estimate that in pineapple processing, 75-85% results in juice and 15-25% are process residues. In addition, pineapple peel is the residue with the greatest potential for bromelain extraction, since the peel constitutes the largest proportion of pineapple processing residues. (KETNAWA et al., 2012). In this perspective, the pineapple peel has the potential for biomarking, especially as a source of lipases, since it is an abundant and very consumed fruit, and that bromelain has the capacity to catalyze transesterification reactions.

Several high value-added substances can be extracted from waste if appropriate technology is used. This material can be converted directly into the product of interest or raw material for secondary processes (LAUFENBERG et al., 2003). In the case of plant lipases, they are generally obtained as crude extract, partially purified or totally purified from different extraction and separation methods, as reported in the literature (MOUNGUENGUI et al., 2013).

The technique of immobilization of enzymes arouses great interest, mainly due to the need of avoiding problems

that adverse environments cause to soluble enzymes (MARDER et al., 2008). Besides improving thermal and chemical stability of lipases, preventing the denaturant effect of temperature, pH and solvents (KNIGHT et al., 2000). The possibility of recycling the biocatalyst also makes the immobilized form attractive, since several methods of immobilization are feasible in the case of lipases (MARDER et al., 2008). One of the greatest benefits of reactions catalyzed by immobilized enzymes, it is the possibility of reuse of the enzyme, which adds value to the biocatalytic process (SOARES et al., 1999; ASSUNÇÃO et al., 2009).

Hydrogel is a superabsorbent polymer, hydrophilic material capable of absorbing large amounts of water or aqueous solutions due to the ability to expand and commercially found as small beads used as supports for enzymatic immobilization (MARDER et al., 2008). To fabricate hydrogel beads suitable for protein encapsulation, several methods have been developed, such as extrusion, emulsion, molding, and phase separation (CASTELLANOS et al., 2006; JOYE; MCCLEMENTS, 2014). For encapsulation of bioactive proteins, hydrogel beads obtained of biopolymers (proteins and/or polysaccharides) are quite suitable, once the preparation of the beads involves mild conditions and they do not alter protein properties (JOYE; MCCLEMENTS, 2014; CHEN et al., 2006).

Biodiesel, a biofuel which is obtained from renewable sources, is an alternative to the oil diesel. It is produced by the transesterification of vegetable oils and/or animal fats (SRIVASTAVA; PRASAD, 2000). In the process of production of biodiesel, monohydric alcohols are used in the presence of a catalyst, which may be homogeneous, heterogeneous or enzymatic. Enzymatic production of biodiesel uses lipases (glycerol ester hydrolases, E.C. 3.1.1.3) whose biological function is to hydrolyze oils and fats, besides catalyzing other types of chemical reactions, such as esterification, transesterification, aminolysis and lactonization (RAMOS et al., 2004; PAQUES et al., 2008).

Thus, the present work looked for subsidies that can support the development of a heterogeneous biocatalyst, formed by bromelain from pineapple peel, immobilized in hydrogel beads, for the synthesis of esters for energetic purposes.

## 2 Material and Methods

### 2.1 Materials

#### 2.1.1 Peels, Substrate and Hydrogel Beads

The peels used in this experiment were obtained from four specimens purchased locally as well as olive oil. The p-nitrophenyl palmitate (p-NPP) was purchased from Sigma-Aldrich Co (Saint Louis, MO, USA), the lauric acid from Merck Millipore and the hydrogel beads were those of the brand XYXIANGXUN with 2-3mm in diameter.

#### 2.1.2 Chemicals

Potassium phosphate monobasic (KH<sub>2</sub>PO<sub>4</sub>), sodium

hydroxide (NaOH), ethanol, n-propanol, methanol, acetone, hexane, phenolphthalein, brilliant blue Coomassie G-250, phosphoric acid, sodium dodecyl sulfate, mercaptoethanol and sodium polyacrylate were acquired in its analytical grade PA form. Bovine albumin (BSA), Triton X100, filter paper for rapid filtration (qualitative 80 g) and MiniProtein cell (Biorad) were used in their commercial form. The sodium hypochlorite (NaClO) employed was the commercial product at 3% and potassium bromide (KBr) was of spectroscopic grade. All the reagents were purchased from Sigma-Aldrich Co (Saint Louis, MO, USA).

## 2.2 Methods

### 2.2.1 Hydrogel Beads Characterization

Hydrogel beads were characterized by infrared spectroscopy technique, in which the samples were homogenized in a mortar with spectrophotometric grade KBr (potassium bromide) in a proportion of approximately 10% of sample. The mixture was then pressed into a 4.5 ton steel die applied by a hydraulic press. Tablets were obtained and then analyzed in a Perkin-Elmer spectrometer (model 1000-FT) in the transmittance mode and in the range of 4000 to 400  $\text{cm}^{-1}$ . In this case, two spectra were obtained, one of the hydrogel beads and the other of a standard sample of sodium polyacrylate.

#### 2.2.2 Study of Hydration of the Hydrogel Beads

Hydration rate of the beads was determined by placing a hydrogel bead in a Becker vessel containing 100 mL of distilled water. The diameter was calibrated with pachymeter every hour, up to 9 hours of immersion. This process was repeated for 6 (six) times and the mean diameter was calculated. Finally, the measurements were confirmed after 48 hours of immersion to determine the equilibrium size of the beads, adapted from Marder et al. (2008).

### 2.2.3 Peel Asepsis

The pineapples were washed in running water with detergent and then washed with distilled water. The fruit peel was then removed and cut into cubes of  $\pm 1.5$  cm of edge and then were immersed in sodium hypochlorite solution (NaClO 3%) for a period of 15 min. After further washing with distilled water, the peels were oven dried at about 30 °C for 4 hours (FEITOSA et al., 2013).

