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Pseudomonas fluorescens lipase immobilization on polysiloxane–polyvinyl alcohol composite chemically modified with epichlorohydrin

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Abstract

The technique based on sol–gel approach was used to generate silica matrices derivatives by hydrolysis of silane compounds. The present work evaluates a hybrid matrix obtained with tetraethoxysilane (TEOS) and polyvinyl alcohol (PVA) on the immobilization yield of lipase from *Pseudomonas fluorescens*. The resulting polysiloxane–polyvinyl alcohol (POS–PVA) matrix combines the property of PVA as a suitable polymer to retain proteins with an excellent optical, thermal and chemical stability of the host silicon oxide matrix. Aiming to render adequate functional groups to the covalent binding with the enzyme the POS–PVA matrix was chemically modified using epichlorohydrin. The results were compared with immobilized derivative on POS–PVA activated with glutaraldehyde. Immobilization yield based on the recovered lipase activity depended on the activating agent and the highest efficiency (32%) was attained when lipase was immobilized on POS–PVA activated with epichlorohydrin, which, probably, provided more linkage points for the covalent bind of the enzyme on the support. This was confirmed by determining the morphological properties using different techniques as X-ray diffraction and scanning electron microscopy (SEM). Comparative studies were carried out to attain optimal activities for free lipase and immobilized systems. For this purpose, a central composite experimental design with different combinations of pH and temperature was performed. Enzymatic hydrolysis with the immobilized enzyme in the framework of the Michaelis–Menten mechanism was also reported. Under optimum conditions, the immobilized derivative on POS–PVA activated with epichlorohydrin showed to have more affinity for the substrate in the hydrolysis of olive oil, with a Michaelis–Menten constant value (K_m) of 293 mM, compared to the value of 401 mM obtained for the immobilized lipase on support activated with glutaraldehyde. Data generated by DSC showed that both immobilized derivatives have similar thermal stabilities.

Keywords: Lipase; Polysiloxane–polyvinyl alcohol; Enzyme immobilization; Glutaraldehyde; Epichlorohydrin

1. Introduction

The use of lipases in organic media is of major commercial importance, and this interest is expected to grow over the coming years as wider range of lipase catalysts become available [1]. For example, a range of fatty acid esters has been synthesized on a commercial scale using lipases in essentially nonaqueous media [2,3]. To further expand their synthetic utility, efficient methods for immobilizing lipases are needed since immobilization allows enzyme reuse and thus reduces overall process costs [4]. Several methods have been reported, such as adsorption on solid supports [5,6], covalent binding [7,8] and entrapment within a polymer matrix or hydrophobic sol–gel material [9,10]. The latter method can be applied to a variety of lipases, yielding immobilized systems with 80-fold esterification activity compared with the free enzyme [11].

The sol–gel process involves the transition of a system from a liquid "sol" (mostly colloidal) into a solid "gel" phase. By applying this methodology, it is possible to fabricate ceramic or glass materials in a wide variety of forms: ultra-fine or spherical shaped powders, thin film coatings, ceramic fibers, micro porous inorganic membranes, monolithic ceramics and glasses, or extremely porous aerogel materials [12].

The sol–gel technique is also an excellent method to prepare hybrid material. The low temperature synthesis enables organic or inorganic species to be incorporated into rigid silicon oxide matrices without degradation. The resulting composite combines the chemical and physical properties of the guest with the excellent optic, thermal, and chemical stability of the host silicon oxide matrices [13]. Recently, we reported a procedure

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for covalent immobilization of several lipase sources on particles of a polysiloxane–polyvinyl alcohol composite prepared by the sol–gel technique [7,8,14]. This simple but effective procedure gave an immobilized lipase preparation with greatly improved activity and stability. The procedure used tretaethoxysilane and polyvinyl alcohol for the matrix formation followed by activation with glutaraldeyde to render compatible surface for covalent immobilization.

However, the support surface activation with glutaraldeyde often leads to a loss of native activity. Besides, large mass fraction, roughly 90% of the solid support used as carrier is free of biocatalytic activity. This leads to lower spacer–time yields and lower productivity [15]. Therefore, the evaluation of other activating agents of the support could be useful to enhance the catalytic activities of the immobilized derivatives. Among the used activating agents, epichlorohydrin seems to overcome such drawbacks, while producing immobilized derivatives with high activity and stability [16,17].

Thus, this work assesses the effect of using epichlorohydrin as functional activating agent for the support (POS–PVA) upon the *Pseudomonas fluorescens* lipase immobilization. This enzyme was chosen due to its potential previously identified to synthesize important products, such as biodiesel [18]. The properties of the support and immobilized derivatives were evaluated by X-ray diffraction, chemical composition (FTIR) and scanning electron microscopy (SEM). The influence of the temperature and pH on the activity of the biocatalysts was determined using surface response methodology. Under the established conditions, the kinetic behavior of the systems was also determined. The results were compared with immobilized derivative on POS–PVA activated with glutaraldehyde.

2. Experimental

2.1. Materials

P. fluorescens lipase (Lipase AK) was from Amano Enzyme Inc. (Nagoya, Japan). Tetraethoxysilane (TEOS) was acquired from Aldrich Chemical Co. (Milwaukee, WI, USA). Glutaraldehyde (25%, w/v), epichlorohydrin, hydrochloric acid (minimum 36%), polyvinyl alcohol (PVA, MW 72,000) and polyethylene glycol (PEG, MW 1500) were supplied by Reagen (Rio de Janeiro, RJ, Brazil). Commercial olive oil (low acidity) was purchased in a local market. All other chemicals were of analytical grade.

