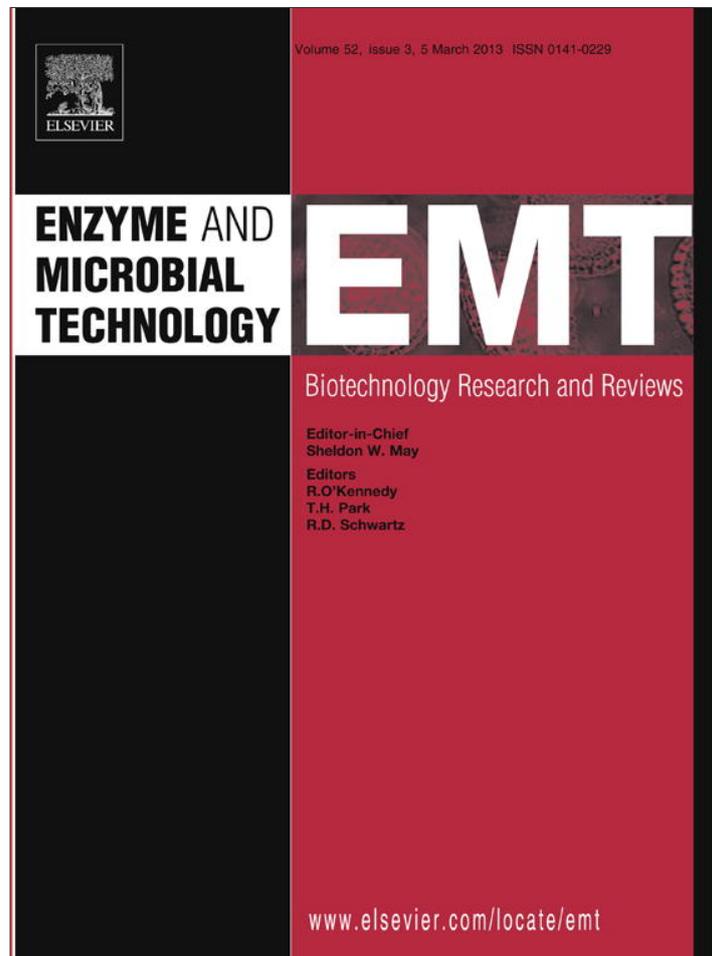


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## Protic ionic liquid as additive on lipase immobilization using silica sol–gel

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## ABSTRACT

Ionic liquids (ILs) have evolved as a new type of non-aqueous solvents for biocatalysis, mainly due to their unique and tunable physical properties. A number of recent review papers have described a variety of enzymatic reactions conducted in IL solutions, on the other hand, to improve the enzyme's activity and stability in ILs; major methods being explored include the enzyme immobilization (on solid support, sol–gel, etc.), protic ionic liquids used as an additive process. The immobilization of the lipase from *Burkholderia cepacia* by the sol–gel technique using protic ionic liquids (PIL) as additives to protect against inactivation of the lipase due to release of alcohol and shrinkage of the gel during the sol–gel process was investigated in this study. The influence of various factors such as the length of the alkyl chain of protic ionic liquids (monoethanolamine-based) and a concentration range between 0.5 and 3.0% (w/v) were evaluated. The resulting hydrophobic matrices and immobilized lipases were characterised with regard to specific surface area, adsorption–desorption isotherms, pore volume ( $V_p$ ) and size ( $d_p$ ) according to nitrogen adsorption and scanning electron microscopy (SEM), physico-chemical properties (thermogravimetric – TG, differential scanning calorimetry – DSC and Fourier transform infrared spectroscopy – FTIR) and the potential for ethyl ester and emulsifier production. The total activity yields ( $Y_a$ ) for matrices of immobilized lipase employing protic ionic liquids as additives always resulted in higher values compared with the sample absent the protic ionic liquids, which represents 35-fold increase in recovery of enzymatic activity using the more hydrophobic protic ionic liquids. Compared with arrays of the immobilized biocatalyst without additive, in general, the immobilized biocatalyst in the presence of protic ionic liquids showed increased values of surface area ( $143\text{--}245\text{ m}^2\text{ g}^{-1}$ ) and pore size ( $19\text{--}38\text{ \AA}$ ). Immobilization with protic ionic liquids also favoured reduced mass loss according to TG curves (always less than 42.9%) when compared to the immobilized matrix without protic ionic liquids (45.1%), except for the sample containing 3.0% protic ionic liquids (46.5%), verified by thermogravimetric analysis. Ionic liquids containing a more hydrophobic alkyl group in the cationic moiety were beneficial for recovery of the activity of the immobilized lipase. The physico-chemical characterization confirmed the presence of the enzyme and its immobilized derivatives obtained in this study by identifying the presence of amino groups, and profiling enthalpy changes of mass loss.

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## 1. Introduction

Lipase (EC 3.1.1.3) is an important enzyme with a wide variety of applications in the food, fine chemical and pharmaceutical industries due to the multiplicity of reactions it catalyses, such

as esterification, transesterification and hydrolysis [1–5]. Among them, the lipase from *Burkholderia cepacia* is distinguished by its ability to carry out organic synthesis, a feature of great interest to industry [6]. The economy of biocatalytic processes can be improved by improving the reuse and/or the enzyme itself, may improve performance of the enzyme under optimal conditions of reaction process (e.g., temperatures of alkalinity, acidity, organic solvents, and high), a requirement that has often retarded enzyme application in industrial chemical synthesis [7,8]. Therefore, numerous efforts have focused on the preparation of lipases

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in immobilized forms, involving a variety of both support materials and immobilization methods [6,9].

Compared to chemical methods to immobilize enzymes, physico-chemical methods, especially sol–gel encapsulation, making it a powerful tool for improving the properties of biomolecules immobilized for use as biosensors or biocatalysts [10,11]. A very large number of biomolecules have been immobilized by sol–gel techniques, and generally give better enzymatic activity and stability; however, there are also some disadvantages in the process of sol–gel immobilization [12]. One is shrinkage of the gel during the process of condensation and drying, which can cause denaturation of the enzyme. In addition, the slow diffusion of molecules of substrate for the enzyme within the sol–gel matrix hinders the catalytic activity of the immobilized enzyme in materials with a pore diameter smaller than 20 Å [13].

One way to overcome these drawbacks could be the use of additives to stabilize enzymes and assist in gel formation within arrays. Recently the use of aprotic ionic liquids as additives in the immobilization process has been reported, which could increase the activity and stability of immobilized enzymes by altering the hydration shell of the enzyme and reducing shrinkage of the gel [14]. They can also affect the physical properties of the gel by participating in condensation reactions with free silanol groups [14].

Studies of various additives such as ionic liquids (ILs) have recently been suggested as agents that can stabilize enzymes, protecting the hydration shell around the enzyme and/or causing conformational changes leading to permanent activation of the enzyme [15]. Lee et al. [16] reported that the process of immobilizing the *Candida rugosa* lipase using the sol–gel technique showed high stability and increased enzyme activity, about 10 times over that of the lipase in free form. The positive influence of the use of aprotic ionic liquids was also reported by Zarcu et al. [17] using [C<sub>8</sub>mim][BF<sub>4</sub>] in the process of immobilizing the lipase from *Pseudomonas fluorescens* in hybrid sol–gel matrices as an additive; the results obtained with the immobilized biocatalyst showed total income generally exceeding 100%. The use of aprotic ionic liquids is also reported as additive on the activity and regioselectivity of *Rhizomucor miehei* lipase immobilized, showing that the affinity of the enzyme for the substrate increased in the presence of ILs more hydrophobic [18]. In fact, aprotic ionic liquids based on cations primarily in imidazolium and pyridinium showed satisfactory results in the immobilization of enzymes; however, the use of ionic liquids still has high costs of synthesis, which hinders the industrial application and can be toxic to enzyme [17,19].

Álvarez et al. [20] report that the ionic liquids obtained from amines, organic and inorganic acids, the so-called protic ionic liquids (PIL), feature low cost and simplicity of synthesis, favouring different applications including industrial applications. Moreover, the protic ionic liquids (PILs) have been demonstrated to be biocompatible with lipases, showing high activity and enantioselectivity in these media [21].

Potential applications have been identified for the use of PIL with proteins, such as dissolving hydrophobic ligands (e.g., ferrocene), to incorporate them into a protein crystal, improving the solubility of some proteins and improving monodispersity of proteins, as a precipitating agent and as an additive [22]. In particular, the potential of ionic liquids leads to implementing them as additives in the process of sol–gel immobilization, for the purpose of protecting against inactivation of enzymes due to release alcohol and shrinking of the gel during the encapsulation process.

