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Lipase entrapment in PVA/Chitosan biodegradable film for reactor coatings

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ABSTRACT

This study reports the development and characterization of novel biodegradable film, based on chitosan and polyvinyl alcohol containing lipase entrapped. The films showed a thickness of 70.4 and 79 μm to PVA/Chitosan and PVA/Chitosan/Lipase, respectively. The entrapment of lipase in PVA/Chitosan film resulted in increasing of 69.4% tensile strength (TS), and 52.4% of elongation. SEM images showed the formation of a continuous film, without pores or cracks. The lipase entrapment efficiency was estimated in 92% and the films were repeatedly used for 25 hydrolytic cycles, maintaining 62% of initial activity. The PVA/Chitosan/Lipase film was used for olive oil hydrolysis of high performance. These results indicate that PVA/Chitosan/Lipase is a promising material for biotechnology applications such as triacylglycerol hydrolysis and biodiesel production.

1. Introduction

Lipases are enzymes that catalyze the hydrolysis of acylglycerides and other esters at the interface between water and insoluble substrates. In organic medium, lipases also catalyze the reverse reaction of synthesis of esters or transesterification [1]. The microbial lipases have attracted considerable attention due to their potential, such as high production, good stability, and many stereo-specific properties. Most of the reports on lipase-catalyzed ester hydrolysis or synthesis involves lipases from *Candida rugosa*, *Rhizomucor miehei*, *Rhizopus oryzae*, *Humicola lanuginosa*, *Thermomyces lanuginosus* and others [2,3].

Lipolase® is a commercial lipase preparation from the fungus *T. lanuginosus*, which is produced on industrial scale using *Aspergillus oryzae* as a host organism [4]. In industrial processes, the feasibility of the use of enzymes is mainly determined by the cost of the biocatalyst, easy recovery, and improvement of its stability [3,5]. Thus, the immobilization of enzymes in supports that allow their recovery, and that may also help to increase the operational stability is very attractive. Immobilization also contributes to stabilize protein structure; thus, reducing biocatalyst inactivation, either thermal or due to interactions with solvents [6–8]. Regarding to lipases, the chemical nature of the material used in immobilization represents an important component in the reaction, once lipases actuate at water/oil interface and the support must have a chemical characteristic that allows the reaction to take place.

In this scenario, blends of chitosan and polyvinyl alcohol (PVA) are promising biodegradable materials due to their highly controllable chemical and physical properties. Chitosan is a deacetylated polysaccharide obtained from chitin, the main exoskeleton component in crustaceans [9]. Chitosan has promising applications in biotechnological fields due to its good biodegradability, nontoxicity, biocompatibility, and ability to form membranes and films, beads, fibers, gels and actuate as gas and aroma barrier [10–13].

Polyvinyl alcohol is a biodegradable, water-soluble synthetic polymer that presents good biocompatible properties and excellent film forming capacity. Owing characteristics such as easy preparation, chemical resistance, and mechanical properties, the polyvinyl alcohol has been used in many biomaterial applications [9,11].

Considering the good biological properties of chitosan and polyvinyl alcohol, a combination of these polymers may have beneficial effects on the biological characteristics of the blended films [14]. Furthermore, the chemical cross-linking in the blend formed between PVA/Chitosan may improve mechanical strength, thermal stability, keeping the intrinsic properties of transparency and swelling ability [15]. Thus, the purpose of this work was to immobilize lipase in PVA-chitosan films for reactor coating, and test this new material as a slow enzyme delivery system for batching reactions.

2. Methodology

2.1. The preparation of PVA/Chitosan and PVA/Chitosan/Lipase film

In order to select the most appropriate combination of lipase and mannitol in the film composition a central composite rotatable design (CCRD) was performed. The ultimate goal was to investigate the effect of lipase and mannitol concentration on the tensile strength and % elongation. For the experimental design, the two variables were confined in two levels: 0.05% (low level) to 0.2% (high level) for mannitol and 25 μL