### 2.2.4 Enzyme Extracts Preparation

The preparation of the enzymatic extracts was done by two methods. In the first, 60 g of dried peels were immersed in 300 mL of distilled water and allowed to stand at 5 °C. In the other process, 60 g of dried peels were ground with 300 mL of distilled water in a domestic blender and stored at 5 °C. Storage time was variable, comprising 1-, 6- and 9-days. After each of these periods, a simple filtration with sieve was carried out, and the aqueous extracts were obtained, denominated: enzyme extract number 1 (EXT1) for the first methodology

and enzyme extract number 2 (EXT2) for the second methodology (KETNAWA et al., 2012; FEITOSA et al., 2013). Residues were discarded.

### 2.2.5 Enzyme Extracts Characterization

Enzymatic extracts were characterized in terms of the molecular masses of the proteins by electrophoresis (SDS-PAGE) under denaturing conditions (LAEMMLI, 1970). A MiniProtein cell (Bio-rad), 12% run gel in a 9 cm x 6 cm separation zone, and a 5% polyacrylamide concentration zone were used. The EXT1 and EXT2 samples were filtered on filter paper for rapid filtration and resuspended in 1 mL of burst buffer (2% SDS and 10% mercaptoethanol); boiled for 5 min and 20  $\mu\text{L}$  aliquot of the supernatant was used in the experiments. The gels were stained with brilliant blue Coomassie G-250. Low molecular weight markers were used (10-200 kDa).

#### 2.2.6 Enzyme Extracts Immobilization on Hydrogels Beads

This procedure followed according to Ketnawa et al. (2012) and the immobilization strategy employed in this study was notably that of physical encapsulation. For this experiment, 60 hydrogel beads (1.0 g) were immersed in 150 mL of enzyme extract for 4 hours at room temperature while stirring at 100 RPM. Then, the beads were separated from the medium by simple filtration. The beads were named: immobilized number 1 (IMOB1), those immersed in EXT1, and immobilized number 2 (IMOB2), those where EXT2 was used. The volume of the remaining enzyme extracts obtained in the filtration processes were denominated supernatant number 1 (SOBN1) and supernatant number 2 (SOBN2), respectively.

### 2.2.7 Immobilized Beads Dehydration

Still according to Ketnawa et al. (2012), part of the immobilized beads was taken to an oven at 37 °C for 48 hours to withdraw the water from the interior. The IMOB1 beads, after dehydration, were called catalytic material number 1 (CAT1) and the IMOB2 beads were called catalytic material number 2 (CAT2).

### 2.2.8 Protein Concentration Determination

Protein concentration of the enzyme preparations was determined by the Bradford colorimetric method (BRADFORD, 1976). Initially, a solution of bovine serum albumin (BSA) at a concentration of 1 mg  $\text{mL}^{-1}$  was prepared. From this solution, dilutions were made with a total volume of 200  $\mu\text{L}$  in Eppendorf tubes, 2000  $\mu\text{L}$  of the Bradford reagent was added and each Eppendorf tube was shaken for 5 seconds. The reaction time was 10 minutes.

Protein concentration determination was performed on EXT1 and EXT2 extracts at 1-, 6- and 9-days storage at 5 °C, as well as on their respective immobilization supernatants (SOBN1 and SOBN2), in order to calculate the approximate amount of protein immobilized on

each hydrogel sphere. For this, 200  $\mu\text{L}$  of the sample was pipetted into eppendorf tubes (in triplicate) and 2000  $\mu\text{L}$  of Bradford reagent was added, repeating the same procedure. The calibration curve is performed with absorbance in relation to concentration.

### 2.2.9 Nitrophenyl Palmitate (p-NPP) Hydrolysis

This method is based on the hydrolysis of p-nitrophenyl palmitate (p-NPP) by the enzyme in aqueous medium and was adapted from Pencreach and Baratti (1996). For this procedure, 50 mL of solution A containing 4.6 mM of p-NPP in n-propanol and 250 mL of a solution B with 0.4% of Triton X 100 and 0.1% of arabic gum in 100 mM sodium phosphate buffer (pH 7.0). Thus, 0.18 mL of solution A and 1.62 mL of solution B are transferred to a cuvette. After stabilizing the temperature at 37 °C, 0.2 mL of the soluble enzyme was added. The release of yellow p-nitrophenol (p-NP) was monitored at 410 nm against a blank without enzyme. The absorbance change was measured every minute up to 5 minutes. In the case of immobilized enzyme, 30 mL of the solution (1 vol. of A + 9 vol. of B) was reacted with 5 hydrogel beads containing immobilized enzymes (IMOB1 and IMOB2). 2 mL of sample was collected every minute up to 5 minutes and measured the absorbance under the same conditions. For both cases, the slope of the “absorbance vs time” curve was obtained. Data not shown.

After that, the reaction rate was determined from the molar extinction coefficient under the described conditions. This value was determined by the absorbance of standard solutions of p-nitrophenol (p-NP) in the reaction mixture (Supplementary Data). One unit of activity (UPNPP) was the amount of enzyme required to release 1  $\mu\text{mol}$  of p-NP per minute under the described conditions.