This study aimed to immobilize the *B. cepacia* lipase in hydrophobic matrices obtained by a sol–gel technique in the presence of various protic ionic liquid alkyl chains with different C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub>, in addition to varying the content of a particular ionic liquid additive – C<sub>5</sub> (0.5–3.0%, w/v), which has hitherto not been reported, with physico-chemical properties of ammonium-based,

assessing the total yield of recovered activity, potential production of ethyl esters and emulsifiers, and physical–chemical properties of the immobilized derivatives.

## 2. Experimental

### 2.1. Materials and reagents

Lipase from *B. cepacia* (Amano Lipase) was purchased from SIGMA – ALDRICH (Japan). The silane precursor tetraethoxysilane (TEOS) was supplied by Across Organic (NJ, USA) and used without further purification. Ethanol (minimum 99% pure), ammonia (minimum 28% pure), hydrochloric acid (minimum 36% pure) and gum Arabic were obtained from Synth (São Paulo, Brazil). Water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. The protic ionic liquids used are shown in Table 1. Other chemicals were of analytical grade and used as received.

### 2.2. Encapsulation of lipase from *B. cepacia* in sol–gel matrices

A methodology previously established by Patent PI0306829-3 [23] was used, and is briefly described as follows: 30 mL of TEOS was dissolved in 36 mL of absolute ethanol under an inert nitrogen atmosphere. To this, 0.22 mL of hydrochloric acid was dissolved in 5 mL of slowly added ultra-pure water, and the mixture was agitated (200 rpm) for 90 min at 35 °C. Lipase from *B. cepacia* (870.71 U) was dissolved in a solution of 10 mL of water, to which were simultaneously and separately added 1% (w/v) of the protic ionic liquids C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub>. As PIL-C<sub>5</sub> has proven to be the best additive (see Section 3.1), we also evaluated the effect of varying the amount of the additive added (0.5–3.0% w/v). 1.0 mL of ammonium hydroxide dissolved in 6.0 mL of ethanol (hydrolysis solution) were added to the sol–gel reaction, and the mixture was kept under static conditions for 24 h to complete polycondensation. The bulk gel was then washed with heptane and acetone and dried under vacuum at room temperature for 72 h. For comparison purposes, the encapsulated lipase (EN) from *B. cepacia* was prepared similarly in the absence of IL (EN-AIL), and pure silica sol–gel (PS) was prepared in the absence of the enzyme or additives. The sol–gel matrix immobilized lipases were designated: EN-C<sub>2</sub>, EN-C<sub>3</sub>, EN-C<sub>4</sub>, EN-C<sub>5</sub> and EN-C<sub>5</sub>-0.5, EN-C<sub>5</sub>-1.0, EN-C<sub>5</sub>-2.0, EN-C<sub>5</sub>-3.0.

### 2.3. Enzymatic activity

Enzymatic activities of the free and immobilized lipase samples were assayed by the olive oil emulsion method according to a modification used by Soares et al. [24]. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of gum Arabic solution (7% w/v). The reaction mixture containing 5 mL of the oil emulsion, 4 mL of sodium phosphate buffer (0.1 M, pH 7.0) and either free (1.0 mL, 0.1 mg mL<sup>-1</sup>) or immobilized (≈250 mg) lipase was incubated in a thermostated batch reactor for either 5 min (free lipase) or 10 min (immobilized lipase) at 37 °C. A blank titration was done using a sample in which the enzyme was replaced with distilled water. The reaction was stopped by the addition of 2 mL of acetone–ethanol–water solution (1:1:1). The liberated fatty acids were titrated with potassium hydroxide solution (0.04 M) in the presence of phenolphthalein as an indicator. All reactions were carried out in triplicate. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per min (μmol min<sup>-1</sup>) under the assay conditions (37 °C, pH 7.0, 80 rpm).

Analyses of hydrolytic activities were carried out on the lipase loading solution and bioencapsulated preparations to determine the total activity recovery yield, Y<sub>a</sub> (%), according to Eq. (1):

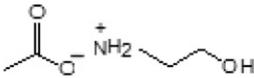
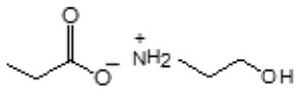
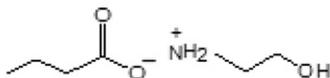
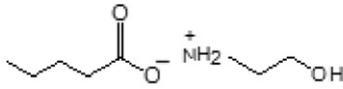
$$Y_a = \frac{U_s}{U_o} \times 100 \quad (1)$$

where U<sub>s</sub> is the total enzyme activity recovered on the support and U<sub>o</sub> is the enzyme units offered for immobilization.

### 2.4. Ethyl esters and emulsifier production

Transesterification reactions catalysed by the lipase from *B. cepacia* were performed under the conditions proposed by Nouredini et al. [10]. The transesterification reactions were carried out in batch reactors, submerged in a thermostatic bath to keep each mixture at constant temperature and under agitation. The reaction was initiated by mixing soybean oil and ethyl alcohol (ratio 1:15.2), 0.075 g water, and lastly the free or immobilized *B. cepacia* (EN-AIL or EN-C<sub>5</sub>), as PIL-C<sub>5</sub> proved to be the most potent additive (see Section 3.1). The equivalent of 38.3 U of enzyme were added to each sample. Aliquots were removed at different time intervals and analysed by a GC gas chromatograph equipped with a CARBOVAX (30 m × 0.25 mm × 0.25 μm) column. Column temperature was initially kept at 140 °C for 1 min, increased to 180 °C at a rate of 4 °C per minute, maintained for 2 min, then increased to 230 °C at a rate of 10 °C per minute, where it was maintained for a further 10 min. The temperatures of the injector and detector were set to 250 °C. Ethyl ester conversion (%) was defined as the observed amount of product divided by the theoretical yield if all the soybean oil was converted.

**Table 1**  
Structure of protic ionic liquid used in this work.

Abbreviation	Organic acid	Structure (nomenclature)
C <sub>2</sub>	Acid acetic	 (N-methylmonoethanolamine acetate)
C <sub>3</sub>	Acid propionic	 (N-methylmonoethanolamine propionate)
C <sub>4</sub>	Acid butyric	 (N-methylmonoethanolamine butyrate)
C <sub>5</sub>	Acid pentanoic	 (N-methylmonoethanolamine pentanoate)

The esterification activity of the immobilized lipase was determined by the formation of isopropyl laurate in a reaction of isopropyl alcohol with lauric acid in heptane in the ratio (1:2.34) at 52.5 °C. The reaction was initiated by the addition of 5.25% (w/v) of immobilized lipase and 10% (w/v) molecular sieves, (3 ×, 3.2 mm, Peppets – Sigma) to the reaction medium (5.0 g) at 150 rpm, followed by agitation for 10 min. The amount of isopropyl laurate formed was quantified by titration of NaOH (0.01 M). One unit of activity (esterification) was defined as the amount of enzyme leading to the formation of 1 mol of isopropyl laurate per minute under the test conditions. The yield of esterification was quantified based on the blank sample.

### 2.5. Morphological and physico-chemical properties

The surface areas of the pure silica gel and immobilized lipase derivatives were calculated using the Brunauer–Emmett–Teller method [25]. Pore volume and average pore diameter based on model developed by Barret, Joyner and Halenda (BJH) calculations for mesoporous samples, for microporous samples by *t*-method. Surface areas were evaluated according to their N<sub>2</sub> adsorption at 77 K using BET apparatus software (Model NOVA 1200e – Surface Area & Pore Size Analyzer, Quantachrome Instruments – version 11.0). Prior to analysis, samples were submitted to a thermal treatment at 120 °C for 48 h, to eliminate any water existing within the pores of the solids.

Thermogravimetric (TG) curves were obtained using a Shimadzu DTG-60H simultaneous DTA-TG apparatus, under a nitrogen atmosphere that started from room temperature and went up to 1000 °C, increasing at a heating rate of 20 °C min<sup>-1</sup>. DSC curves were obtained in a Shimadzu DSC-60, under a nitrogen atmosphere that started from room temperature and was then increased to 500 °C at a heating rate of 10 °C min<sup>-1</sup>. Scanning electron microscopy (SEM; model Hitachi SU-70) was also used to characterise the surfaces of the samples.

The samples of immobilized lipase in the presence and absence of additive (EN-AIL and EN-C<sub>5</sub>-1.0) were submitted to FTIR analysis (spectrophotometer FTIR BOMEMMB-100). Spectra were obtained in the wavelength range from 400 to 4000 cm<sup>-1</sup>.

SEM was used to characterize the morphologies of the immobilization products of the EN-AIL and EN-C<sub>5</sub>-1.0 samples.