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(low level) to 175 μL (high level) for lipase. A central point (0.1%; 100 μL) with two replicates was also included for statistical evaluation (at the 95% confidence level), resulting in ten experiments performed for the CCRD (Table 1). The PVA/Chitosan film was prepared by casting on the glass molds a solution containing a mixture of 3 mL of 5% (w/v) acidic PVA solution (pH 2.0), 1 mL of 2% (w/v) chitosan solutions in 2% (v/v) acetic acid, 0.05–0.2% (w/v) mannitol as plasticizer and 0.125% (v/v) glutaraldehyde as cross-linker, followed by water evaporation at room temperature (25 °C) for 24 h (Fig. 1). This resulted in a 78.5 cm^2 film. The PVA/Chitosan/Lipase film was produced as described except that 25–175 μL of Lipolase® (2.3–16 U) was added before glutaraldehyde addition.

The dry films were stored in plastic bags before all subsequent characterization procedures. A template was used to cut 1 cm^2 testing strips from the films.

2.1.1. Film characterization

2.1.1.1. Thickness

The film thickness was determined using a manual micrometer (Mitutoyo, São Paulo, Brazil). The final thickness was determined by twenty random determinations in all film areas.

2.1.1.2. Water solubility

The water solubility of the PVA/Chitosan film was determined according to Gontard et al. [16]. The dry matter was determined by heating the sample at 105 °C for 24 h. 500 mg of dried film was immersed in 50 mL of distilled water and incubated at 20 °C under agitation for 24 h. After that, the solution was filtered and the material retained in the filter was dried at 105 °C for 24 h. The film solubility was expressed as percentage of soluble mass in relation to the total mass according to the following equation:

$$\text{Solubility}(\%) = ((\text{Wi}–\text{Wf})/\text{Wi}) \times 100$$

Where: *Wi* is the initial weight of the sample (mg) and *Wf* is the weight of dried material retained in the filter (mg)

2.1.1.3. Mechanical properties

Mechanical properties were determined in a Texture Analyzer TA.TX2 (Stable Micro Systems, Surrey, UK), with a 50 N load cell equipped with tensile grips (A/TG model). Samples of the film were cut into strips of 20 mm wide and 40 mm long, according to the ASTM D-638M-93 standard [17]. The grip separation was set at 25 mm, with a cross-head speed of 500 mm min^{-1}. Tensile strength (TS), percentage of elongation (%E) at break, and elastic modulus (EM) were evaluated. Each sample used was previously inspected and those containing any defect such as air bubbles, holes, and tears or showing average thickness variation superior to 5% were rejected.

Table 1
Experimental design and results according to the CCRD[2].

Run	Variable level		Responses	
	Mannitol (%) X_1	Lipase (μL) X_2	Tensile strength (MPa)	Elongation (%)
1	(−) 0.05	(−) 25	25.39	187.68
2	(−) 0.05	(+) 175	48.59	236.66
3	(+) 0.20	(−) 25	49.4	224.48
4	(+) 0.20	(+) 175	47.52	158.17
5	(−) 0.05	(0) 100	72.60	209.62
6	(+) 0.20	(0) 100	71.28	141.88
7	(0) 0.10	(−) 25	59.07	164.35
8	(0) 0.10	(+) 175	55.17	198.14
9 (C)	(0) 0.10	(0) 100	82.39	149.52
10 (C)	(0) 0.10	(0) 100	83.86	149.84