### 2.2.10 Immobilization Parameters

The percentage of immobilized protein (Rimmob) is calculated by Equation (1) according to Santos et al. (2017). The volume of the supernatant at the end of the immobilization process is different from the initial volume of the enzyme extract.  $C_i$  is the total protein concentration of the extract after immobilization (supernatant) in ( $\text{mg mL}^{-1}$ ) and  $C_f$  is the total protein concentration of the extract ( $\text{mg mL}^{-1}$ ),  $V_i$  is the initial volume of the enzyme extract  $V_f$  is the volume of the supernatant (mL).

$$R_{immob}(\%) = \frac{C_i \cdot V_i - C_f \cdot V_f}{C_i \cdot V_i} \cdot 100 \quad \text{equation 1}$$

The immobilization yield in terms of activity was calculated by Equation (2).

$$At_{immob}(\%) = \frac{U_i \cdot V_i - U_f \cdot V_f}{U_i \cdot V_i} \cdot 100 \quad \text{equation 1}$$

Where  $U_i$  is the initial activity of the enzyme extract ( $\mu\text{mol min}^{-1} \text{mL}^{-1}$ ),  $V_i$  is the initial volume of the enzyme extract (mL),  $U_f$  is the enzyme activity measured in the supernatant at the end of immobilization ( $\mu\text{mol min}^{-1}$ ),  $V_f$  is the volume of the supernatant (mL).

### 2.2.11 Esterification activity determination

The esterification activity was calculated from the reaction of lauric acid and n-propanol. It was carried out according to Pierozan (2014). Thus, 1 g of lauric acid was reacted with 15 mL of n-propanol catalyzed per 10 mL of enzyme extract or 12 beads of hydrogel, in the case of the immobilized enzyme. The reaction medium was maintained at 40 °C for 40 minutes. 200  $\mu\text{L}$  aliquots were withdrawn from the reaction medium at time zero and at 40 minutes, diluted into 20 mL of acetone/ethanol (1:1 v/v) to stop the reaction. The amount of lauric acid consumed was determined by titration with 0.025M NaOH using phenolphthalein as indicator. One unit of esterification activity was defined as the amount of enzyme capable of consuming 1  $\mu\text{mol}$  of lauric acid per minute under the described conditions. Productivity was the parameter used to evaluate the lipase extraction potential of pineapple peels.

## 2.3 Immobilized Pineapple Peels for the Esters Production

### 2.3.1 Esterification Reaction for the -CH<sub>3</sub>, -C<sub>2</sub>H<sub>5</sub> and -C<sub>3</sub>H<sub>7</sub> Dodecanoates

This experiment, adapted from Marder et al. (2008), was carried out in erlenmeyer flasks. The formation of n-propyl dodecanoate, ethyl and methyl, was developed by the mixture of dodecanoic acid with n-propyl, ethyl and methyl alcohol, respectively, in the molar ratio of (1:5). The reaction was catalyzed by the derivative catalysts (CAT1 and CAT2) at the rate of 6% (w/v) and shaken at 150 rpm for 5-days at room temperature. A blank without catalyst was monitored for comparison. 200  $\mu\text{L}$  aliquots were withdrawn from the reaction medium with 24 hours, 48 hours and 120 hours and packed in eppendorf tubes containing 200  $\mu\text{L}$  of hexane at room temperature.

The mass spectra of the esterification products were obtained on the Hewlett-Packard mass spectrometer, model HP-5971 A, coupled to a gas-liquid chromatograph, model HP-5890 A, series II (CGL/MS), fitted with a capillary column of methyl-phenyl silicone (25.0 m x 0.20 mm x 0.25  $\mu\text{m}$ ) using helium at 1.0  $\text{mL min}^{-1}$  as carrier gas in split mode, operating with temperature gradients varying depending on the chemical nature of the compounds analyzed. For the low molecular mass compounds, a gradient of 10 °C  $\text{min}^{-1}$  (40-180 °C) and 40 °C  $\text{min}^{-1}$  (180-300 °C) was used and for those of higher molecular mass a gradient of 10 °C  $\text{min}^{-1}$  (100-180 °C) and 40 °C  $\text{min}^{-1}$  (180-300 °C), with the injector temperature of 250 °C and detector of 280 °C.

### 2.3.2 Transesterification Reaction for the -CH<sub>3</sub>, -C<sub>2</sub>H<sub>5</sub> and -C<sub>3</sub>H<sub>7</sub> Oleates

The transesterification reaction was performed similarly to esterification but this time the raw material used was extra virgin olive oil. 10 mL of the oil was added to 6.5 mL of n-propanol (0.1 mmol) and the reaction medium was catalyzed by both CAT1 and CAT2 beads. This same procedure was performed for methanol and ethanol, where the same amounts were used in moles, 5 mL and 3.5 mL, respectively.

## 3 Results and Discussion

### 3.1 Hydrogel Beads

#### 3.1.1 Characterization

In the comparative analysis between the absorption spectra in the infrared region obtained, hydrogel beads and sodium polyacrylate showed big similarities (SILVERSTEIN et al., 2005). The peaks that identify molecules clusters have shown that the hydrogel beads used are effectively sodium polyacrylate. Some distortion between them is probably due to the degree of purity

of the beads; however, it was not possible to verify any quantitative analysis of purity. The wavelength values that characterize the sodium polyacrylate where deformations and stretches occur in the spectrum are: 3400 cm<sup>-1</sup> (OH-), 2950 cm<sup>-1</sup> (alkanes: CH<sub>2</sub>), 1655 cm<sup>-1</sup> (carbonyl), 1450 cm<sup>-1</sup>, 1400 cm<sup>-1</sup> and 1320 cm<sup>-1</sup> (alkanes: CH, CH<sub>2</sub>, CH<sub>3</sub>). The spectra of hydrogel can be seen in Figure 1.