## 3. Results and discussion

### 3.1. Enzymatic activity of the immobilized sol–gel

The use of PIL as an additive was found to have various effects both in the process and in retaining the catalytic activity of the immobilized enzyme. The results show that the increased alkyl side chain of PIL, added at 1.0% (w/v), enhanced the yield of

immobilization ( $Y_a$ ) with values always higher than 1000%. The absence of additives enabled us to detect only a 43% yield of the enzymatic activity (Fig. 1a). The increase in enzyme activity also occurred in immobilized lipase using additives; however, increasing the number of carbon atoms (in the additive) allowed values near the enzymatic activity (Table 2). According to Moniruzzaman and co-workers [26] and Zhao and co-workers [27], this is due to the use of an additive which acts as a mold, coating the surface of silica and protecting channels and the enzyme during shrinkage and collapse the gel pores as well as behaving as a stabilizer to protect the enzyme from inactivation, the release of alcohol during hydrolysis of a silicon alkoxide.

The best recovery of activity was observed in the sample containing the ionic liquid with the most hydrophobic character (IL-C<sub>5</sub>) employed during the immobilization process. The results demonstrate this positive effect, evidenced by the excellent values in the recovery of activity ( $Y_a = 1526\%$ ) representing an increase of 35 times compared with a sample EN-AIL. The increased recovery of activity was a positive effect up to a level of 1% (w/v) of the content as added IL-C<sub>5</sub> in the immobilization process.

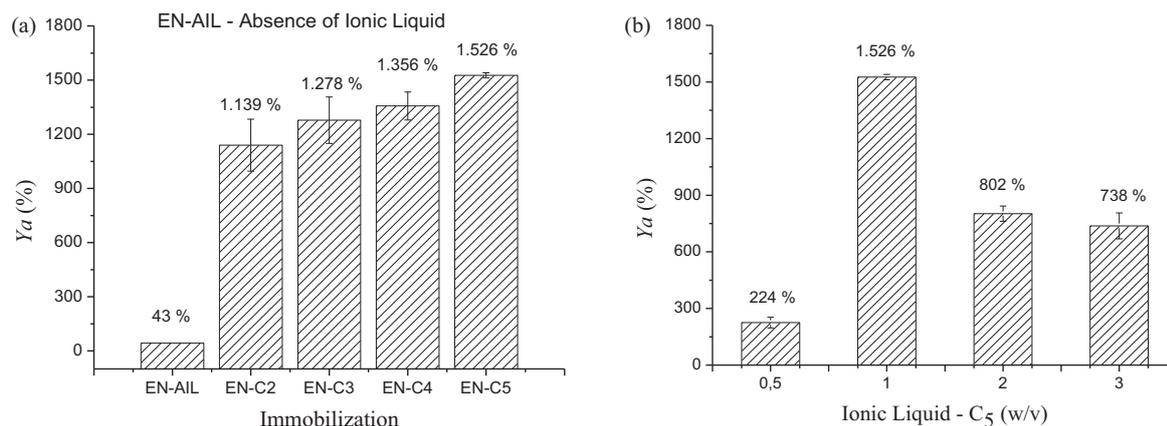
Other favourable results in the use of additives employed for sol–gel immobilization of lipases have also been reported;

**Table 2**  
Influence of alkyl chain lengths of the PIL, and concentration on recovery of total activity recovery yield of the immobilized samples.

Sample	Water content (%)	A. Total (U)
EN-AIL	21.88	374.4
EN-C <sub>2</sub> <sup>a</sup>	11.10	9923.7
EN-C <sub>3</sub> <sup>a</sup>	15.77	12,136.1
EN-C <sub>4</sub> <sup>a</sup>	14.20	11,812.9
EN-C <sub>5</sub> <sup>a</sup>	10.70	13,291.5
EN-C <sub>5</sub> -0.5 <sup>b</sup>	7.71	1958.6
EN-C <sub>5</sub> -1.0 <sup>b</sup>	10.70	13,291.5
EN-C <sub>5</sub> -2.0 <sup>b</sup>	13.69	6987.9
EN-C <sub>5</sub> -3.0 <sup>b</sup>	18.21	6428.1

<sup>a</sup> Immobilized with different alkyl chain lengths with PIL.

<sup>b</sup> Immobilized in different concentrations of more hydrophobic character of PIL.



**Fig. 1.** Total activity recovery yield for enzyme *Burkholderia cepacia* encapsulated in sol–gel matrices for hydrolysis reaction: (a) immobilized in the absence and presence of IL with different numbers of carbon atoms and (b) immobilized in different concentrations of IL-C<sub>5</sub>.

however, the values reported were much lower than those found in our work. Soares et al. [2] found a  $Y_a$  of 60% for *C. rugosa* lipase on silica using PEG-1500 as the additive. Zarcula et al. [17] also observed the recovery of lipase from *P. fluorescens* was always over 100% in hybrid sol–gel matrices using an aprotic ionic liquid as an additive.

Immobilization studies of the according with researches may produce alterations observed in their activity, specificity or selectivity. Although in many cases an impoverishment of the enzyme properties is observed upon immobilization (caused by the distortion of the enzyme due to the interaction with the support) in some instances such properties may be enhanced by this immobilization. These alterations in enzyme properties are sometimes associated with changes in the enzyme structure. Occasionally, these variations will be positive. For example, they may be related to the stabilization of a hyperactivated form of the enzyme, like in the case of lipases immobilized on hydrophobic supports via interfacial activation. Furthermore, immobilization of enzymes on a support, mainly on a porous support, may in many cases also have a positive impact on the observed enzyme behaviour, not really related to structural changes. For example, the promotion of diffusional problems (e.g., pH gradients, substrate or product gradients), partition (towards or away from the enzyme environment, for substrate or products), or the blocking of some areas (e.g., reducing inhibitions) may greatly improve enzyme performance [28]. In this work we studied the influence of changes in the number of carbon atoms in the ionic liquid (monoethanolamine-based) and verified that the best total activity yield used the most hydrophobic additive (IL-C<sub>5</sub>) to immobilize the enzyme from *B. cepacia*. In Table 2, derivatives immobilized with additive always presented values for water content below those of the fixed assets without additive (21.88%). These results demonstrate the effects caused by the use of an ionic liquid of hydrophobic character, in fact, when the enzyme immobilized within a porous solid (such as ionic liquid and the porous inert support) some inactivating the enzyme sources, such as aggregation and adsorption of hydrophobic surface are minimized [8], however, the presence of water is very important because it forms a hydration shell around the enzyme, inducing lipolytic activity. That can be achieved by the presence of hydrophobic ionic liquids. Possibly the hydrophobicity of the PIL provided this environment, inducing the presence of water next the enzyme. Since the hydrophilic nature of ionic liquids can inactivate lipases in a nonaqueous medium by taking away the water that is essential for maintaining the active conformation of the enzyme [2,29]. Palomo et al. [18] also relate to the positive effect on the hydrolysis reaction indicating that the affinity of the enzyme for the substrate increased in the presence of ILs more hydrophobic.

This effect has been reported for *Candida antarctica* lipase B [30]. In this work it has also been possible to verify the effect of varying the concentration of a particular additive (IL-C<sub>5</sub>) on the process of immobilization; we observed that upon addition of up to 1.0% (w/v), there was an increase in the total activity recovery yield, but further increases in the amount added only decreased this value. This fact can be explained by the excess PIL saturation providing the additive process (Fig. 1b).

### 3.2. Ethyl esters and emulsifier production

After the excellent results obtained for sample recovery yield of enzyme activity (EN-C<sub>5</sub>-1.0) were observed, the potential reactions of esterification and transesterification for the synthesis of ethyl esters and emulsifiers were examined (Table 3). For the production of ethyl esters, the lipase in its native form showed 74.7% conversion in 48 h of reaction, whereas the sample immobilized in the presence of 1.0% PIL achieved the maximum conversion of 46.5% within 72 h; however, the assets without the presence of PIL showed drastic decreases in the conversion of triglycerides into ethyl esters, reaching only 1.3% in 48 h.

The same reaction conditions for ethyl ester production were used with *B. cepacia* immobilized in silica to obtain hydrolysis of tetramethoxysilane (TMOS) and showed an improved ethyl ester conversion rate of 65% [10]. The use of ionic liquids in transesterification reactions with lipases has been reported by several research groups [31,26]. Ha et al. [32] reported that the hydrophobic nature of ionic liquids favours the enzymatic transesterification reactions of soybean oil, increasing the solubility of the reaction.

