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Journal of Molecular Catalysis B: Enzymatic 29 (2004) 69–79
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Influence of the alkyl-substituted silane precursor on sol–gel encapsulated lipase activity

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Received 3 July 2003; received in revised form 16 February 2004; accepted 16 February 2004

Available online 22 April 2004

Abstract

Lipase from *Candida rugosa* was encapsulated within a chemically inert sol–gel support prepared by polycondensation of three precursor types (tetraethoxysilane (TEOS), methyltrimethoxysilane (MTMS) and polydimethylsilane (PDMS)) in the presence and absence of polyethylene glycol (PEG) and polyvinyl alcohol (PVA) as additives. Silica and their derivatives were characterised with regard to mean pore diameter, specific surface area, pore size distribution (BET method), weight loss upon heating thermogramivemetric analysis (TGA), chemical composition Fourier transform infrared spectroscopy (FT-IR), and catalytic activities. Immobilisation yields based on the recovered lipase activity vary from 3.02 to 31.98% and the highest efficiency was attained when lipase was encapsulated using TEOS in the presence of the PEG. Further information was obtained by testing the derivatives in esterification reactions and a different reactivity profile was found. Better performance was obtained with derivatives containing lipase encapsulated within gels prepared with MTMS as precursor in the presence of PEG. This lipase preparation exhibits increased esterification activity (155 μmol g^{-1} min^{-1}), up to of three times greater than that prepared with TEOS (52 μmol g^{-1} min^{-1}), and almost twice that prepared with MTMS/PDMS (89 μmol g^{-1} min^{-1}) as precursors.
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Keywords: Lipase; Sol–gel; Entrapped lipase; Silane; Additive

1. Introduction

Lipases are enzymes that catalyses a variety of reactions, such esterifications, interesterification and hydrolysis. Because of their selectivity, lipases are important biocatalysts in several applications, such as the synthesis of chiral drug intermediates and nutraceutical lipids [1–3]. To further expand their synthetic utility, efficient methods of immobilisation for lipases are needed as immobilisation promotes enzyme reuse and thus reduces overall process cost [4–6]. Several methods have been reported for the immobilisation of lipases, such as deposition onto solid supports, covalent binding and encapsulation within a polymer matrix or silica glasses obtained by sol–gel techniques [3,7–10]. Using sol–gel materials for mechanical entrapment of enzymes allowed stabilisation of the proteins tertiary structure because of the tight gel network [11,12].

The sol–gel process involves the transition of a system from a liquid "sol" (mostly colloidal) into a solid "gel" phase. By applying the sol–gel process, 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 fibres, microporous inorganic membranes, monolithic ceramics and glasses, or extremely porous aerogel materials [6,12,13]. Sol–gel technique includes hydrolysis and condensation of metal alkoxides in adequate solvents followed by thermal treatment. The biomaterial to be encapsulated is added to sol after partial hydrolysis of the alkoxides. Typical laboratory procedure includes hydrolysis and polycondensation of tetraethoxysilane or tetramethoxysilane catalysed by an acid. After partial evaporation of generated alcohol the prepolymer is cooled and the biological material is introduced in a suitable buffer. By this method it is essential to prepare a homogeneous solution contain-

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doi:10.1016/j.molcatb.2004.02.014

ing the catalytic precursor in order to uniform coagulate the solution.

Since the precursors are extremely different in relation to the rate of polymerisation, it is necessary to test them with respect to their reactive levels in polar solvents to form the matrix, or to increase their condensation rate, with the addition of an acid or base [14–16]. Moreover, the easy insertion of substituent groups into silicate matrix may provide entrapped enzymes with beneficial microenvironments [9].

Systems already tested for lipases from either microbial or animal sources include the utilisation of different precursors (tetramethoxysilane, methyltrimethoxysilane (MTMS), ethyltrimethoxysilane, polydimethylsilane (PDMS), and others), stabilising additives (alcohol polyvinyl, albumin, gelatine and others), and solvents (methanol, ethanol and others) [9,13–15,17].

The sol–gel technique was used in this work to generate silica matrices by acid or base-catalysed hydrolysis of silane compounds as tetraethoxysilane (TEOS), methyltrimethoxysilane, and polydimethylsilane in the presence and absence of additives such as polyethyleneglycol (PEG) and polyvinylacohol (PVA). For the precursor TEOS a novel methodology was used based on a procedure that consisted in dissolving TEOS in ethanol followed by condensation with ammonium solution. This was a modified methodology previous established for a chemical catalyst [12] and to our knowledge it has not been used before for encapsulating the enzyme lipase. For the gel precursors MTMS and PDMS, a typical immobilisation procedure as described by Reetz et al. [9] was used in order to have a comparison profile with the results obtained in the present study. The support and immobilised derivatives were characterised with respect to their catalytic activities in aqueous and non-aqueous media, and morphologic properties, i.e. mean pore diameter, surface area and pore size distribution.

2. Experimental procedures

2.1. Enzyme and chemicals

Commercial *Candida rugosa* lipase (Type VII—Product No. L1754) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). This lipase is substantially free of α-amylase and protease, and contains lactose as an extender. Nominal specific lipase activity was 104.94 U mg^{-1} protein. Polyethyleneglycol (PEG, MW 1450, Merck) and polyvinylalcohol (PVA, MW 15,000, Sigma) were used as stabilising agents. The silane precursors: tetraethoxysilane (TEOS), methyltrimethoxysilane (MTMS) and polydimethylsilane (PDMS) were supplied by Across Organic (NJ, USA) and used without further purification. Ethanol (minimum 99%), ammonia (minimum 28%), hydrochloric acid (minimum 36%) and gum Arabic were from Synth (São Paulo, Brazil). Olive oil (low acidity) was purchased at a local market. Water was purified by reverse osmosis and deionised through a Milli-Q four-cartridge organic-free water purification system. Other chemicals were of analytical grade and used as received.