#### 3.1.2 Hydration

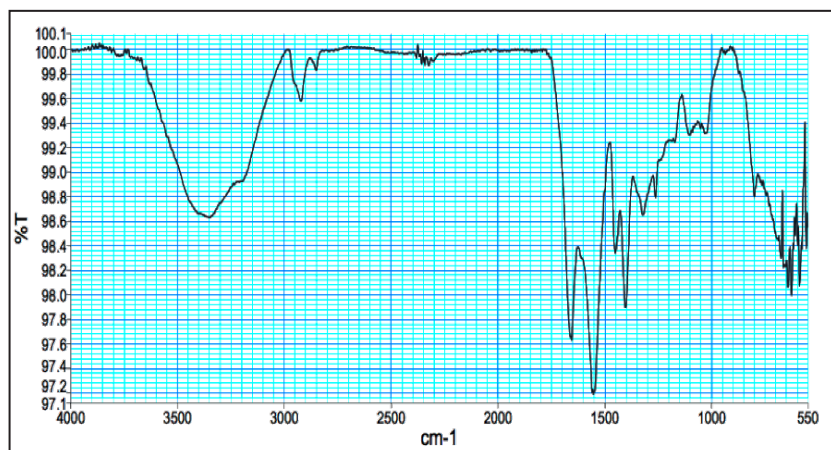
The initial mean diameter of beads was 2.3 mm and mean mass was 0.01757 g/bead. At equilibrium, these values reached 16.4 mm and 0.74 g/beads. A more intense growth rate was observed in the first four (4) hours of immersion, corresponding to the region of maximum slope in the Graph 1.

At the end of this period, the beads had a mean diameter of 15.0 mm (91.11% of the equilibrium diameter).

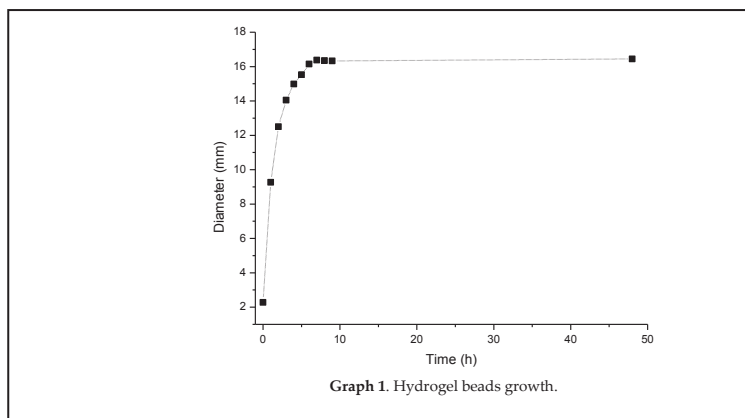
Based on these results, four hours were considered as the optimal time for the immersion of the beads in the enzymatic extract, once, after this time, there was not considerable increase in the volume of the beads and, consequently, no considerable amount of the enzymatic extract inside the support.

The mathematical model that best represents the

Figure 1. Absorption spectrum in the infrared region of the hydrogel.



Graph 1. Hydrogel beads growth.



phenomenon of hydration of hydrogel beads, with confidence level  $R^2 = 0.9832$ , is described in Equation (3).

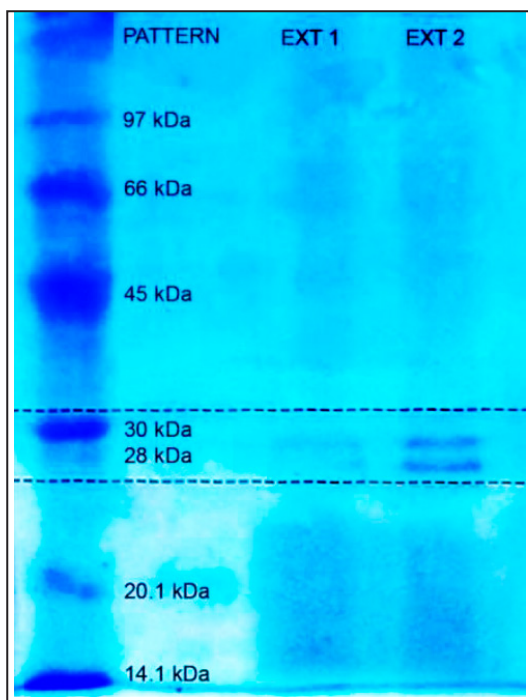
$$d(t) = \frac{18.2t}{0.94 + t}$$

equation 3

### 3.2 Preparation, characterization and immobilization of enzyme extract

The enzyme extracts obtained were very different, whereas the first one (enzyme extract number 1: EXT1), prepared by the simple immersion of the aseptically peeled in distilled water, has shown a clear and less intense color appearance, the second one (enzyme extract number 2: EXT2), which underwent mechanical grinding, has shown a stronger color and higher turbidity. Plant tissues not retained in the filtration were also perceived in this case, which justifies at first, the increase of turbidity. The protein patterns for the enzyme extracts (EXT1, EXT2) are set forth in Figure 2.

Figure 2. Molecular mass patterns of proteins of the enzymatic extracts. Lane 1: pattern; Lane 2: enzyme extract number 1; Lane 3: enzyme extract number 2.