2.2. Support synthesis and activation

A polysiloxane–polyvinyl alcohol (POS–PVA) hybrid composite was prepared by the hydrolysis and polycondensation of tetraethoxysilane according to the methodology previously established by Bruno et al. [7] as briefly described. The reagents TEOS (5 mL), ethanol (5 mL), and PVA solution 2% (w/v) (6 mL) were carefully mixed and stirred for 5 min at 60 °C, followed by addition of two or three drops of concentrated HCl, in order to catalyze the reaction. After an incubation period of 40 min, the material was transferred to micro wells of tissue culture plates (disc shape) and kept at 25 °C until complete gel solidification (formation of an interpenetrated network of POS–PVA). Then, the spheres were ground in a ball mill and classified to attain particles with 0.308 mm of diameter (−40/+60 MESH Tyler standard sieves). Activation of POS–PVA particles was carried out with either epichlorohydrin or glutaraldehyde at 2.5% (w/v) pH 7.0 for 1 h at room temperature, followed by exhaustive washings with distilled water.

2.3. Lipase immobilization onto POS–PVA particles

Activated POS–PVA particles were soaked into hexane under stirring (100 rpm) for 1 h at 25 °C. Then, excess hexane was removed and powder lipase preparation was added at a ratio of 1:5 g of enzyme per gram of support. PEG-1500 was added together with the enzyme at a fixed amount (5 mg/g of support). Lipase-support system was maintained in contact for 16 h at 4 °C under static conditions. The immobilized lipase derivatives were filtered (nylon membrane 62HD from Scheiz Seidengazefabrik AG, Thal Schweiz, Switzerland) and thoroughly rinsed with hexane. Hydrolytic activities of free and immobilized lipase derivatives were assayed by the olive oil emulsion method according to the modification proposed by Soares et al. [19]. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of free fatty acid per minute under the assay conditions (35 °C and pH 7.5). The results were expressed in activity units per milligram of solid (free enzyme preparation or immobilized derivative). The immobilization efficiency (η%) was calculated according to Eq. (1):

$$\eta(\%) = \frac{\mathrm{Us}}{\mathrm{Uo}} \times 100 \quad (1)$$

where Us is the total enzyme activity recovered in the support and Uo is the enzyme units offered for immobilization.

2.4. Determination of optimum enzymatic activities and kinetic parameters

Optimum enzymatic activities (free and immobilized lipases) were investigated by running enzymatic analyses according to an experimental design with five different temperatures (35, 40, 53, 65 and 70 °C) and five pH values (6.1, 6.5, 7.5, 8.5 and 8.9). The activities were assayed by the hydrolysis of olive oil emulsion at a fixed proportion oil/water 1:1 [19]. The pH values were achieved using appropriate phosphate buffer solutions. The influence of substrate concentration on hydrolytic activities was also analyzed in the olive oil hydrolysis assay varying the proportion of oil in the emulsion from 10 to 50%. Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max}) were calculated by the computational program Enzfitter version 1.05 published by Elsevier-Biosoft, 1987.

2.5. Statistical design analysis

Results were analyzed using Statistica version 5 (StatSoft Inc., USA) and Design-Expert 6.0 (Stat-Ease Corporation, USA) softwares. The statistical significance of the regression coeffi-