In this sense, we observed the positive effect caused by the addition of protic opening groups in the conversion of triglycerides into ethyl esters for the immobilization technique utilized in this work. However, the potential production of emulsifiers, verified by the esterification reaction catalysed by derivatives and immobilized lipase from *B. cepacia*, represented less than 5% conversion in the formation of isopropyl laurate.

**Table 3**

Transesterification reaction of soybean oil and ethanol, catalysed by sol–gel immobilized lipase from *Burkholderia cepacia* absent of ionic liquids, the presence of 1.0% (w/v) ionic liquid and free enzyme.

Sample	Ethyl esters (%)	Reaction time <sup>a</sup> (h)
Free enzyme	74.7	48
EN-AIL	1.3	48
EN-C <sub>5</sub> -1.0	46.5	72

<sup>a</sup> Reaction time with higher conversion.

**Table 4**  
Influence of concentration and the alkyl chain protic ionic liquid used as an additive in sol–gel immobilized lipase in the textural properties of adsorption–desorption of nitrogen.

Sample	Surface area (m <sup>2</sup> g <sup>-1</sup> )	<sup>a</sup> Pore volume (cm <sup>3</sup> g <sup>-1</sup> )	Pore diameter (Å)
Pure silica	224	0.21	35
EN-AIL	143	0.05	19
EN-C <sub>2</sub>	72	0.005	19
EN-C <sub>3</sub>	176	0.02	24
EN-C <sub>4</sub>	241	0.04	24
EN-C <sub>5</sub>	245	0.08	30
EN-C <sub>5</sub> -0.5	164	0.02	26
EN-C <sub>5</sub> -1.0	245	0.08	30
EN-C <sub>5</sub> -2.0	181	0.15	38
EN-C <sub>5</sub> -3.0	121	0.08	38

<sup>a</sup> Pore volume calculated from nitrogen desorption.

### 3.3. Morphological characterization of samples of lipase

#### 3.3.1. Specific surface area and porous properties

The addition of PILs changed the morphological structure of the immobilized biocatalysts (Table 4). Only IL addition of more hydrophobic groups (C<sub>4</sub> and C<sub>5</sub>) correlated with increases in surface area when compared with the pure silica matrix; however, compared with immobilized matrices without additive (EN-AIL), all immobilized derivatives showed increases in surface area, pore volume and average pore diameter. The largest pore size and surface area were obtained when the additive was increased to C<sub>5</sub> in the sol–gel process. The effect of the concentration of the IL-C<sub>5</sub> biocatalyst can be seen in Table 4. Concentrations above 2.0% (w/v) had positive effects on the pore diameter (38 Å); however, the immobilized matrices also showed reduced surface area (245 m<sup>2</sup> g<sup>-1</sup>) compared with 1.0% (w/v). In this case, the use of a support coated with the ionic liquid may allow penetration of the enzyme inside the bed (because the pores increase), while increasing the number of coupled enzymes and consequently increasing the yield of immobilisation [33]. This type of support (with ionic additives) has been used to stabilize  $\alpha$ -galactosidase and  $\beta$ -galactosidase from *Thermus* sp. strain T2 [34,35], different D-amino acid oxidases [36], formate dehydrogenase from *Candida boidinii* [37], different alcohol oxidases [38], etc. Therefore, in EN-C<sub>5</sub>-1.0, the magnitude of the volume adsorbed became larger, indicating that an optimal amount of the additive was being absorbed in the preparation of the immobilized samples, leading to materials with increased surface areas, up to a maximum, and then decreasing; in fact, excess PIL may block the pores, causing the decrease in surface area (Table 4).

Thus, from the nitrogen adsorption–desorption measurements, it is clear that the additive acted as an agent of pore formation. A similar profile was observed by Vila-Real et al. [29], who reported that the addition of IL in the process of sol–gel immobilization had an important role in the performance of the enzyme, affecting the structural characteristics of the immobilized biocatalyst. Souza et al. [39] reported that the addition of Aliquat 336 (a quaternary salt) during sol–gel immobilization of the lipase from *Bacillus* sp. ITP-001 modified the porous structure of the immobilized derivatives, including enlarging the pores (92 Å) and increasing the surface area (183 m<sup>2</sup> g<sup>-1</sup>) and pore volume (0.36 cm<sup>3</sup> g<sup>-1</sup>). This behaviour was also reported by Zarcu et al. [17] during encapsulation of lipase in a hydrophobic matrix with a large number of hydrophobic groups, since the hydrophobic liquids induced significant changes in the porous structure of the biocatalyst.

The consistency of these results is in good alignment with the well-known fact that a more hydrophobic microenvironment is beneficial to lipase activity [2]. The higher values of total activity recovery yield may also be associated with the pore size and surface area, the rate of enzymatic reaction depending on the accessibility

of the substrate to the enzyme, considering the rate of diffusion of the substrate for the enzyme within the structure of the silica cage. Once the enzyme is already within the channel pore after gelatinization, the pore channels need to be just large enough to allow diffusion of molecules of substrate and product molecules to reach the enzyme within the matrix cage [2]. Thus, circumvents the limitations suggested by Brady and Jordaan [7], where the application of immobilized enzymes may be limited by the size of substrate molecules prone to mass transfer limitations. Therefore, with the addition of 1.0% (w/v) IL-C<sub>5</sub> in the process of immobilizing the lipase from *B. cepacia*, enlarging of the pores and increase in the surface area can be clearly seen in relation to the biocatalyst without the ionic additive.

The sample of pure silica (Fig. 2a) showed a type IV isotherm with a hysteresis loop, which is usually exhibited by mesoporous solids [40]. The results observed for pure silica in the N<sub>2</sub> adsorption–desorption tests agree with those of the isotherms with H2 hysteresis loops, which is a characteristic of mesoporous materials and generally associated with pores with narrow necks and wide bodies [41]. For the samples EN-AIL and EN-C<sub>2</sub> (Fig. 2b and c respectively), the isotherm is of type I, showing a rapid increase of gas adsorbed as the pressure is increased until a plateau is reached. This isotherm is characteristic of microporous solids having relatively small external surface areas, and is also obtained when the adsorption forms only a monolayer [42].

The matrix sol–gel immobilized lipase from *B. cepacia*, in the presence of PIL with different numbers of carbon atoms in its structure (Fig. 2) and changes in the concentration of the most hydrophobic ionic liquid (IL-C<sub>5</sub>), showed the type IV isotherms and loop hysteresis characteristic of type H2, which in most cases displays a prominent region in which the relative pressure and volume vary little but the adsorption increases sharply [43]. However, as shown in Fig. 3, the increased concentration of IL present in fixed assets produced an enlargement of the hysteresis associated with the secondary process of capillary condensation, and as a result enabling more complete filling of the mesopores at relative pressures less than 1 [39,42].

#### 3.3.2. Thermogravimetric analysis – TG

The mass losses of samples of pure silica (PS), biocatalyst free (free), immobilized with additive (EN-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub>, C<sub>5</sub> and EN-C<sub>5</sub>-0.5 to 3.0) and without additive (EN-AIL) were determined by thermogravimetric analysis (TGA). The weight losses obtained after heating each sample to 1000 °C are reported as TGA weight loss in Table 5. The weight loss observed is due to mostly degradation of the enzyme present in the samples. The TGA curves for all the immobilized samples with the different additives are shown in Fig. 4. It can be observed that the PS sample showed a weight loss of only 22%. This weight loss can be attributed to the presence of un-reacted silanol groups from the TEOS, which are present in the silica because of incomplete sol–gel reactions [43]. A part of this weight loss is also due to the removal of water molecules, which were tightly bound to the silica matrix [44]. Soares et al. [24] correlated a lower mass loss obtained by an immobilized additive with an increased thermal stability of the matrix, resulting from interactions between silane precursors and organic components (additives and lipase).