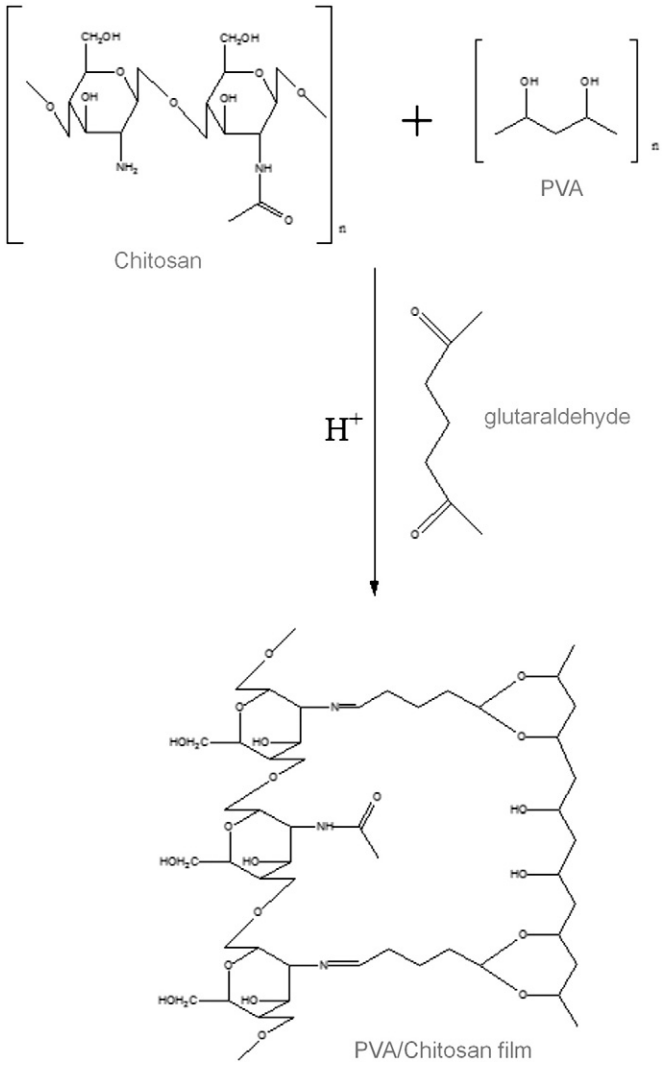

Fig. 1. Scheme representing the mechanism of PVA/Chitosan reaction.

2.2. Determination of hydrolytic activity

The hydrolytic activity of PVA/Chitosan/Lipase film (cm^2) was assayed using p-nitrophenyl palmitate (PNP-palmitate) or olive oil as substrate. The PNP-palmitate hydrolysis was carried out according to the methodology described by Winkler and Stuckmann [18]. The substrate was prepared as an emulsion by mixing 1 mL of PNP-palmitate solution (0.15% w/v in isopropyl alcohol) with 9 mL of arabic gum solution (0.1% w/v in 50 mmol L^{-1} Tris buffer pH 8.0). One milliliter of this emulsion was added to the film (1 cm^2) and the system was incubated for 5 min at room temperature (25 °C). The p-nitrophenol was used to construct a standard curve and one unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of p-nitrophenol per minute. The course of pNP palmitate hydrolysis reaction for free lipase and PVA/Chitosan/Lipase was evaluated.

The hydrolysis of olive oil was carried out according to Soares et al. [19]. Briefly, the substrate was prepared by mixing 2.5 mL of olive oil with 2.5 mL of arabic gum solution (7% w/v in 50 mmol L^{-1} Tris buffer pH 8.0). Five milliliters of this substrate emulsion was added to the reactor covered with PVA/Chitosan/lipase film and the reaction proceeded for 30 min under string. The released fatty acid was titrated with 20 mM potassium hydroxide solution. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free

fatty acid/min under the assay conditions, calculated according to the following equation:

$$EU = \frac{(V_1 - V_2)\cdot M \cdot 106}{t \cdot v}.$$

Where, V_1 represents the volume (mL) of KOH used in the titration of olive oil after hydrolyze; V_2 represents the volume (mL) of KOH used in the titration of olive oil before hydrolyze; M is the molar concentration (mol L^{-1}) of KOH; t is the reaction time (min) and v is the volume (mL) of enzyme used for immobilization.

2.3. Operational stability and reusability

The reuse test was conducted by coating the interior of a glass reactor (12.56 cm^2) with a layer of 70 μm thickness of PVA/Chitosan/Lipase film. The coated reactor was used for PNP-palmitate hydrolysis in the experimental conditions described above. After each 30 min reaction, the reactor was washed twice with 50 mmol L^{-1} Tris buffer pH 8.0 and then reused. After 10 reaction/wash cycles, the reactor was stored at 4 °C overnight.