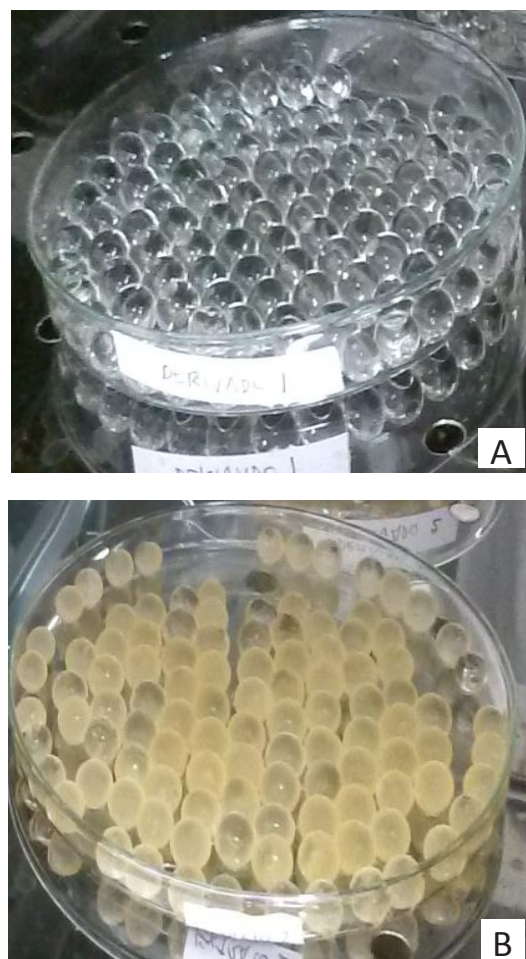
These results have shown that the protein components of both extracts were practically the same, except in the intensity, which suggests that the EXT2 has a higher amount of protein. The major protein components identified in both extracts have presented molecular mass between 28 and 30 kDa. Therefore, both extracts probably contain bromelain in their composition, once Ketnawa, Chaiwut and Rawdkuen (2012) obtained in their experiment's values of approximately 28 kDa for the commercial extract of bromelain. This result is

particularly relevant because it shows that the two extraction processes were able to remove bromelain from the pineapple peel.

The immobilized number 1 (IMOB1) retained in its interior  $0.82 \pm 0.08$  mL (standard deviations  $n = 3$ ) of crude enzyme extract for each hydrogel sphere, while for immobilized number 2 (IMOB2) this value was  $0.76 \pm 0.14$  mL/bead (standard deviations  $n = 3$ ). However, by the standard deviations obtained, it was not possible to infer the existence of some relation between the two types of extraction and the volume of extract encapsulated by the hydrogel spheres.

Regarding the physical aspect, the immobilized spheres with the enzymatic extract number 2 (IMOB2) presented, as well as the extract (EXT2), a stronger color tending to "yellow-green" and less transparency. It was also noticed the increase in the mass of the spheres; for IMOB1 this increase was on average 45.53 times, and 38.13 times for IMOB2. See Figure 3.

Figure 3. Immobilized hydrogel beads - IMOB1 (A): Immobilized number 1 immersed in EXT1; IMOB2 (B): immobilized number 2 immersed in EXT2.



After water removal in the oven, there was an increase of 24.6% for catalytic material number 1 (CAT1) and 25.8% for catalytic material number 2 (CAT2) in relation to the original beads mass, indicating that the substances contained in the extracts, among them bromelain, remained fixed into the support and only the water was removed. Catalytic derivatives (CAT1 and CAT2) also exhibited different staining than in natura hydrogel beads.

### 3.3 Protein Concentration

The protein concentration for the crude extract obtained without grinding (EXT1) was 0.13 mg/mL for 1-day of immersion, after 5-days, this value increased to 0.96 mg mL<sup>-1</sup>. When the immersion time reached 9-days, the protein content in the extract dropped to 0.90 mg mL<sup>-1</sup>. When the enzyme extract number 2 (EXT2) was used, the same trend was observed, but all protein contents were much higher than the values of the first extract, which suggests that the blender grinding favors the extraction of proteins from pineapple peels. See Table 1.

Table 1. Protein concentration of enzyme extracts and corresponding immobilized beads.

Protein Concentration	Extraction time (days)		
	1	6	9
EXT1 (mg mL <sup>-1</sup> )	0.13 ± 0.01*	0.96 ± 0.03*	0.90 ± 0.02*
IMOB1 (mg g <sup>-1</sup> )	6.26 ± 1.70*	24.48 ± 10.24*	18.21 ± 3.98*
EXT2 (mg mL <sup>-1</sup> )	1.26 ± 0.05*	1.95 ± 0.06*	1.93 ± 0.05*
IMOB2 (mg g <sup>-1</sup> )	68.87 ± 16.40*	53.73 ± 8.48*	40.00 ± 13.09*

\*Standard deviation (n = 3)

Table 2. Activity of p-NPP hydrolysis and immobilization parameters for 1-day extraction.

Pineapple Peels	Enzymatic extract activity1 (U mL <sup>-1</sup> )	Immobilized beads activity2 (U g <sup>-1</sup> )	Yields in terms of protein(%)	Yields in terms of activity (%)
Extraction 1	0.0087 ± 0.0017*	0.0107 ± 0.0007*	24.0	5.1
Extraction 2	0.0125 ± 0.0012*	0.0225 ± 0.0051*	28.8	7.8

1Refers to what was termed enzyme extract (EXT1 and EXT2) after 1-day of extraction. 2Immobilized (IMOB1 and IMOB2). \*Standard deviation (n = 3).

Table 3. Activity of p-NPP hydrolysis and immobilization parameters for 9-days extraction

Pineapple Peels	Enzymatic extract activity1 (U mL <sup>-1</sup> )	Immobilized beads activity2 (U g <sup>-1</sup> )	Yields in terms of protein(%)	Yields in terms of activity (%)
Extraction 1	0.0119 ± 0.0051*	0.0152 ± 0.0010*	9.9	40.6
Extraction 2	0.0117 ± 0.0007*	0.0200 ± 0.0101*	10.1	18.7

1Refers to what was termed enzyme extract (EXT1 and EXT2) after 9-days of extraction. 2Immobilized (IMOB1 and IMOB2). \*Standard deviation (n = 3).