The matrices with immobilized IL-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub> showed similar values of mass loss; however, in the samples with various concentrations of IL-C<sub>5</sub> (Fig. 5), we observed a trend of increasing mass loss, possibly influenced by the additional volume of protic ionic liquid. The thermographs were divided into three regions. Region I in the weight loss thermographs is mainly associated with extraction of the water in the surface and decomposition of amino groups, usually of organic groups, up to a temperature of 200 °C. Weight loss associated with this region may also

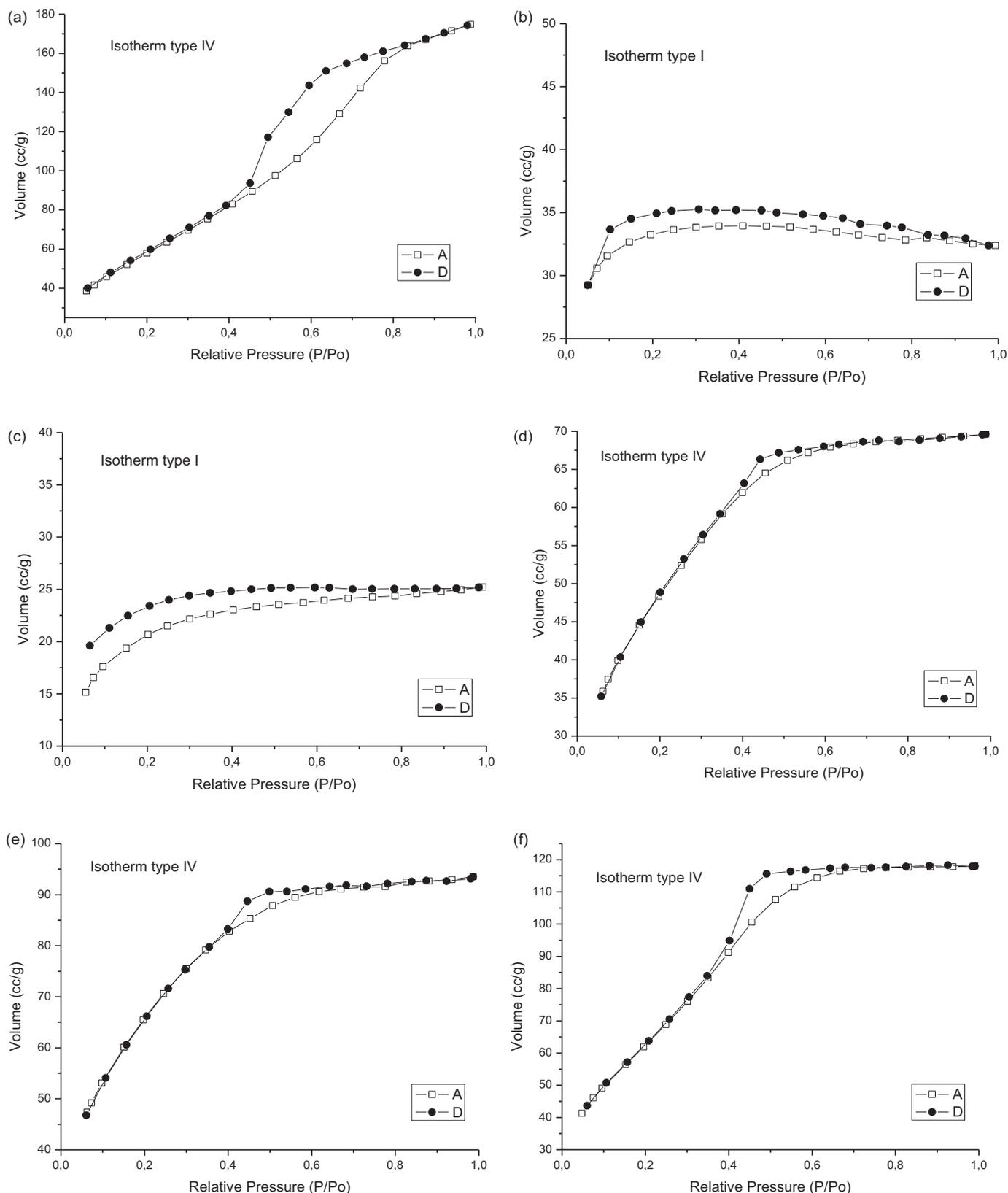


Fig. 2. Nitrogen adsorption–desorption isotherms of the pure silica gel and immobilized samples: (a) PS, (b) EN-A1L, (c) EN-C<sub>2</sub>, (d) EN-C<sub>3</sub>, (e) EN-C<sub>4</sub>, and (f) EN-C<sub>5</sub>.

be related to the presence of water ionically associated stoichiometrically to the cation or anion; this water may be either fixed or random, occupying positions in the lattice of the sol-gel matrix. Region II, comprising the range between 200 and 600 °C,

is associated with condensation of silanol groups and some loss of organic constituents (C, H, O and N) in the form of volatiles either present or formed by the beginning of organic decomposition, including lipase. In Region III, the weight loss is associated

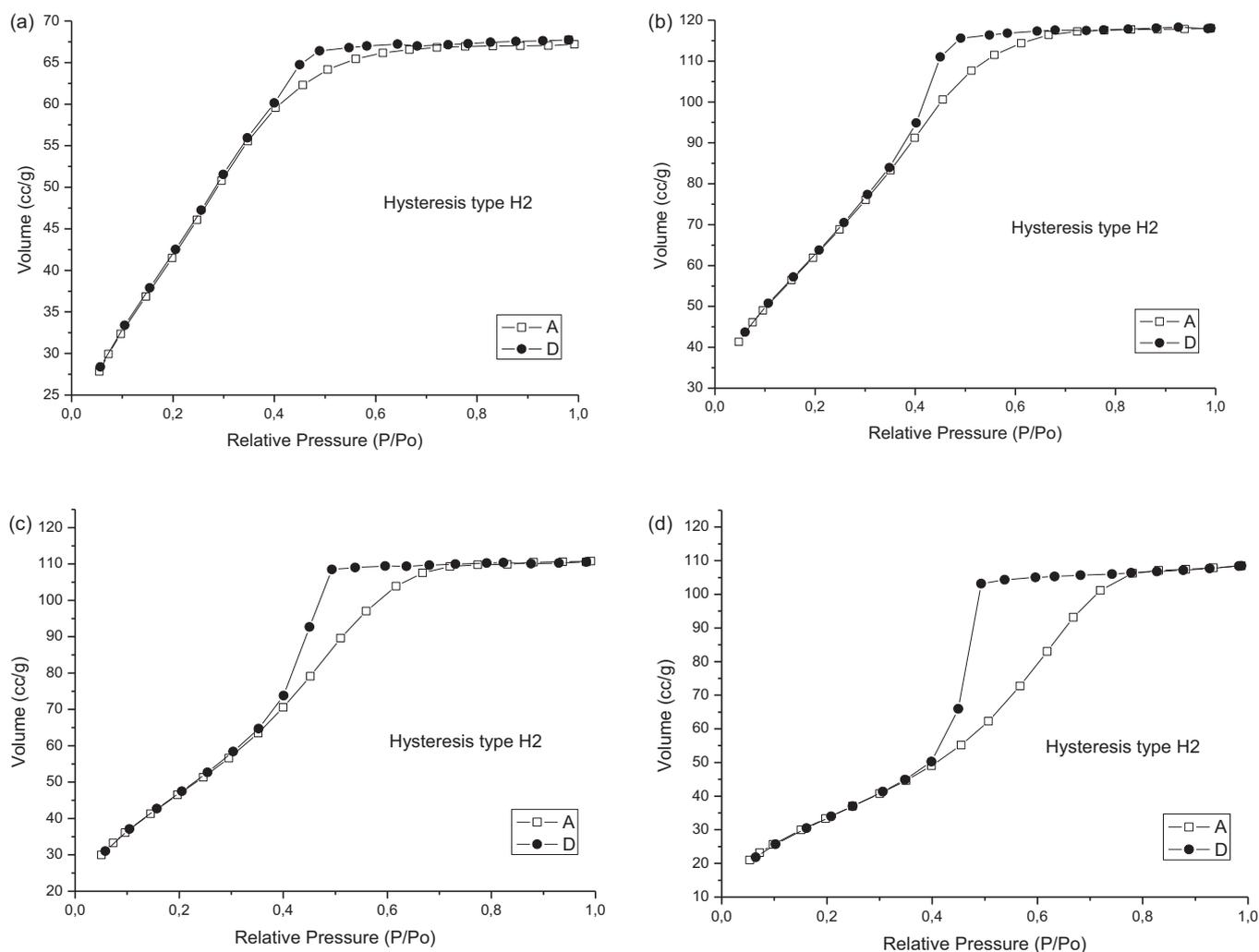


Fig. 3. Nitrogen adsorption–desorption isotherms of the pure silica gel and immobilized samples: (a) EN-C<sub>5</sub>-0.5, (b) EN-C<sub>5</sub>-1.0, (c) EN-C<sub>5</sub>-2.0, and (d) EN-C<sub>5</sub>-3.0.

with final dehydroxylation reactions and definitive carbonisation of organic compounds, including the lipase [24]. In the range above 750 °C, either the thermal stability of the material is reached or it completely breaks down, as occurred with the sample of free biocatalyst.

Therefore, the lower values obtained for the weight loss associated with the lipase of *B. cepacia* encapsulated derivatives are the result of an increased matrix thermal stability resulting from interactions among silane precursors and organic components (lipase and ionic liquids), as observed by Soares et al. [24].