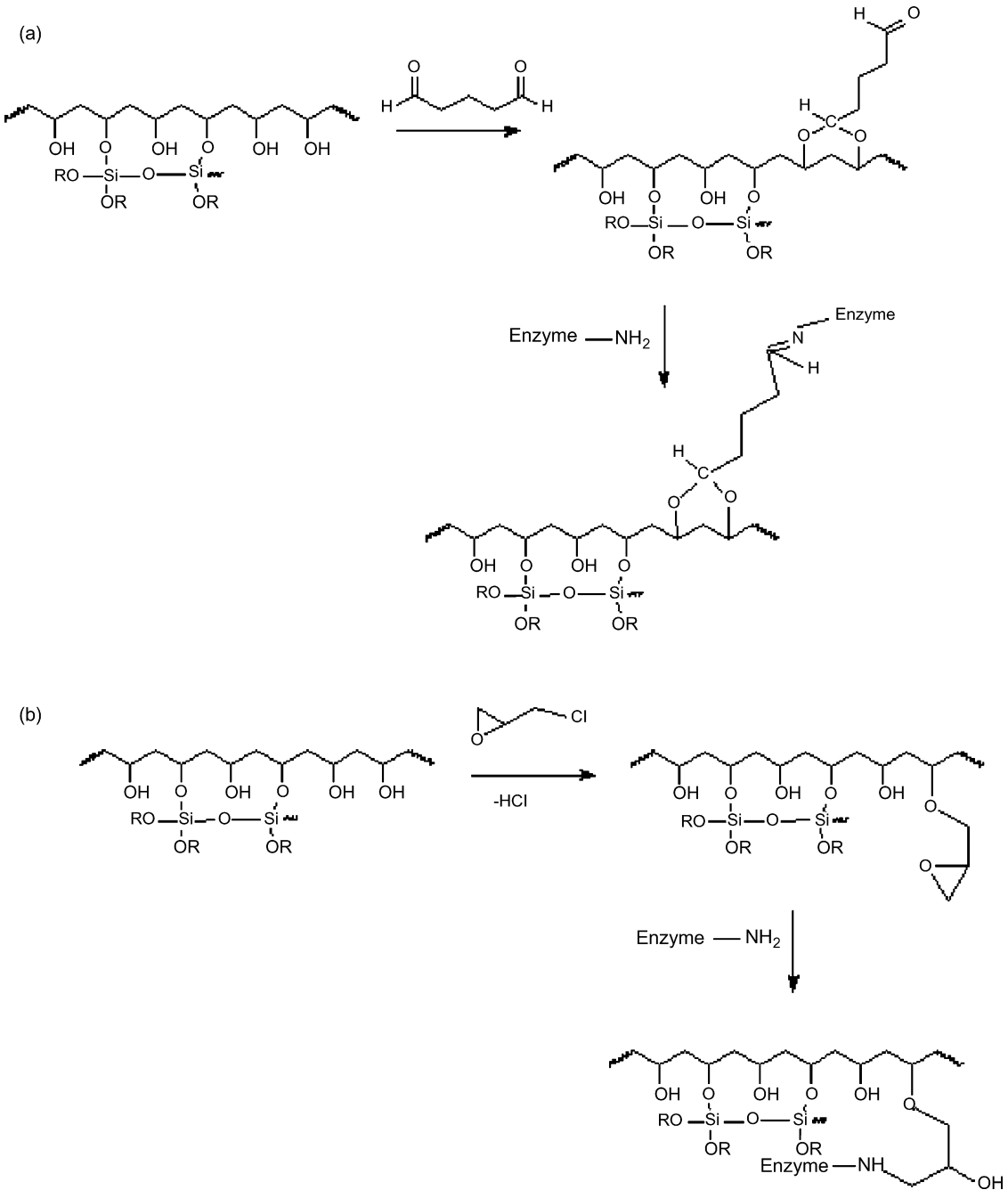

Fig. 1. Mechanism of POS–PVA activation and enzyme immobilization using glutaraldehyde (a) or epichlorohydrin (b) as activating agents.

cients was determined by Student's test, the second order model equation was determined by Fischer's test and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2. Optima variable values were obtained by the analysis of the response curve levels graphics and by the numerical optimization of "Design expert" program, based on the criteria of desirability [20].

2.6. Fourier transform infrared spectroscopy (FTIR)

Samples were submitted to FTIR analysis using the equipment Spectrum One, PerkinElmer (Waltham, USA). Spectra were obtained in the 400–4000 cm^{-1} wave number range.

2.7. X-ray diffraction

X-ray diffraction patterns were collected on a XRD 6000 X-ray difractometer Shimadzu (Shimadzu from Brazil, São Paulo, SP, Brazil). The identification of the support morphological structure was observed by X-ray diffraction; using CuK radiation source, with 2θ angle varying from 10 to 70 °C.

2.8. Scanning electron microscopy (SEM)

Conformational changes, such as surface cavities in the support, set by the activating agents insertion were observed by

scanning electron microscopy. Micrographs of support surfaces were obtained by using a LEO1450VP scanning electron microscope (Schott Zeiss from Brazil, São Paulo, SP, Brazil).

2.9. Differential scanning calorimetry (DSC)

DSC curves were obtained in a Thermal Analyzer SDT 2960-Q10, TA Instruments (New Castle, Delaware, USA). Samples were weighed out into aluminum "crucible" pans (~5–10 mg); the heat flux was 10 °C/min in the range from −10 to 450 °C and an empty aluminum pan served as reference.

3. Results and discussion

3.1. Immobilization efficiency

The support obtained by sol–gel technique was activated with either glutaraldehyde or epichlorohydrin and used to immobilize *Pseudomonas fluorescence* lipase (AK). Between the tested activating gents, the epichlorohydrin showed the best performance, producing immobilized lipase samples with average activity of 740 U/mg, which corresponded to a yield of 32%. The use of glutaraldehyde as activating agent decreased the hydrolytic activity by about 50% (360 U/mg) indicating that different chemical modifications were produced by each activating agent. Concerning the activation with glutaraldehyde, the most probably reaction occurred between the aldehyde extremity of this compound and hydroxyl groups of the PVA via acetal mechanism (Fig. 1a) as proposed by Araujo et al. [21]. In this case, two hydroxyl groups from the PVA are needed to react with an aldehyde extremity of the glutaraldehyde that will be inserted into the support surface, and the remaining free extremity to bind with a $-NH_2$ group of the enzyme.

On the other hand, when epichlorohydrin is used, epoxy groups are formed in the support surface, as showed in Fig. 1b according to the mechanism proposed by Porath and Fornestedt [22]. Thus, by using this activating agent, only one hydroxyl group is needed to the reaction, compared to the two groups required by glutaraldehyde. As a consequence, it is possible that a higher number of active sites for enzyme binding are available in the POS–PVA activated with epichlorohydrin. This fact, associated to the known higher reactivity of epoxy groups, can explain the higher immobilization yield achieved when this activating agent was used.

This hypothesis was further confirmed by analyzing the infrared (IR) spectra as displayed in Fig. 2a and b. For the non-activated support POS–PVA (Fig. 2a), the spectrum showed fewer bands, as the 1110–1000 cm^{-1} peak, corresponding to Si–O–R asymmetric stretching. Other characteristic bands were observed at 3200–3600 cm^{-1} (hydroxyl groups), 950 cm^{-1} (Si–O–Si axial deformation), 810 cm^{-1} (Si–O–Si axial deformation) and 600 cm^{-1} (Si–O–Si angular deformation) [23]. After chemical modifications, the peak correspondent to the hydroxyl group decreased and such reduction was more intense for epichlorohydrin than glutaraldehyde.

Free enzyme showed a typical spectrum of proteins (Fig. 2b); with bands associated to their characteristic amide group

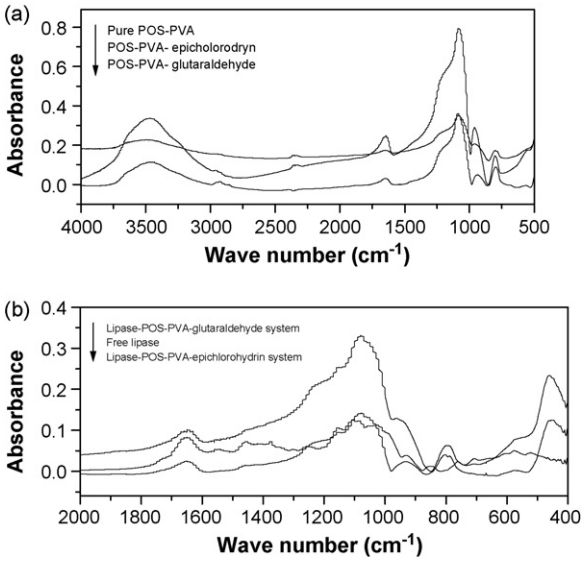

Fig. 2. Infrared spectra for the POS–PVA before and after the activation (a) and for the free and immobilized lipase derivatives (b).