These results corroborate the work of Ketnawa, Chaiwhit and Rawdkuen (2012), which showed a total of proteins extracted equal to 132.4 mg per 100g of pineapple peel, equivalent to 0.26 mg mL<sup>-1</sup> of aqueous extract. It is noteworthy that in this case, the enzymatic extract was obtained immediately after mechanical grinding, there was no immersion time. The fact that the protein concentrations obtained for EXT2 are higher may justify a direct influence of the extraction time with the protein concentration obtained in the aqueous extracts.

According to the values shown in Table 1, although longer extraction times have produced higher protein content in the crude enzyme extract, this did not imply in a support with more immobilized proteins or, on the other hand, the maintenance of the water-immersed pineapple peel at 5 °C for a time greater than 1-day, only resulted in wasted resources. The reasons for this phenomenon are unclear; however, it must be linked to the ability of aqueous solutions to be absorbed by the support. Thus, IMOB2 presented the best protein content, that is, the one obtained from the extract (EXT2), with extraction time of 1-day.

### 3.4 Hydrolysis activity: p-nitrophenyl palmitate (p-NPP)

As can be seen in Table 2, the results concerning the hydrolysis activity of p-nitrophenyl palmitate (p-NPP) were satisfactory as it was possible to immobilize enzymatic activity in the beads with only one day. These values show that the mechanical grinding favored the extraction of lipases that hydrolyze this type of substrate.

Regarding immobilization yields, it was obtained 24.0% and 28.8% in terms of protein and 5.1% and 7.8% in terms of activity to produce IMOB1 and IMOB2, respectively. These results show that extracts prepared from mechanical grinding result in a better immobilization efficiency both in terms of proteins and in activity (see Table 2).

The enzymatic extracts with 6-days extraction achieved values of 0.0109 ± 0.0070 U mL<sup>-1</sup> and 0.0120 ± 0.0040 U

mL<sup>-1</sup>, as well as their respective immobilized ones that showed p-NPP hydrolysis activity of  $0.0147 \pm 0.0019$  U g<sup>-1</sup> and  $0.0140 \pm 0.0005$  U g<sup>-1</sup>. It was possible to achieve a yield of 32.4% in terms of activity to produce IMOB2.

After 9-days, the yield in terms of protein was 9.9% for the first immobilization and 10.1% for the second, yields in terms of activities reached 40.6% and 18.7%, respectively (see Table 3).

Regarding the results shown above, although hydrolysis activity values of p-NPP can be considered low compared to the peel (1.02 U g<sup>-1</sup>) and orange frit - outer part of the epicarp of *Citrus sinensis* L. Osbeck in which there is a higher concentration of oil glands (3.12 U g<sup>-1</sup>) shown by Delgado (2014), they are still quite expressive, since no purification technique of the lipase extract was used. In the same work, Delgado (2014) did not identify hydrolysis activity of the p-NPP for the peel, pulp and mango seeds (*Mangifera indica* L.) and orange pomace (*Citrus sinensis* L. Osbeck).

Moreover, it was observed that mechanical grinding favored an increase in lipolytic activity both in the crude extracts and in the respective immobilized ones; in addition, the extraction time had an opposite influence on the yields. While yield in terms of protein decreased with increasing time, yield in terms of activity increased as a function of the increase in extraction time. However, despite the standard deviations, the best condition was the one in which there was mechanical grinding of the peels in the blender and time of extraction of 1-day.

### 3.5 Esterification Activity

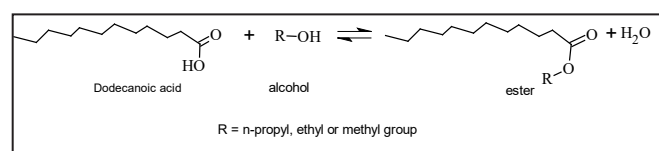
The extracts of pineapple peel showed esterification activity between lauric acid and n-propanol. In contrast to the hydrolysis, catalysts from the extraction process (EXT1, IMOB1 and CAT1) showed higher esterification activity than those with mechanical grinding (EXT2, IMOB2 and CAT2), although these values are very close. It is noteworthy that, apparently, the dehydrated beads obtained an increase of activity in relation to the hydrated ones; however, it is worth mentioning that the beads IMOB1 and IMOB2 have a mass much higher than the catalysts CAT1 and CAT2 (see table 4).

Pierozan (2014) obtained esterification activity of lauric acid with n-propanol between 7.06 and 57.88 U g<sup>-1</sup> for

wheat seed extract (*Triticum aestivum*). These results have evidenced that the pineapple peels produced satisfactory values, since they yielded maximum activity of 9.10 U g<sup>-1</sup> of dehydrated pineapple peels. The result is even greater if it is considered that cereals and oilseeds are the main sources of plant lipases (PAQUES; MACEDO, 2009).

### 3.6 Esters Production

The production of n-propyl dodecanoate has shown that CAT1 did not achieve higher yields than the uncatalyzed reaction; however, the CAT2 catalyst has shown a promising result, since it reached a maximum yield of 95.1% for 48 hours of reaction, and, in the three investigated moments, it always maintained better yields than those for the CAT1 and the non-catalyzed reaction. In addition, it was observed that, after 48 hours of reaction, there was a shift of the reaction equilibrium toward the reactants in the three reactions (Scheme 1).