### 3.3.3. Differential scanning calorimetry – DSC

Differential scanning calorimetry, or DSC, is a technique in which a sample is subjected to controlled heating, and heat flow in and out of the sample is measured. Through this technique, phase transitions in different materials can be studied. These transitions

give rise to endothermic or exothermic peaks over the temperature range in a DSC scan.

The sample containing lipase from *B. cepacia* as the free enzyme (Fig. 6a) showed a first endothermic peak temperature of 98 °C and an enthalpy of 177.7 J g<sup>-1</sup>; the other peaks were less significant and were associated with decomposition of organic matter and loss of water. The sample of pure silica (PS) showed only one endothermic transition, with a peak temperature of 65 °C and an enthalpy of 364.3 J g<sup>-1</sup>. Comparing the immobilized samples with additives C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub> to the sample without additive (EN-AIL) presented the results of changes in temperature of the exothermic peaks, since the increase in the number of carbon atoms of the ionic liquids in the process of immobilization increased the temperature of the exothermic peaks of the samples (EN-C<sub>2</sub> = 364.9 °C, EN-C<sub>3</sub> = 392.5 °C EN-C<sub>4</sub> = 404.1 °C and EN-C<sub>5</sub> = 443.2 °C). These DSC curves are shown in Fig. 6(a).

Table 5

Total loss of mass of the pure silica samples, free enzyme and immobilized of lipase from *B. cepacia*.

Samples	Total loss of mass (%)	Samples	Total loss of mass (%)	Samples	Total loss of mass (%)
Biocatalyst free	99.1	EN-C <sub>2</sub>	38.1	EN-C <sub>5</sub> -0.5	37.1
Pure silica	22.6	EN-C <sub>3</sub>	40.8	EN-C <sub>5</sub> -1.0	41.4
EN-AIL	45.1	EN-C <sub>4</sub>	36.6	EN-C <sub>5</sub> -2.0	42.9
		EN-C <sub>5</sub>	41.4	EN-C <sub>5</sub> -3.0	46.5

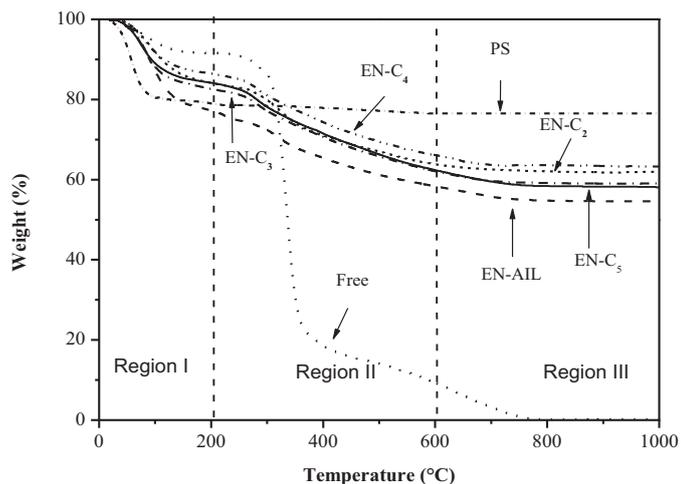


Fig. 4. Thermogravimetric curve of samples with the biocatalyst free (Free), without additive (EN-AIL) and immobilized with ionic liquid EN-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub> at 20 °C min<sup>-1</sup> under nitrogen atmosphere.

However, the main change caused by the increase in the concentration of IL-C<sub>5</sub> immobilized in the matrix is related to the exothermic peak, which increased the energy of enthalpy of these peaks (EN-C<sub>5</sub>-0.5 = 233.3 J g<sup>-1</sup>, EN-C<sub>5</sub>-1.0 = 277.4 J g<sup>-1</sup>, EN-C<sub>5</sub>-2.0 = 287.5 J g<sup>-1</sup>, EN-C<sub>5</sub>-3.0 = 351.2 J g<sup>-1</sup>). This behaviour could be associated with the loss of water still present in the sample by means of so-called “water of hydration,” and strongly present in the lattice of fixed assets, since it is possible that water can be

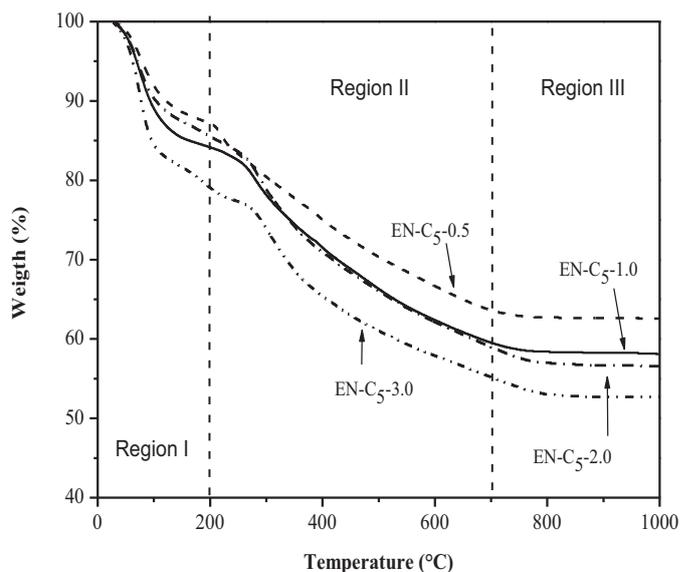


Fig. 5. TG curve of samples immobilized with C<sub>5</sub> ionic liquid at 20 °C min<sup>-1</sup> under nitrogen atmosphere.

maintained on the surface of silica gel by different forces of interaction: “dispersive forces” (e.g., physically connected water), “polar forces” (e.g., hydrogen from water ions), or by “chemical forces” (e.g., silanol groups that condense siloxane bonds with the release of water) [24]. This fact contributed to the choice of additives with the ionic and hydrophobic properties studied in this work, as well

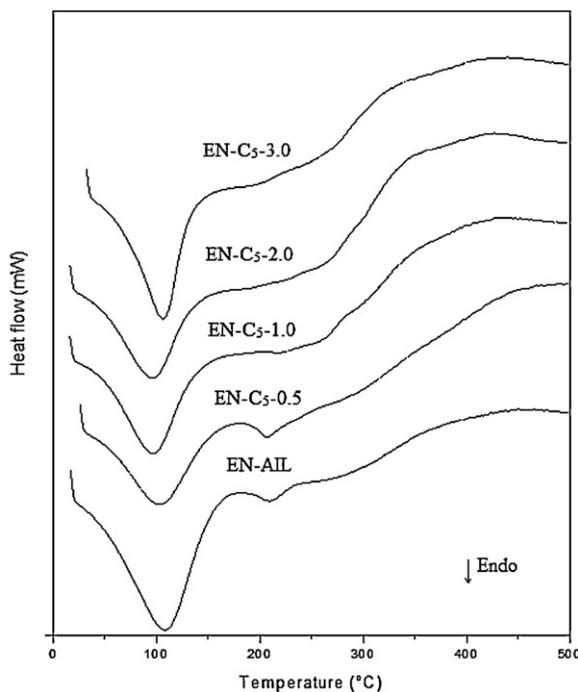
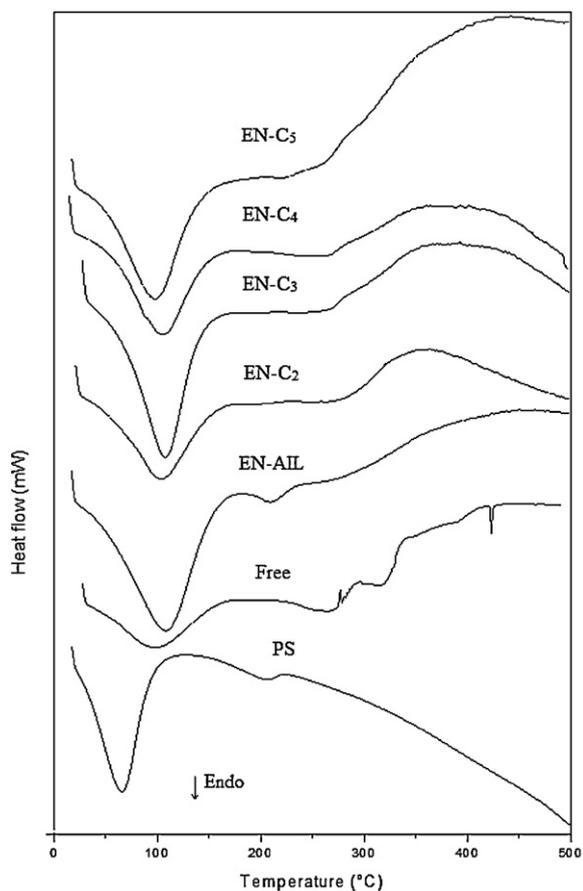


Fig. 6. DSC curves at 10 °C min<sup>-1</sup> under nitrogen atmosphere of samples: (a) pure silica (PS), biocatalyst free (free), absence of IL (AIL) and EN-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub> and (b) immobilized in different concentrations of IL-C<sub>5</sub>.