(CONH). In this case, in the range of 1600–1700 cm^{-1}, there is a amide I band due to the double bond CO stretching, the CN stretching and NH bending [23]. For the immobilized derivatives (Fig. 2b), no additional bands in the spectrum were observed, indicating that the covalent bond between the enzyme and support is of same nature of typical protein or showed overlap bands in relation to the support POS–PVA. However, there are some indications on Fig. 2b regarding to the enzyme incorporation into the matrix. By comparing the spectra for the activated supports with the immobilized derivatives, it is possible to correlate to enlargement of peaks at 900–1100 cm^{-1} and 1400–1500 cm^{-1} to bands in the spectrum for the free enzyme.

3.2. Morphological characterization of support and immobilized derivatives

Diffraction of the X-ray information data on the support structure (non-activated and activated) and free lipase and immobilized derivatives as shown in Fig. 3a–f. The diffractogram obtained from the pure support (Fig. 3a), shows peaks of low intensity and expressive width, indicating a predominantly amorphous structure of the hybrid composition. The activation treatments resulted on support structure modifications as showed by the diffractograms (Fig. 3b and c) which clear indicated insertion of crystalline regions into the POS–PVA and can be associated with the presence of the activating agents. Those modifications displayed different patterns for each activating agent, with narrow and intense peaks corresponding to different angles. Intense and narrow peaks evidenced in Fig. 3d for the high crystalline structure of the free lipase were also identified in the immobilized derivatives (Fig. 3e and f) showing higher intensity when epichlorohydrin was used as activating agent (Fig. 3f).

Scanning electron microscopy allowed the verification of morphological differences between the support in the non-

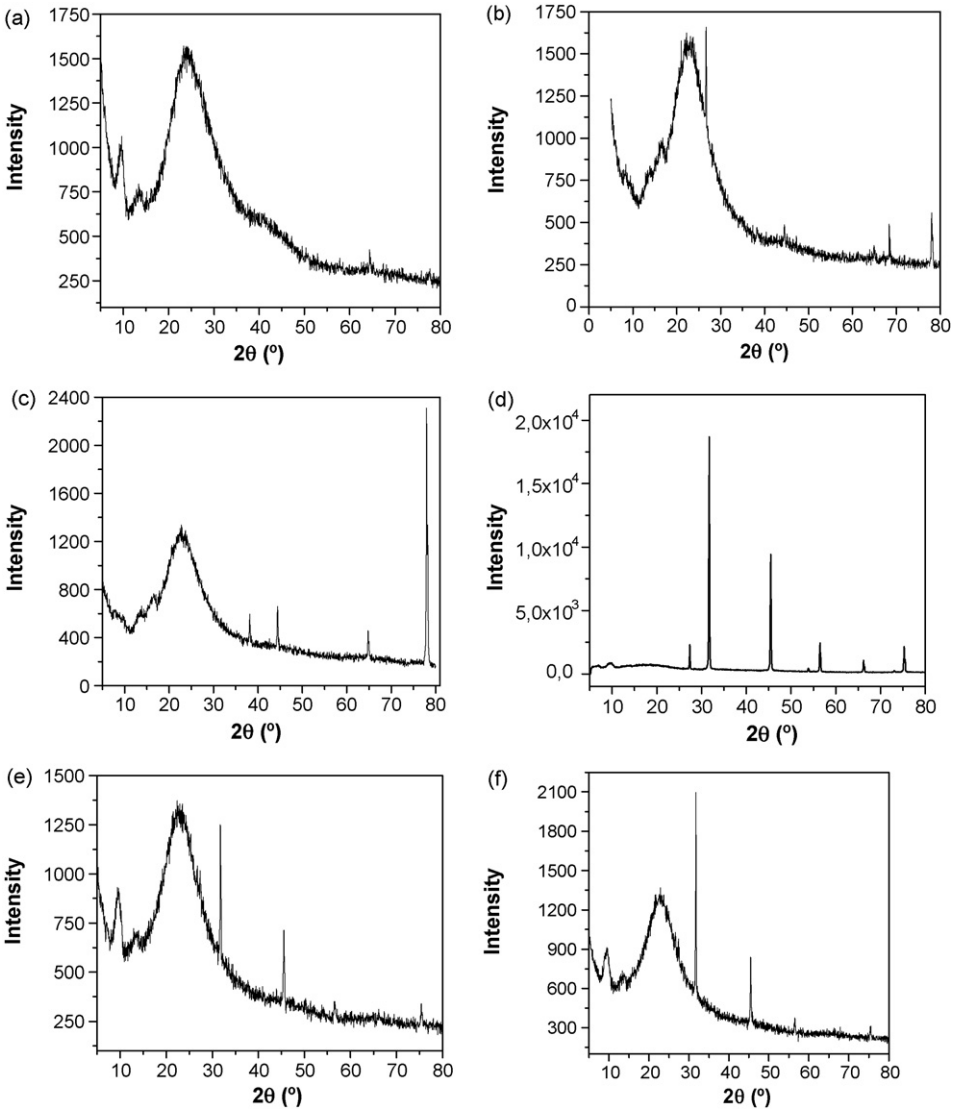

Fig. 3. X-ray diffractograms for non-activated POS–PVA (a), support activated with glutaraldehyde (b) and epichlorohydrin (c), free enzyme (d), immobilized lipase on POS–PVA activated with glutaraldehyde (e) and epichlorohydrin (f).

activated and activated states (Fig. 4a–c). For the non-activated support, a typical flat surface of amorphous material was observed (Fig. 4a). After the activation, small fissures were verified, which can facilitate the fixation of the enzyme on the support (Fig. 4b and c).