Scheme 1. The Esterification equation of dodecanoic acid.

In the production of ethyl dodecanoate, both CAT1 and CAT2 achieved higher yields than those obtained without the use of catalysts. This shows that the catalysts shifted the reaction towards the products. However, unlike what happened with n-propanol, CAT1 performed better than CAT2. After 120 hours of reaction, CAT1 achieved 90.7% yield and CAT2 79.0% yield. In this experiment, the reactions reached maximum extension for 120 hours for the three forms.

When methanol was used in the reaction to produce methyl dodecanoate, better yields were observed with 24 hours, and thereafter, there was a gradual shift towards the reactants in the three configurations. This experiment indicated that the use of methanol possibly inactivated the effect of the catalytic derivatives CAT1 and CAT2, since these obtained inferior results to the reaction

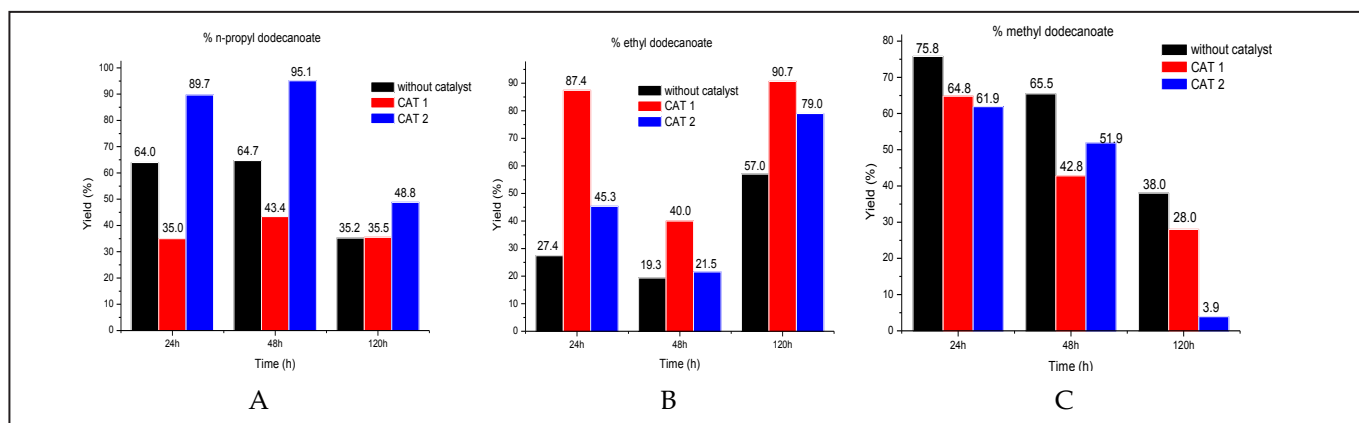
Table 4— Lipase productivity and esterification activity of the enzyme extract, immobilized beads and catalytic derivative

Pineapple Peels	Productivity (U g <sup>-1</sup> dehydrated peel)	Enzymatic extract activity <sup>1</sup> (U mL <sup>-1</sup> )	Immobilized beads activity <sup>2</sup> (U g <sup>-1</sup> )*	Immobilized dehydrated beads activity <sup>3</sup> (U g <sup>-1</sup> )**
Extraction 1	$9.10 \pm 1.80$	$1.82 \pm 0.36$	$4.72 \pm 0.23$	$7.14 \pm 0.50$
Extraction 2	$8.59 \pm 1.75$	$1.72 \pm 0.35$	$4.10 \pm 0.48$	$7.01 \pm 0.49$

<sup>1</sup>Refers to what was termed enzyme extract (EXT1 and EXT2) after 1-day extraction. <sup>2</sup>Immobilized (IMOB1 and IMOB2), respectively. <sup>3</sup>Catalytic derivative (CAT1 and CAT2), respectively. \*The mass of the catalyst for the hydrated beads was used. \*\*The mass of the catalyst for the dehydrated beads was used. Standard deviation (n = 3).



Figure 4. Yield of the esterification of n-propyl dodecanoate (A), ethyl (B) and methyl (C).



without catalyst. This phenomenon can be explained by analogy to the work of Shimada et al. (1999), in which it is stated that the use of methanol increases the loss of lipase activity (Novozym 435), what may also be occurring for the plant lipases of pineapple peel (See Figure 4).

Figure 5 shows the yield percentages for the transesterification of olive oil with the n-propanol, ethanol and methanol alcohols in terms of oleic esters. This experiment has shown that there was no formation of n-propyl oleate in any of the three cases (without catalyst, CAT1, CAT2) even after 120 hours of reaction.

For the reaction conducted with ethanol, ethyl oleate ester formation was detected for the three cases, but the most expressive values were after 120 hours of reaction, in which CAT2 obtained the best yield of 18.1%, followed by CAT1 with 12.8%. At this point, a positive influence on the shift of the reaction towards the products was noted, indicating that the immobilized lipases had an effect.

The methyl oleate formation was better favored using CAT2, after 120 hours, and it achieved 47.3% yield, while in the other two configurations the results were 7.8 and 9.5% for reaction without catalyst and with CAT1, respectively. Although these results are not quite uniform, it can be understood that the catalytic derivatives, in comparison to the sample without catalyst, influenced the shift towards the products of the reaction.

The use of methanol for transesterification has shown

that the catalytic derivatives CAT1 and CAT2 influenced the equilibrium of the reaction towards the products, reinforcing the idea that the catalytic derivatives made from the pineapple peels have lipolytic activity with respect to the transesterification.