2.4. Statistical analysis

The results were expressed as average ± standard deviation (X ± SD). The variance analysis and Tukey's test were used to define differences in mean values of the data from 3 to 6 replicates. Results from CCRD were analyzed using the software Statistica 6.0 (Statsoft, Inc., Tulsa, USA, 1997). The adjustment of the experimental data for the independent variables was represented by the second-order polynomial equation:

$$y = \beta_0 + \sum_j \beta_j x_j + \sum_{i<j} \beta_{ij} x_i x_j + \sum_j \beta_{jj} x_j^2 + e$$

where y is the dependent variable to be modeled; β_0, β_j, β_{ij} and β_{jj} are regression coefficients; x_i and x_j are independent variables and e is the error. The model was simplified by dropping terms that were not statistically significant ($p > 0.05$) by ANOVA.

3. Results and discussion

3.1. Film production and characterization

The experimental design and results obtained from the experiments are shown in Table 1. The data from tensile strength (TS) were converted into a second-order polynomial equation with two independent variables. Consequently, the polynomial model describing the correlation between the response and the variables was presented as follows:

$$TS(MPa) = -27.70 + 852.53X_1 - 2843.49X_1^2 + 1.14X_2 - 0.004X_2^2 \quad (1)$$

where X_1 and X_2 denoted mannitol and lipase content, respectively. High proportion of variability ($R^2 = 0.94$) in the response model can be explained successfully by the experimental model.

Eq. (1) was used and three dimensional plots were drawn. Fig. 2A shows a well-defined region of optimum values for the tensile strength. The response surface showed a maximum region corresponding to 0.1% of mannitol and 100 μL of lipase with TS of 83.12 MPa.

Regarding to elongation, the regression analysis showed an adequate fit ($R^2 = 0.91$) of experimental values to the second-order polynomial model as a function of significant factors. The mathematical model is represented in the following equation:

$$Elongation(\%) = 246.95 - 1156.90X_1 - 5792.89X_1^2 - 0.35X_2 + 0.005X_2^2 - 5.34X_1X_2 \quad (2)$$

where X_1 and X_2 denoted mannitol and lipase content, respectively. The fitness of the model was expressed by the R^2 value, which indicates that 91% of the variability in the response can be explained by the model. This suggested that the model accurately represented the data in the experimental region.

Fig. 2B shows the response surface obtained from Eq. (2). As can be seen, an opposite behavior to that shown in Fig. 2A was obtained. These results allowed defining the best conditions to produce PVA/Chitosan/Lipase film as those used in the central point. Therefore, the properties of PVA/Chitosan/Lipase film containing 0.1% of mannitol and 100 μL of lipase were compared to a film with the same compounds, except lipase. Comparing the mechanical properties of PVA/Chitosan and PVA/Chitosan/Lipase films, it was observed that the film containing immobilized lipase presented higher values in all evaluated parameters.

The tensile strength (TS) is defined as the capacity of resistance to rupture as presented by the material when submitted to pressure force [20]. The TS observed in the PVA/Chitosan/Lipase was 69.4% higher than that in the PVA/Chitosan film (48.63 MPa). Moreover, the inclusion of lipase resulted in a film with increased malleability, evidenced by the higher elongation percentage of PVA/Chitosan/Lipase film (149.91%) compared to the PVA/Chitosan film (98.34%). The improvement in TS and %E probably occurred as a consequence of a more cohesive matrix formation. TS of PVA/Chitosan films is higher

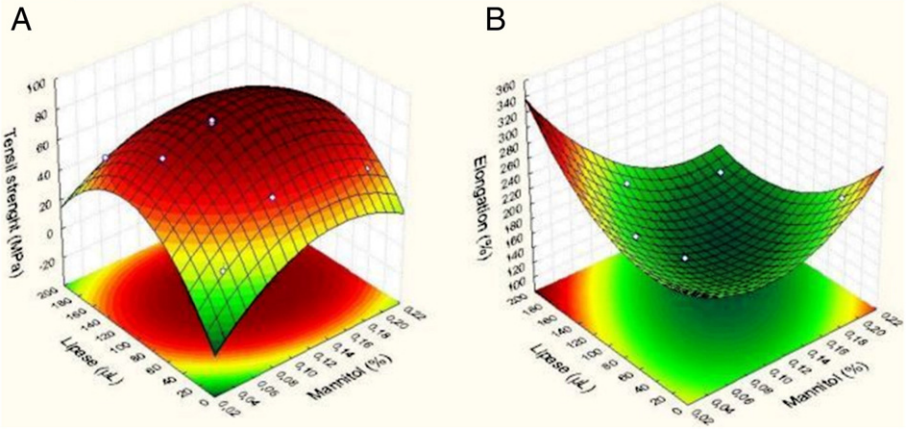

Fig. 2. Response surfaces of the effect of mannitol and lipase content in the (A) tensile strength and (B) elongation of the PVA/Chitosan/Lipase films.