3.3. Biochemical properties and kinetic constants

The influence of the variables pH and temperature in the hydrolytic activity of the free lipase and immobilized derivatives was evaluated in experiments carried out according to

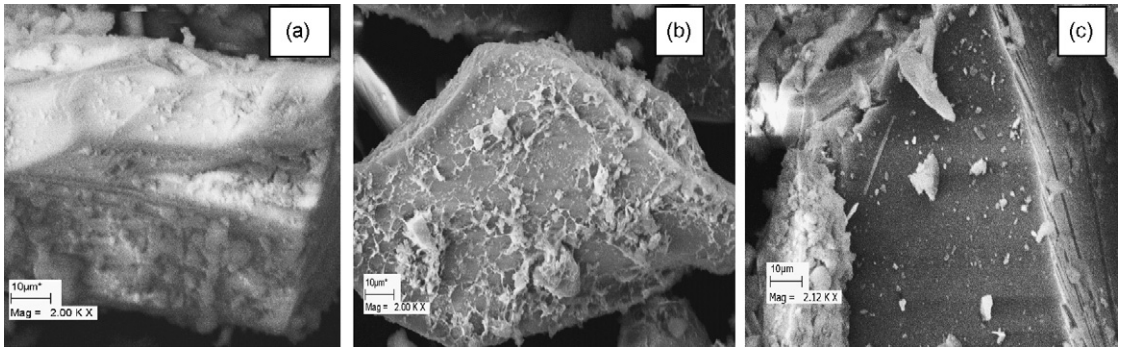

Fig. 4. Micrography of POS–PVA non-activated (a) and activated with glutaraldehyde (b) and epichlorohydrin (c).

Table 1
Experimental design and results according to the central composite "2^2 + star" factorial design

Runs	Independent variables		Free lipase	Hydrolytic activity (U/mg)	
	Temperature (X_1) (°C)	pH (X_2)		Lipase–POS–PVA–glutaraldehyde	Lipase–POS–PVA–epichlorohydrin system
1	40	6.5	12,138	406	1,104
2	40	8.5	17,766	320	722
3	65	6.5	10,290	277	874
4	65	8.5	18,606	418	775
5	53	7.5	16,632	418	744
6	53	6.1	9,282	404	1,109
7	53	8.9	17,934	352	917
8	35	7.5	13,398	331	658
9	70	7.5	18,564	313	646
10	53	7.5	17,136	402	785
11	53	7.5	17,640	370	798
12	53	7.5	18,648	381	834
13	53	7.5	19,320	384	873
14	53	7.5	19,488	376	885
15	53	7.5	16,296	411	905

Table 2
Estimated effects, standard errors and Student's *t*-test for the hydrolytic activity of the lipase *Pseudomonas fluorescens* in free form and immobilized in POS–PVA activated with glutaraldehyde and epichlorohydrin using the central composite "2^2 + star" factorial design

Variables	Free lipase			Lipase–POS–PVA–glutaraldehyde system			Lipase–POS–PVA–epichlorohydrin system		
	Effects	Standard error	*p*-value	Effects	Standard error	*p*-value	Effects	Standard error	*p*-value
Mean	17,880	±548	0.00[a]	392	±8	0.00[a]	832	±23	0.00[a]
X_1 (Linear)	1,574	±513	0.16	−14	±15	0.37	−48	±42	0.28
X_1 (Quadratic)	−1,939	±522	0.09[b]	−67	±16	0.00[a]	−162	±43	0.00[a]
X_2 (Linear)	6,555	±513	0.00[a]	−5	±15	0.77	−189	±42	0.00[a]
X_2 (Quadratic)	−4,339	±524	0.00[a]	−11	±16	0.50	200	±43	0.00[a]
X_1X_2	1,344	±725	0.38	114	±22	0.00[a]	142	±60	0.04[a]

[a] Significant at 95% confidence level.
[b] Significant at 90% confidence level.

a central composite "2^2+star" rotatable with seven replicates in the center point. The design of this experiment is given in Table 1, together with the experimental results. As expected, the studied variables have great influence in the activity of the free and immobilized systems. For the free enzyme, the activity values varied from 9,282 to 19,488 U/mg, and for the immobilized enzymes, the ranges of observed activities were 277–418 U/mg and 722–1,109 U/mg for the systems activated with glutaraldehyde and epichlorohydrin, respectively. For all tested conditions, the determined hydrolytic activities for the lipase-POS–PVA-epichlorohydrin system were at least 80% higher compared to the lipase–glutaraldehyde–POS–PVA system.

Table 2 displays the correspondent estimative of the effects of the variables, standard error and *p*-values. The influence of the studied variables on the hydrolytic activities of the lipase was different for the free and immobilized forms. For all lipase preparations, the quadratic effect of the temperature had a significant influence ($p < 0.10$). With relation to the variable pH, both the linear and quadratic effects were significant for the free enzyme and the lipase–POS–PVA–epichlorohydrin system ($p < 0.05$). Moreover, the interaction between the variables had a significant effect ($p < 0.05$) for both immobilized systems.

From these results, statistical models could be composed with the coefficients correspondent to the significant effects. The coefficients related to non-significant effects were excluded from these models, except when it was necessary to maintain hierarchy [24]. The resulting models are shown in Table 3.