### 3.7 Enzyme Reuse

In order to analyze the reuse potential of the polyacrylamide immobilized enzyme, the oil acetylation reaction was performed four times at 30 °C, using the optimized conditions discussed above, an interval between

Figure 6. Immobilized enzyme reuse for four cycles of oil acetylation at 30 °C.

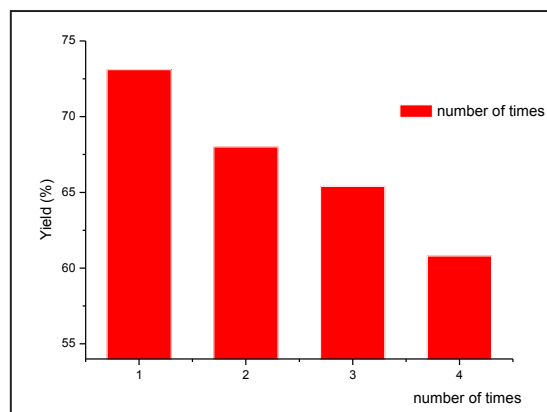
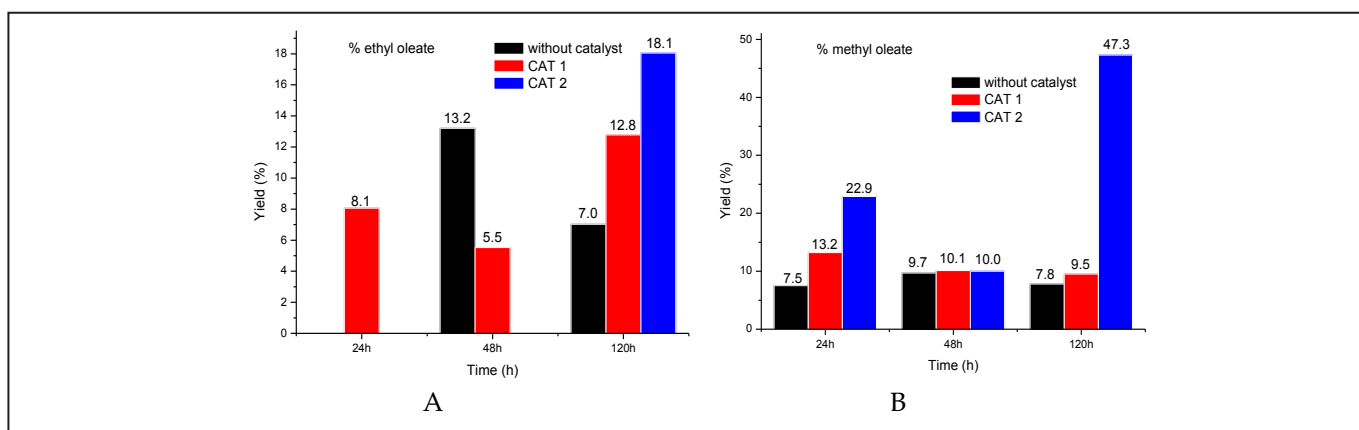


Figure 5. Yield of the transesterification of ethyl oleate (A) and methyl oleate (B).



one reaction and another of 2 hours was considered. The results (shown in Figure 6) made it possible to verify that the reaction conversion rate varied very little (4.0-5.0%) between the first and the last reaction. Based on the 3.6 section, with 120h, CAT1.

## 4 Conclusion

By analyzing the results of this work, it can be stated that pineapple peels are sources of bromelain and that a simple immersion of these peels in water is enough to allow the migration of these molecules to the liquid phase. The utilization of mechanical grinding and water has improved the lipolytic potential of the extract produced. Regarding the time of extraction, the period of 1-day has proved to be effective for the objectives of this study.

For the extracts immobilized on the hydrogel beads, it was observed the fixation of this lipolytic activity on the support, since a peak of 40.6% fixation yield in terms of activity was achieved for the immobilized number 1 after 9-days of extraction and p-NPP hydrolysis activity was detected for the beads immobilized with both EXT1 and EXT2.

In the reactions of transesterification and esterification, the catalytic derivatives CAT1 and CAT2 have shown to favor the displacement of the reaction towards the products. In the formation of the dodecanoic acid esters, the catalytic derivatives obtained good results for n-propanol and for ethanol, CAT2 and CAT1, respectively. The formation of n-propyl dodecanoate reached a maximum value of 95.1% conversion after 48 hours of reaction, while CAT1 achieved 90.7% for ethyl dodecanoate after 120 hours of reaction.

In the transesterification, the catalytic derivatives did not show influence for n-propanol, that is, there was no formation of n-propyl oleate in any of the cases. Differently from the first case, the formation of ethyl and methyl esters with some influence of the catalytic derivatives in the increase of the displacement of the reaction was detected. The maximum conversion obtained was 47.3% for methyl oleate and 18.1% for ethyl oleate for 120 hours of reaction.

To sum up, it was possible to notice that the heterogeneous catalyst formed by the pineapple peel extract with mechanical grinding and hydrogel beads has proved to be promising as a biocatalyst in the production of esters of commercial interest.

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Conceptualization, Resources, Data Curation, Investigation Aluísio M. Fonseca and Mauro M. de Oliveira; Methodology, Writing-Original Draft Preparation, Writing-Review & Editing, José Cleiton S. dos Santos and Rodolpho R. C. Monteiro; Juan Carlos A. Alcócer, Software, Regilany P. Colares; Validation, Aiala V. Amorim and Rinaldo S. Araújo; Formal Analysis, Maria C. M. de Souza and Olienaiide R. de O. Pinto.

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