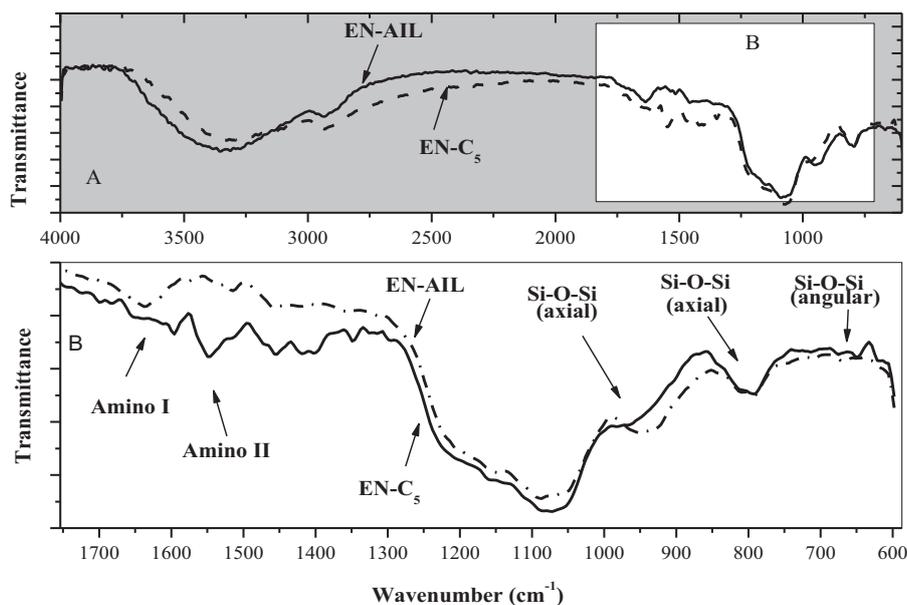


Fig. 7. FTIR spectra for the immobilized lipase from *B. cepacia* on silica gel in the absence and presence of protic ionic liquid (EN-AIL and EN-C<sub>5</sub>-1.0, respectively).

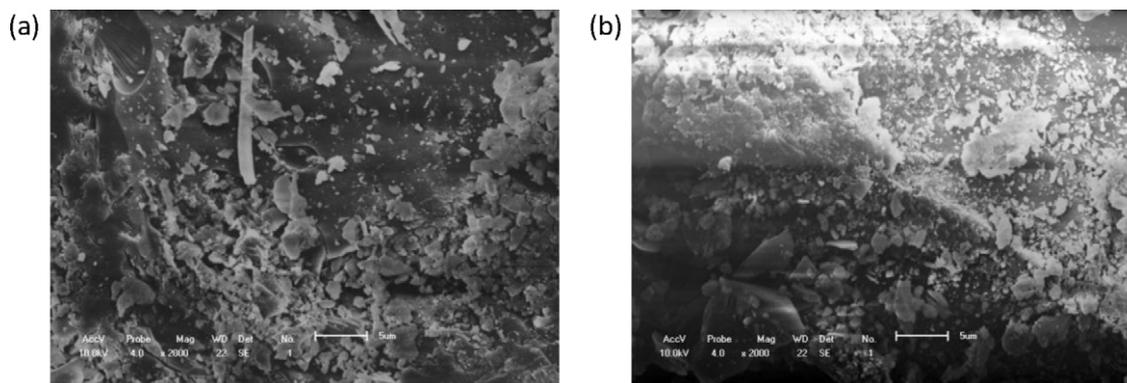


Fig. 8. Scanning electron micrographs for (a) EN-AIL ( $\times 2000$ ) and (b) EN-C<sub>5</sub>-1.0 ( $\times 2000$ ).

as the influence of hydration layers and the strength of interactions between the enzyme and the support (Fig. 6b).

### 3.3.4. Fourier transform infrared spectroscopy – FTIR analysis

When samples of the *B. cepacia* lipase encapsulated in either the absence (EN-AIL) or presence (EN-C<sub>5</sub>-1.0) of additive were characterized by FTIR, both samples showed the same characteristic FTIR spectrum, with bands at 650 cm<sup>-1</sup> (Si–O–Si silica), 800 cm<sup>-1</sup> (Si–O–Si silica) and 950 cm<sup>-1</sup> (Si–O–Si silica) (Fig. 7). As described in the literature [24], the lipase has two characteristic bands at 1600 and 1650 cm<sup>-1</sup> (primary and secondary amino groups) as exhibited in Fig. 7. Those bands are also displayed in the spectra for the immobilized derivatives, revealing the presence of primary and secondary amino groups (lipase), particularly in samples EN-AIL and EN-C<sub>5</sub>-1.0; they are more evident in sample EN-C<sub>5</sub>-1.0, probably due to the positive effect of the ionic liquid.

The consistency of the results, which showed higher peak intensity amino (corresponding to the presence of enzymes) in the sample of immobilized lipase sol gel employing PIL as an additive, compared to the sample in the absence of additive (EN-AIL), can be verified by observing the values for conversion of ethyl esters seen in Table 3, where the results show higher values for samples immobilized using the ionic liquid. This same effect can also be verified with regard to the analysis of enzymatic activity, previously seen in Table 2.

### 3.3.5. Scanning electron microscopy (SEM)

SEM micrographs of the pure silica gel and immobilized lipases, in the presence or absence of ionic liquid, are shown in (Fig. 8a and b). Conditions of porosity, surface area and pore volume in gels containing lipase activity resulted in a favourable environment for the catalytic reactions. The SEM studies only deliver information regarding the general morphology of the particles, and not on the actual conformation of the internal porous structure, but that was confirmed by analysis of nitrogen adsorption. It can be observed that matrix immobilized in the absence of additive showed low surface porosity (Fig. 8a). On the other hand, the encapsulated lipase in the presence of protic ionic liquid (Fig. 8b) showed a surface that appears to be more porous.

## 4. Conclusions

The lipase from *B. cepacia* immobilized by a sol–gel was successfully encapsulated technique in the presence of protic ionic liquids. Sol–gels produced in the presence of PIL, in general, showed total activity yields of more than 1000% compared with the immobilized enzyme without additive. However, the number of carbon atoms characteristic of each IL was the key determining factor for these results, since the immobilized lipase together with the most hydrophobic PIL (C<sub>5</sub>) at a concentration of 1.0% (w/v) showed the best total activity recovery yield representing an increase of 35

times compared with a sample absent PIL, an increase in surface area ( $245 \text{ m}^2 \text{ g}^{-1}$ ) and presented 46.2% conversion of triglycerides into ethyl esters. Therefore, a positive effect on the morphological structure and interactions in the biocatalytic immobilized catalyst in the presence of a protic ionic liquid was observed with increasing hydrophobic character, making it an important additive for immobilization, since the ease of synthesis, their lower ecotoxicity, low cost and different applications of this IL new family, favour industrial applications.