than that reported for commercial plastics such as PVA [14], polypropylenes [21] and polyethylene [22].

Lipases are known to have a hydrophobic spot at the protein surface that interacts with oil, exposing the lid that protects the active site. This hydrophobic spot may interact with hydrophobic structures of chitosan by one side, while hydrophilic portion of lipase interacts with PVA by the other side. This amphipathic interaction established by lipase is probably responsible for enhancing the interaction between chitosan and PVA polymeric chains distant from each other, reinforcing the tridimensional structure of the film. The consequence was an increase in the tensile strength and higher elongation capacity, which are very important characteristics in the manufacture of films for bioreactor coating.

3.2. Appearance of the films

The produced film presented no fractures or breaks, high flexibility and easy handling. The coloration was uniform, presenting satisfactory transparency and brightness (Fig. 3A). However, similar to other films containing glutaraldehyde, the PVA/Chitosan film was yellow. This phenomenon was observed previously by Yu et al. [15], and it can be due to the reaction between aldehyde groups found in the glutaraldehyde molecule with the amino group on chitosan, forming the chromophore group –C═N–. Yu et al. [15] also explain that the absorption band of –C═N– is in the extreme ultraviolet. However, in glucosamine containing groups –OH and/or –OR, the –C═N– acts as an auxochrome. In this case, the absorption band of –C═N– shifting to the range of long wave, and chromogenic in the range of visible light. This phenomenon is responsible by darker color of PVA/Chitosan film in comparison with films without glutaraldehyde as cross-linker.

3.3. Water solubility

The PVA/Chitosan/Lipase films presented thickness values between 70.4 and 79 μm. These films presented 72.6% water solubility, value higher than presented by xylan/wheat gluten films [23]; soy protein isolate films [24]; and oxidized cassava starch biodegradable films [25]. The water solubility is related to the content of free hydroxyl groups in the polymeric matrix, which allows the establishment of hydrogen interactions between film and water [26]. The high solubility is a desirable characteristic for biodegradable films, once the increase in the solubility leads to an increased biodegradability [27].

3.4. Scanning electron microscopy

Scanning electron micrographs (SEMs) of PVA/Chitosan and PVA/Chitosan/Lipase film are shown in Fig. 3. The SEM showed the formation of continuous films. The surface of films presented good structural integrity, without any pores or cracks. Chitosan microdomains are homogeneously dispersed within PVA matrix in the film, with excellent interfacial adhesion between the two components. The entrapment of lipase into PVA/Chitosan resulted in a film with very similar characteristics (Fig. 3C), confirming that the lipase did not interfere in the interfacial adhesion or structural integrity.

3.5. Lipase activity in PVA/Chitosan films

The entrapment of lipase in PVA/Chitosan was very efficient. The activity observed for PVA/Chitosan/Lipase film was of 0.107 U cm^{-2}. This means that 92% of lipase offered for immobilization was actively retained. In comparison with literature, this immobilization was very efficient, superior to 29% lipase immobilization by absorption in PVA/Chitosan membrane reactor [28], 81% entrapment into Carboxymethylcellulose/PVA [29] or covalent linking procedures, such as 37% immobilization in styrene-divinylbenzene (STY-DVB) copolymers [30], and 51% immobilization in chitosan beads [31].

Furthermore, the high water solubility of PVA/Chitosan/Lipase film makes the film surface renewable, continuously exposing new layers of film containing active lipase. This characteristic will be very useful for continuous use in reactors.