The statistical analysis of the models indicated that they were significant at 99% confidence level, without significant lack of fit ($p > 0.10$). Moreover, the R^2 values indicated that the models

Table 3
Model equations for the response surfaces fitted to the experimental data points, considering the enzyme activity (A, U/mg) as a function of the temperature (X_1) and pH (X_2) coded values

System	Model equations	R^2
Free lipase	$A = 17,850 + 786X_1 + 3,290X_2 - 958X_1^2 - 2,184X_2^2$	0.87
Lipase–POS–PVA–glutaraldehyde	$A = 389 - 7.16X_1 - 3.34X_2 - 34.06X_1^2 + 56.19X_1X_2$	0.82
Lipase–POS–PVA–epichlorohydrin	$A = 833 - 24.63X_1 - 96.27X_2 - 83.07X_1^2 + 101.74X_2^2 + 71.85X_1X_2$	0.88

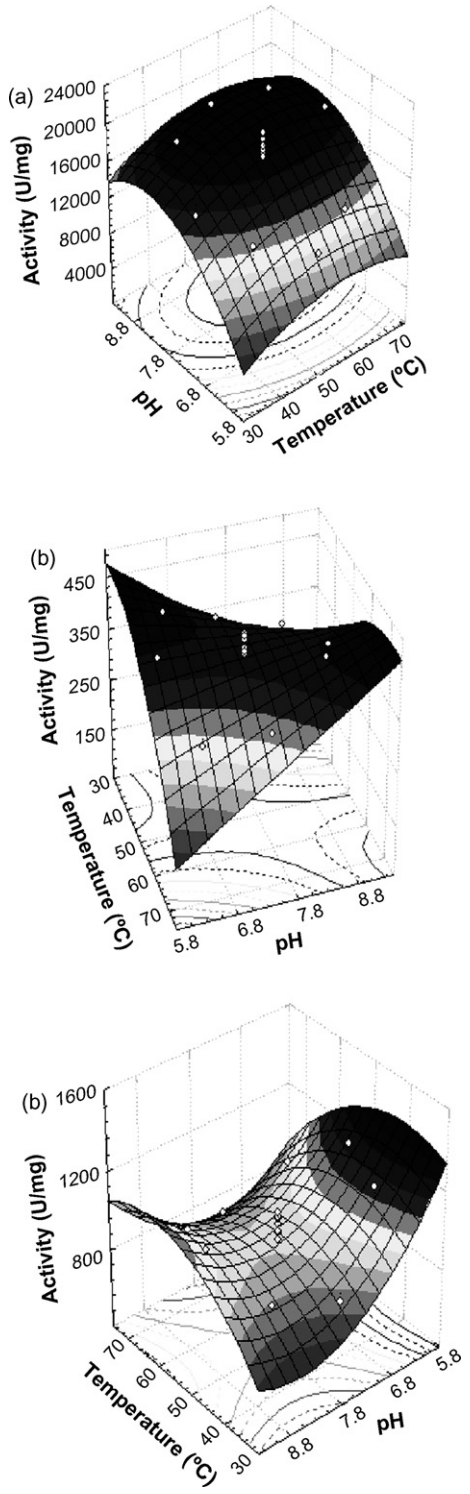

Fig. 5. Surface responses for the hydrolytic activity of the *Pseudomonas fluorescens* lipase in the free form (a) and immobilized on POS–PVA activated with glutaraldehyde (b) and epichlorohydrin (c), as a function of the variables pH and temperature.

can explain more than 80% of the experimental variability. Thus, the equations displayed in Table 3 were considered adequate for describing the activity as a function of the studied variables and were used to plot the response surfaces as showed in Fig. 5a–c.

The response surface corresponding to the free lipase (Fig. 5a) showed a top corresponding to a temperature of 58 °C and pH 8.3. These maximum values of the studied variables in relation to the hydrolytic activity were similar to those related by Angkawidjaja and Kanaya [25] for different strains of *P. fluorescens*, but our results showed slight higher temperature. For the immobilized systems, saddle-shaped response surfaces were obtained (Fig. 5b and c). This kind of response surface is complex and shows maximum and minimum points for various combinations of the independent variables. The surface correspondent to the lipase–POS–PVA–glutaraldehyde system showed the contribution of the quadratic effect of the temperature and the interaction between the variables. The response related to the lipase–POS–PVA–epichlorohydrin system showed also the contribution of the quadratic effect of pH.

Normally, when enzymes are immobilized, there is an increase in the optimum value for temperature. This fact is due to the interaction between the enzyme and the support surface that can result in a more rigid structure for the protein molecule, resulting in a more stable active globular form of the biocatalyst at higher temperatures [26,27]. This increase in the optimum temperature value can be observed in Fig. 5 for both immobilized derivatives, mainly in the more alkaline pH range.

Based on the results of these analyses, the interaction effect between the studied variables is evident. When glutaraldehyde was used to activate the support, higher values for hydrolytic activities were obtained for both alkaline and acid pH values, but at lower temperatures. The interaction effect is also evident for the lipase–POS–PVA–epichlorohydrin system, attaining high activities in either more acid or more alkaline pH values.

A numeric optimization of the hydrolytic activity as a function of the pH and temperature was carried out using the software Design-Expert 6.0. Considering the possibility of using higher temperatures under more alkaline conditions for the lipase–POS–PVA–glutaraldehyde system, this optimization was carried out using pH value at the highest level.

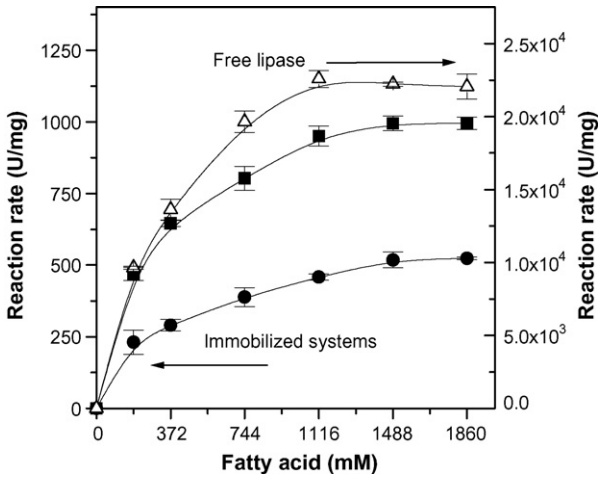

Fig. 6. Hydrolytic activities of the lipase in free form (△, right axis) and immobilized on POS–PVA (left axis) activated with glutaraldehyde (●) and epichlorohydrin (■) as a function of the substrate concentration (expressed in total fatty acids content in olive oil/water emulsions).