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## References

- [1] Kose O, Tuter M, Aksoy HA. Immobilized *Candida antarctica* lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. *Bioresource Technology* 2002;83:125–9.
- [2] Soares CMF, Santos OA, Castro HF, Moraes FF, Zanin GM. Characterization of sol-gel encapsulated lipase using tetraethoxysilane as precursor. *Journal of Molecular Catalysis B: Enzymatic* 2006;39:69–76.
- [3] Chang SW, Shaw JF, Yang KH, Chang SF, Shieh CJ. Studies of optimum conditions for covalent immobilization of *Candida rugosa* lipase on poly( $\gamma$ -glutamic acid) by RSM. *Bioresource Technology* 2008;99:2800–5.
- [4] Souza RL, Barbosa JMP, Zanin GM, Lobão MWN, Soares CMF, Lima AS. Partitioning of porcine pancreatic lipase in a two-phase systems of polyethylene glycol/potassium phosphate aqueous. *Applied Biochemistry and Biotechnology* 2010;161:288–300.
- [5] Barbosa JMP, Souza RL, Fricks AT, Zanin GM, Soares CMF, Lima AS. Purification of lipase produced by a new source of *Bacillus* in submerged fermentation using an aqueous two-phase system. *Journal of Molecular Catalysis B: Enzymatic* 2011;879:3853–8.
- [6] Bon EPS, Maria A, Ferrera MA, Corvo ML, Vermelho AB, Paiva CLA, et al. *Enzimas em biotecnologia: produção, aplicação e mercado*. ed. Rio de Janeiro: Interciência; 2008.
- [7] Brady D, Jordaan J. Advances in enzyme immobilisation. *Biotechnology Letters* 2009;3:1639–50.
- [8] Cowan DA, Fernandez-Lafuente R. Enhancing the functional properties of thermophilic enzymes by chemical modification and immobilization. *Enzyme and Microbial Technology* 2011;49:326–46.
- [9] Yang P, Teo WK, Ting YP. *Bioresource Technology* 2006;97:39–46.
- [10] Noureddini H, Gao X, Philkana RS. Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. *Bioresource Technology* 2005;96:769–77.
- [11] Hernandez K, Fernandez-Lafuente R. Control of protein immobilization: coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. *Enzyme and Microbial Technology* 2011;48:107–22.
- [12] Huang XJ, Yu AG, Xu ZK. Covalent immobilization of lipase from *Candida rugosa* onto poly(acrylonitrile-co-2-hydroxyethyl methacrylate) electrospun fibrous membranes for potential bioreactor application. *Bioresource Technology* 2008;99:5459–65.
- [13] Reetz MT, Zonta A, Simpelkamp J, Könen W. Efficient immobilization of lipase by entrapment in hydrophobic sol-gel materials. *Journal of Sol-Gel Science and Technology* 1996;49:1397–8.
- [14] Guisan JM. *Immobilization of enzymes and cells*. 2nd ed. Madrid: Humana Press; 2006.
- [15] Hara P, Mikkola J-P, Murzin DY, Kanerva LT. Supported ionic liquids in *Burkholderia cepacia* lipase-catalyzed asymmetric acylation. *Journal of Molecular Catalysis B: Enzymatic* 2010;67:129–34.
- [16] Lee SH, Doan TTN, Ha SHH, Chang WJ. Influence of ionic liquids as additives on sol-gel immobilized lipase. *Journal of Molecular Catalysis B: Enzymatic* 2007;47:129–34.
- [17] Zarcula C, Corfci L, Croitoru R, Ursoiu A, Peter F. Preparation and properties of xerogels obtained by ionic liquid incorporation during the immobilization of lipase by the sol-gel method. *Journal of Molecular Catalysis B: Enzymatic* 2010;65:79–86.
- [18] Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology* 2007;40:1451–63.
- [19] Álvarez VH, Mattedi S, Martin-Pastord M, Aznarb M, Iglesias M. Synthesis and thermophysical properties of two new protic long-chain ionic liquids with the oleate anion. *Fluid Phase Equilibria* 2010;299:42–50.
- [20] Álvarez VH, Dosil N, Gonzalez-Cabeleiro R, Mattedi S, Martin-Pastord M, Iglesias M, et al. Brønsted ionic liquids for sustainable processes: synthesis and physical properties. *Journal of Chemical Engineering* 2010;55:625–32.
- [21] Ríos AP, Rantwijk F, Sheldon RA. Effective resolution of 1-phenyl ethanol by *Candida antarctica* lipase B catalysed acylation with vinyl acetate in protic ionic liquids (PILs). *Green Chemistry* 2012;14:1584–8.
- [22] Greaves TL, Drummond CJ. Protic ionic liquids: properties and applications. *Chemical Reviews* 2008;108:206–37.
- [23] INPI Patent submission No. PI0306829-3, 11 September 2003.
- [24] Soares CMF, Santos OA, Castro HF, Moraes FF, Zanin GM. Influence of the alkyl-substituted silane precursor on sol-gel encapsulated lipase activity. *Applied Biochemistry and Biotechnology* 2004;113:307–19.
- [25] Brunauer S, Emmett PH, Teller E. Adsorption of gases in multimolecular layers. *Journal of the American Chemical Society* 1938;60:309–19.
- [26] Moniruzzaman M, Nakashima K, Kamiya N, Goto M. Recent advances of enzymatic reactions in ionic liquids. *Biochemical Engineering Journal* 2010;48:295–314.
- [27] Zhao H. Methods for stabilizing and activating enzymes in ionic liquids – a review. *Journal of Chemical Technology and Biotechnology* 2010;85:891–907.
- [28] Rodrigues RC, Ortiz C, Berenguer-Murcia A, Torres R, Fernández-Lafuente R.  $\alpha$ -Modifying enzyme activity and selectivity by immobilization. *Chemical Society Reviews* 2013; on line.
- [29] Vila-Real H, Alfaia AJ, Rosa JN, Gois PMP, Rosa ME, Calado ART, et al.  $\alpha$ -Rhamnosidase and  $\beta$ -glucosidase expressed by naringinase immobilized on new ionic liquid sol-gel matrices: activity and stability studies. *Journal of Biotechnology* 2011;152:147–58.
- [30] Ríos AP, Hernández-Fernández FJ, Martínez FA, Rubio M, Villora G. The effect of ionic liquid media on activity, selectivity and stability of *Candida antarctica* lipase B in transesterification reactions. *Biocatalysis and Biotransformation* 2007;25:151–6.
- [31] Gamba M, Lapis AAM, Dupont J. Supported ionic liquid enzymatic catalysis for the production of biodiesel. *Advanced Synthesis and Catalysis* 2008;350:160–4.
- [32] Ha SH, Lan MN, Lee SH, Hwang SM, Koo YM. Lipase-catalyzed biodiesel production from soybean oil in ionic liquids. *Enzyme and Microbial Technology* 2007;41:480–3.
- [33] Fernandez-Lafuente R. Stabilization of multimeric enzymes: strategies to prevent subunit dissociation. *Enzyme and Microbial Technology* 2009;45:405–18.
- [34] Filho M, Pessela BC, Mateo C, Carrascosa AV, Fernandez-Lafuente R, Guisán JM. Reversible immobilization of a hexameric  $\alpha$ -galactosidase from *Thermus* sp. strain T2 on polymeric ionic exchangers. *Process Biochemistry* 2008;43:1142–6.
- [35] Pessela BCC, Fernández-Lafuente R, Fuentes M, Vián A, García JL, Carrascosa AV, et al. Reversible immobilization of a thermophilic  $\beta$ -galactosidase via ionic adsorption on PEI-coated Sepabeads. *Enzyme and Microbial Technology* 2003;32:369–74.
- [36] Mateo C, Abian O, Fernandez-Lafuente R, Guisan JM. Reversible enzyme immobilization via a very strong and nondistorting ionic adsorption on support-polyethylenimine composites. *Biotechnology and Bioengineering* 2000;68:98–105.
- [37] Bolivar JM, Wilson L, Ferrarotti SA, Fernandez-Lafuente R, Guisan JM, Mateo C. Evaluation of different immobilization strategies to prepare an industrial biocatalyst of formate dehydrogenase from *Candida boidinii*. *Enzyme and Microbial Technology* 2007;40:540–6.
- [38] Lopez-Gallego F, Betancor L, Hidalgo A, Dellamora-Ortiz G, Mateo C, Fernández-Lafuente R, et al. Stabilization of different alcohol oxidases via immobilization and post immobilization techniques. *Enzyme and Microbial Technology* 2007;40:278–84.
- [39] Souza RL, Resende WCS, Barão CE, Zanin GM, Castro HF, Santos OAA, et al. Influence of the use of Aliquat 336 in the immobilization procedure in sol-gel of lipase from *Bacillus* sp. ITP-001. *Journal of Molecular Catalysis B: Enzymatic* 2012;84:152–9.
- [40] Das S. Immobilization of enzymes in sol-gel mesoporous silica, enzymatic digestion of biomass, and silica-curcumin hybrid materials. Thesis, the Faculty of Drexel University, March 2011.
- [41] Oscik J. *Adsorption*. New York: Wiley; 1982.
- [42] Guan-Sajonz H, Guiochon G, Davis E, Gulakowski K, Smith DW. Study of the physico-chemical properties of some packing materials: III. Pore size and surface area distribution. *Journal of Chromatography A* 1997;773:33–51.
- [43] Mukherjee I, Mylonakis A, Guo Y, Samuel SP, Li S, Wei RY, et al. Effect of nonsurfactant template content on the particle size and surface area of monodisperse mesoporous silica nanospheres. *Microporous and Mesoporous Materials* 2009;122:168–74.
- [44] Wei Y, Xu J, Dong H, Dong JH, Qiu K, Jansen-Varnum SA. Preparation and physisorption characterization of D-glucose-templated mesoporous silica materials via base-catalyzed sol-gel process. *Chemistry of Materials* 1999;11:2023–9.