The course of pNP palmitate hydrolysis reaction for free or PVA/Chitosan/Lipase was evaluated (Fig. 4). Increasing amount of pNP is produced until substrate is consumed. In spite of the very close reaction profile, free lipase reaches maximum product formation after 90 min reaction while PVA/Chitosan/Lipase reaction is slower. This effect on the reaction velocity is commonly observed in systems containing immobilized enzymes [32,33] and may be a consequence of the highly hydrophilic nature of PVA/Chitosan support exerting a partition effect on the reaction microenvironment. However, both free and immobilized lipases produced the same maximum pNP amount at the end of reaction.

3.6. Reusability

The reusability of immobilized catalyst is an important parameter, which can determine the economic viability of any biosynthetic process [2,29]. The PVA/Chitosan/Lipase was tested repeatedly for hydrolysis of PNP-palmitate. The reusability was examined because of its importance in batch reaction and in continuous use reactor for bioconversion processes. To check this parameter, a reactor with 12.56 cm^2 of superficial area was coated with PVA/Chitosan/Lipase film. In this reactor, 30 reaction cycles of PNP-palmitate hydrolysis were performed. The results are shown in Fig. 5.

The high operational stability of PVA/Chitosan/Lipase film was another characteristic that indicates large-scale application for the

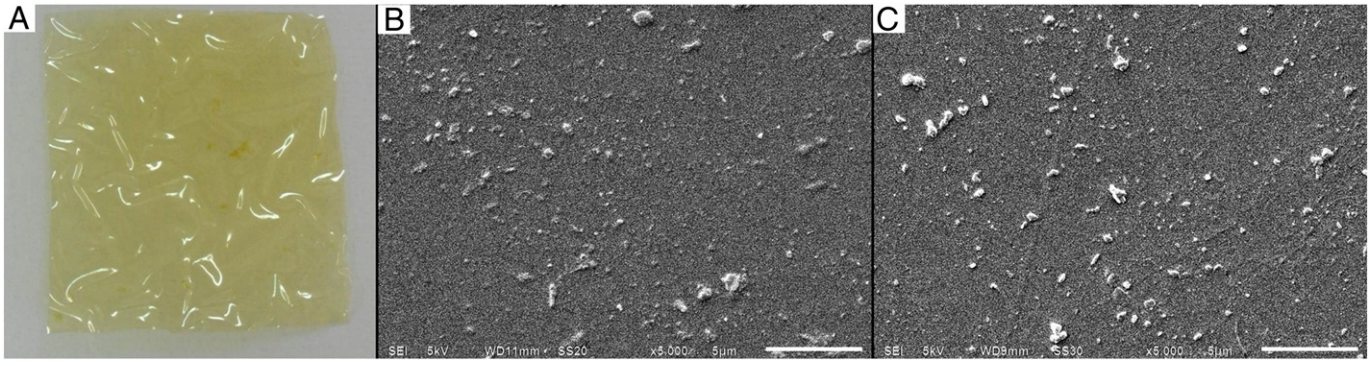

Fig. 3. Photographs and SEM micrographs of films. (A) PVA/Chitosan/lipase film; (B) SEM PVA/Chitosan film; (C) SEM PVA/Chitosan/Lipase film.