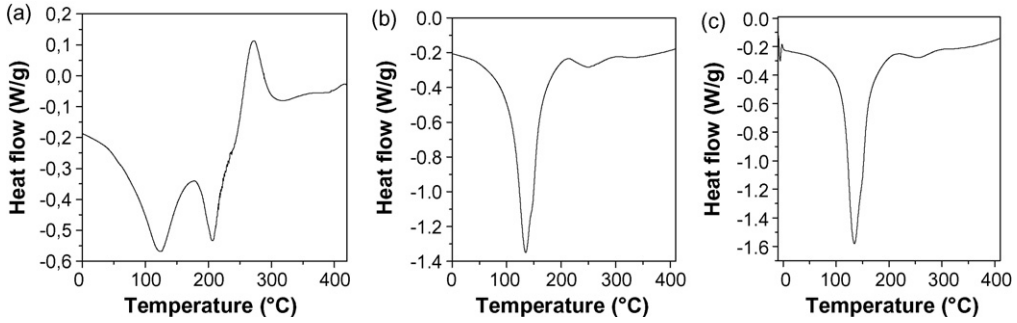

Fig. 7. DSC curves for the free lipase (a) and lipase immobilized on POS–PVA activated with glutaraldehyde (b) and epichlorohydrin (c). Negative heat flows were considered as correspondent to the endothermic direction.

To compare, the same procedure was carried out for the lipase–POS–PVA–epichlorohydrin system. The optimized conditions determined for each lipase preparation were: free enzyme: 58 °C and pH 8.3; lipase–POS–PVA–glutaraldehyde system: 65 °C and pH 8.8; lipase–POS–PVA–epichlorohydrin system: 58 °C and pH 8.9. To confirm these models, experiments were carried out under the established conditions and the differences between the experimental and theoretical values were lower than 8%.

Under the chosen conditions, the kinetic parameters were determined for each lipase preparation. Hydrolytic activities as a function of substrate concentrations are shown in Fig. 6, and the curve profile indicated that the Michaelis–Menten equation can be used to adjust data to calculate K_m and V_{max} values. Results were as follows: for free lipase ($K_m = 329 \pm 99$ mM and $V_{max} = 26{,}965 \pm 2{,}343$ U/mg); for the lipase–POS–PVA–glutaraldehyde system ($K_m = 401 \pm 14$ mM and $V_{max} = 633 \pm 68$ U/mg) and for the lipase–POS–PVA–epichlorohydrin system ($K_m = 293 \pm 58$ mM and $V_{max} = 1{,}169 \pm 62$ U/mg).

The different kinetic parameter values determined for the immobilized systems can be related with different interactions enzyme–support due to the use of either glutaraldehyde or epichlorohydrin in the activation procedure. When the support was activated with glutaraldehyde, lower enzyme–substrate affinity was observed (higher K_m value) compared to the immobilized derivative on POS–PVA activated with epichlorohydrin, although no statistical difference have been found in relation to the free lipase. Glutaraldehyde has higher carbon number in its chain and, in its commercial form, there are oligomeric α, β-unsaturated aldehydes [28]. These oligomeric forms can act as a higher and flexible spacer-arm and, due to this flexibility, the enzyme molecules could be exposed to deleterious interaction with the support surface, explaining the lower affinity observed in the lipase–POS–PVA–glutaraldehyde system. Another possibility is related to the presence of oligomers in the pores of the support after activation that may be responsible for the loss of specificity toward the substrate due to mass transfer and partition resistances. With relation to the lipase–POS–PVA–epichlorohydrin system, the immobilization itself did not affect the lipase affinity for this substrate. Similar behavior was observed by Paula et al. [8] for pancreatic lipase immobilized in POS–PVA. In that work, the K_m value was slightly lower for the immobilized lipase and this fact was explained by a possible selective immobilization of the active protein.

3.4. Thermal stability analysis by DSC

The lipase preparations were also analyzed by differential scanning calorimetry to evaluate the thermal stability of the immobilized derivatives. This technique provides information on the thermal stability of the protein by means of the transition temperatures resulting in the conformational changes the protein experiences when undergoing transition from the native state to the denatured state. DSC curves were obtained as showed in Fig. 7a–c. For free lipase (Fig. 7a), the curve exhibited two endothermic peaks at 123 and 208 °C and one exothermic peak at 274 °C. The endothermic ones could be attributed to the enzyme denaturing, corresponding to a complete transition of the enzyme structure from an ordered to an unordered form that occurred in two stages. On the other hand, the exothermic peak corresponded to the complete decomposition of the lipase structure. For the immobilized derivatives (Fig. 7b and c), the denaturing temperatures were shifted for higher values, in the order of 135 and 250 °C for both systems. Moreover, the exothermic peaks were not observed for these systems. Thus, the immobilization onto the support made lipase more resistant to heat, implying that the covalent bonds generated conveyed a higher conformational stability to the enzyme, independent of the activation agent used.