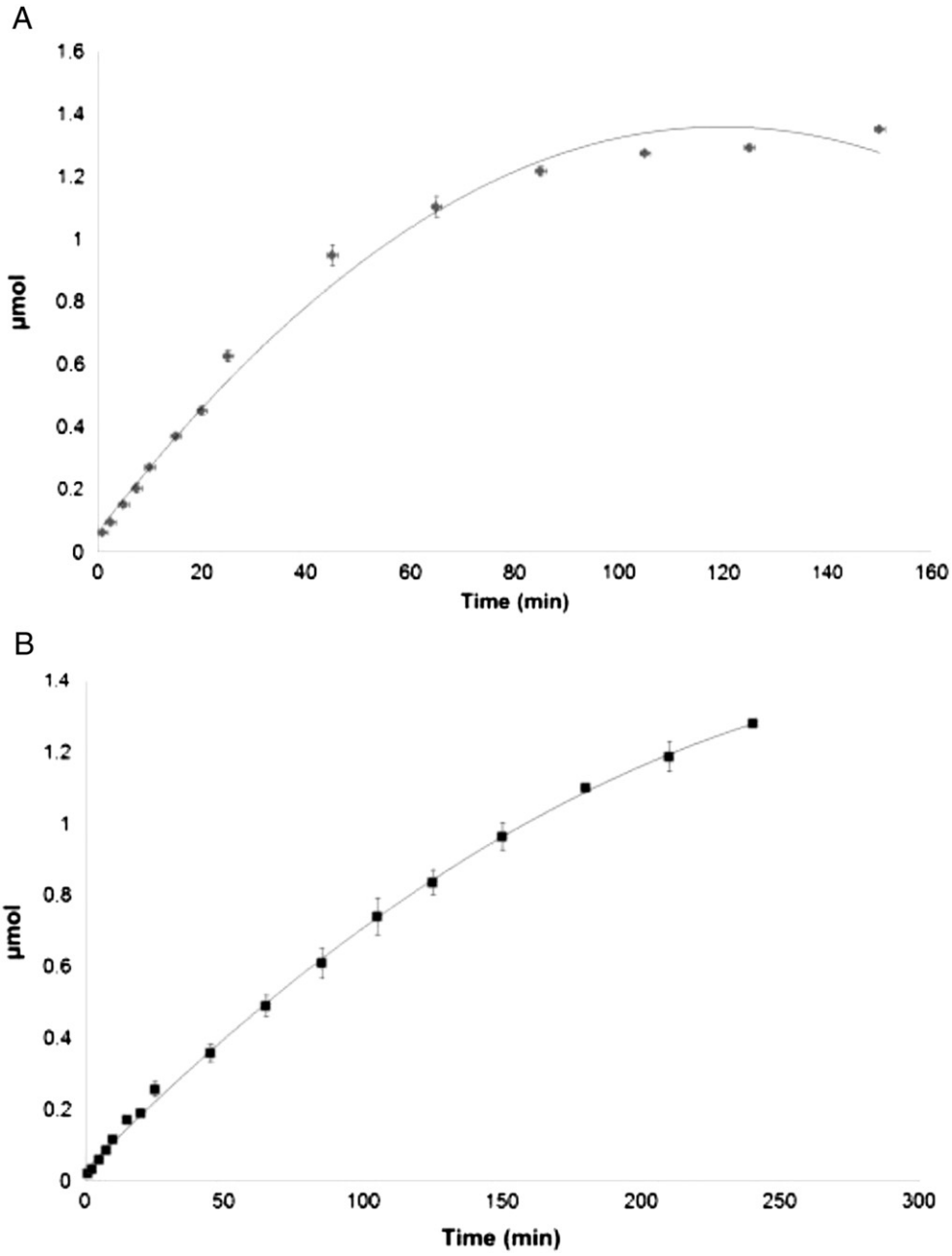

Fig. 4. Kinetics of pNP production for the free lipase and PVA/Chitosan/Lipase film. Activity determined as content of pNP production (µmol).

PVA/Chitosan/lipase film. According to Fig. 5, the immobilized enzyme displays a good reusability, maintaining 62% of the initial hydrolytic activity at 25th cycle. The maintenance of the enzyme activity for 25 cycles can be related to the continuous exposure of film layers containing active lipase, initially found in deeper layers. Such reusability is advantageous for the continuous use of this enzyme in industrial applications. After the 25th cycle, the abrupt loss of activity observed in Fig. 5 was due to the PVA/Chitosan/Lipase film degradation and release from the reactor wall. In this sense, increasing the reactor coating can be a strategy to increase reactor lifetime and reaction cycles. The possibility to store the reactor covered with PVA/Chitosan/Lipase, without loss of enzyme activity, is also a very interesting finding of this work.

The use of PVA/Chitosan/Lipase reactor for olive oil hydrolysis was tested and the results showed that the immobilized lipase was able to hydrolyze this substrate producing considerable amounts of fatty acid. Each cycle of hydrolysis produced about 3.2 µmol $(\text{min}\cdot\text{cm}^2)^{-1}$ of fatty acid. The absence of steric hindrance and the preserved capacity of hydrolysis of its natural substrate were another important results of this work, indicating that this material can be used in several different applications.

Amorim et al. [34] immobilized lipase in chitosan film and reported a residual activity of 42% after 4 cycles. On other hand, Ye et al. [35], using chitosan-tethered poly(acrylonitrile-co-maleic acid) membrane and dual-layer biomimetic membrane obtained, respectively, 62% and 53% of active enzyme after 10 cycles. Studying chitosan-modified nanofiber membrane, Ye et al. [35] found 55% residual activity after 10 cycles, while Romdhane et al. [1] obtained retention of 80% enzyme activity after 10 cycles of reusability of chitosan beads. Compared to these results, the PVA/Chitosan/Lipase film was more effective, retaining higher enzyme activity (62%) after 25 cycles. The adherence of PVA/Chitosan/Lipase to glass, metal and paper, and the possibility of increasing the layer thickness make it a promising material for industrial applications.

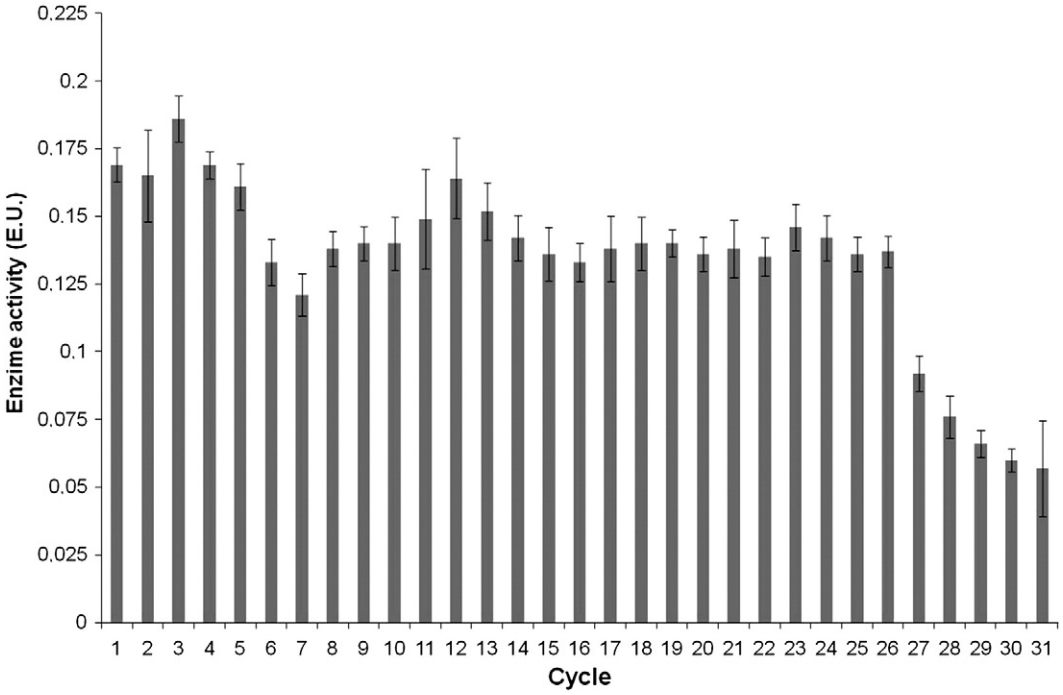

Fig. 5. Reusability of the immobilized lipase.

4. Conclusions

In this work a PVA/Chitosan/Lipase film was produced blending PVA/Chitosan and entrapping the lipase in the polymeric matrix. The conditions employed to produce this bioactive material resulted in an entrapped enzyme that is highly active. The high stability of the PVA/Chitosan/Lipase film was demonstrated by repeated use in a bath reactor. The chemical characterization of PVA/Chitosan/Lipase film indicates that this material has a good stability in organic environment. This is a desirable characteristic for esterification and trans-esterification reactions. The mechanical properties observed in the PVA/Chitosan/Lipase film make this material very robust. In addition to mechanical properties, the high transparency of this biomaterial and high water solubility enable it to several applications, in particular as bioactive biodegradable packaging component.

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