Table 4 summarizes the properties for each immobilized derivative. Although the use of glutaraldehyde has resulted in

Table 4
Biochemical and kinetic properties of *Pseudomonas fluorescens* lipase immobilized on POS–PVA activated with glutaraldehyde and epichlorohydrin

Parameters	Activating agent	
	Glutaraldehyde	Epichlorohydrin
Activity (U/mg)	370	740
Immobilization yield (%)	16	32
Optimum pH	8.8	8.9
Optimum temperature (°C)	65	58
Kinetic parameters		
K_m (mM)	401	293
V_{max} (U/mg)	633	1169

a slightly higher optimum temperature value, the correspondent immobilization efficiency was twice lower compared to the use of epichlorohydrin. Moreover, the lower K_m value evidenced the higher enzyme affinity of the system activated with epichlorohydrin, indicating that the use of this compound, instead of glutaraldehyde, to active POS–PVA before the immobilization of *P. fluorescens* lipase can be advantageous.

4. Conclusions

The characteristics of the lipase from *P. fluorescens* immobilized on POS–PVA were influenced by the activating agent used to chemically modify the support for the enzyme binding. The use of techniques as X-ray diffraction, scanning electron microscopy and FTIR allowed, besides, confirming the enzyme incorporation onto the support matrix, to obtain useful information that explains the differences due to the activation with glutaraldehyde or epichlorohydrin. From the results of the experimental design, statistical models were composed and the conditions of pH and temperature were established for the lipase preparations. Under the chosen conditions, the kinetic parameters K_m and V_{max} were determined, showing that the use of epichlorohydrin resulted in an immobilized derivative with higher affinity for the substrate. Therefore, and considering the higher immobilization efficiency obtained by using this compound, it seems a better choice compared to glutaraldehyde.

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References

[1] U.T. Bornscheuer, C. Bessler, R. Srinivas, S.H. Krishna, Trends Biotechnol. 20 (2002) 433–437.
[2] F. Hasan, A.A. Shah, A. Hameed, Enzyme Microb. Technol. 39 (2006) 235–251.
[3] A.R.M. Yahya, W.A. Anderson, M. Moo-Young, Enzyme Microb. Technol. 23 (1998) 438–450.
[4] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Enzyme Microb. Technol. 40 (2007) 1451–1463.
[5] J.C. Santos, H.F. de Castro, World J. Microbiol. Biotechnol. 22 (2006) 1007–1011.
[6] M. Petkar, A. Lali, P. Caimi, M. Daminati, J. Mol. Catal. B: Enzym. 39 (2006) 83–90.
[7] L.M. Bruno, J.S. Coelho, E.H.M. Melo, J.L. Lima-Filho, World J. Microbiol. Biotechnol. 21 (2005) 189–192.
[8] A.V. Paula, D. Urioste, J.C. Santos, H.F. de Castro, J. Chem. Technol. Biotechnol. 82 (2007) 281–288.
[9] C.M.F. Soares, O.A.A. Santos, J.E. Olivo, H.F. de Castro, F.F. Moraes, G.M. Zanin, J. Mol. Catal. B: Enzym. 29 (2004) 69–79.
[10] C.M.F. Soares, O.A.A. Santos, H.F. de Castro, F.F. Moraes, G.M. Zanin, J. Mol. Catal. B: Enzym. 39 (2006) 69–76.
[11] M.T. Reetz, P. Tielmann, W. Wilsenhoofer, W. Konen, A. Zonta, Adv. Synth. Catal. 345 (2003) 717–728.
[12] M.N. José, L.A.S.A. Prado, Quim. Nova 28 (2005) 281–288.
[13] A.E. Lima Barros, A.M.P. Almeida, L.B. Carvalho Jr., W.M. Azevedo, Braz. J. Med. Biol. Res. 35 (2002) 459–463.
[14] L. Freitas, V.H. Pérez, J.C. Santos, H.F. de Castro, J. Braz. Chem. Soc. 18 (2007) 1360–1366.
[15] C. Ratledge, B. Kristiansen (Eds.), Basic Biotechnology, Chapter 24, Biotransformations, Cambridge University Press, England, 2006, pp. 577–626.
[16] S.A. Costa, R.L. Reis, J. Mater. Sci.: Mater. Med. 15 (2004) 336–342.
[17] T. Tan, F. Wang, H. Zhang, J. Mol. Catal. B: Enzym. 18 (2002) 325–331.
[18] A.B.R. Moreira, V.H. Perez, G.M. Zanin, H.F. de Castro, Energy Fuels 21 (2007) 3689–3694.
[19] C.M. Soares, H.F. de Castro, F.F. Moraes, G.M. Zanin, Appl. Biochem. Biotechnol. 77–79 (1999) 745–757.
[20] R.H. Myers, D.C. Montgomery, Response Surface Methodology: Process and Product Optimization Using Designed Experiment, John & Sons Inc., New York, 1995.
[21] A.M. Araujo, M.T. Neves Jr., W.M. Azevedo, G.G. Oliveira, D.L. Ferreira Jr., R.A.L. Coelho, E.A.P. Figueiredo, L.B. Carvalho Jr., Biotechnol. Tech. 11 (1997) 67–70.
[22] J. Porath, N. Fornstedt, J. Chromatogr. 51 (1970) 489–497.
[23] B. Stuart, W.O. George, P.S. Mcintyre, Modern Infrared Spectroscopy, John Wiley & Sons, England, 1996.
[24] J.A. Nelder, Am. Stat. 52 (1998) 315–318.
[25] C. Angkawidjaja, S. Kanaya, Cell. Mol. Life Sci. 63 (2006) 2804–2817.
[26] V.M. Balcão, A.L. Paiva, F.X. Malcata, Enzyme Microb. Technol. 18 (1996) 392–416.
[27] P. Villeneuve, J.M. Muderhwa, J.M. Graille, M.J. Haas, J. Mol. Catal. B: Enzym. 9 (2000) 113–148.
[28] H.K. Purss, G.G. Oiaio, D.H. Solomom, J. Appl. Polym. Sci. 96 (2005) 780–792.