2.2. Encapsulation of lipase from Candida rugosa (CRL) in sol–gel matrices

2.2.1. Matrices prepared with tetraethoxysilane (TEOS)

The methodology previously established by Dudoit et al. [12] was used with slight modifications as follows: 30 ml of TEOS were dissolved in 36 ml of absolute ethanol under nitrogen inert atmosphere. To this, 0.22 ml of hydrochloric acid dissolved in 5 ml of ultra-pure water was slowly added and the mixture agitated (200 rpm) for 90 min at 35 °C. Then, 10 ml of lipase solution (18.29 mg ml^{-1}), PEG solution (5 mg ml^{-1}, 8 ml added) and 1 ml of ammonium hydroxide dissolved in 6 ml of ethanol were added (hydrolysis solution) and the mixture was kept under static conditions for 24 h to complete the chemical condensation. The bulk gel was washed with heptane and acetone and dried under vacuum at room temperature for 24 h. *Candida rugosa* lipase (CRL) was entrapped in silicate sol–gel prepared by hydrolysis of TEOS in the absence and presence of PEG, and the dried gels were crushed to a powder with particle size in the range of 180–250 μm, resulting in the encapsulated lipase in the absence of additive (EN-TEOS) and in the presence of additive (EN1-TEOS) derivatives (Table 1).

2.2.2. Matrices prepared with methyltrimethoxysilane (MTMS)

A modification of a published method [9] was used. CRL was suspended in water (13.55 mg ml^{-1}), shaken for 15 min and centrifuged to remove insoluble components. Ten millilitres of the supernatant of lipase solution was added to a mixture containing aqueous sodium fluoride (1 M, 1 ml added), PVA (4% (w/w) in water, 2 ml added) or PEG (4% (w/w) in water, 2 ml added) and water (1.64 ml). The solution was shaken and MTMS (8.57 ml) was added. The reaction mixture was vigorously shaken for 5 s on a vortex mixer and gently shaken by hand. After 30 s, when the mixture formed a clear homogeneous solution and warmed up, it was placed in an ice bath until gelation occurred (1 h). The reaction vessel was left to stand closed for 24 h, then it was opened and the gel was dried under vacuum at room temperature for 3 days. The bulk gel was washed with water, acetone and pentane and dried as described in Section 2.2.1. CRL was entrapped in silicate sol–gel monoliths prepared by hydrolysis of MTMS in the absence and presence of PEG or PVA, and the dried gels were crushed to a powder with particle size in the range of 180–250 μm, resulting in the encapsulated lipase in the absence of additive (EN-MTMS), in the presence of PEG (EN1-MTMS) and in the presence of PVA (EN2-MTMS) derivatives (Table 1).

Table 1
Characterisation of the pure silica gel matrix and immobilised derivatives

Precursor	Samples	Surface area[a] ($m^2 g^{-1}$)	Mean pore diameter[a] (Å)	Pore volume[a] ($cm^3 g^{-1}$)
TEOS	PS	607	18.23	0.37
	EN	382	49.99	0.48
	EN1	348	56.59	0.43
MTMS	PS	173	59.08	0.25
	EN	1.33	144.50	0.005
	EN1	0.27	784.70	0.004
	EN2	239	149.90	0.009
MTMS/PDMS	PS	305	21.27	0.002
	EN	430	36.05	0.004
	EN1	3.62	30.99	0.003
	EN2	2.88	51.41	0.004

[a] Evaluated from BET.

2.2.3. Matrices prepared with methyltrimethoxysilane and polydimethylsilane (MTMS/PDMS)

A modification of a published method [9] was used for encapsulating CRL in a sol–gel derived from MTMS/PDMS as follows: *Candida rugosa* lipase was suspended in water (13.60 mg ml^{-1}), shaken for 15 min and centrifuged to remove insoluble components. The supernatant was added to a mixture of aqueous sodium fluoride (1 M, 1 ml added), PVA (4% (w/w) in water, 2 ml added) or PEG (4% (w/w) in water, 2 ml added) and water (0.67 ml). The solution was shaken and PDMS (4.34 ml) was added followed by MTMS (6.43 ml). The reaction mixture was vigorously shaken for 5 s on a vortex mixer and gently shaken by hand. After about 30 s, when the mixture formed a clear homogeneous solution and warmed up, it was placed in an ice bath until gelation occurred (1 h). The reaction vessel was left to stand closed for 24 h, then it was opened and the gel was dried under vacuum at room temperature for 3 days. The bulk gel was washed with water, acetone and pentane and dried as described in Section 2.2.1. CRL was entrapped in silicate sol–gel monoliths prepared by hydrolysis of MTMS/PDMS in the absence and presence of PEG or PVA, and the dried gels were crushed to a powder with particle size in the range of 180–250 μm, resulting in the encapsulated lipase in the absence of additive (EN-MTMS), in the presence of PEG (EN1-MTMS/PDMS) and in the presence of PVA (EN2-MTMS/PDMS) derivatives (Table 1).

2.3. Activity of lipase in the hydrolysis of emulsified olive oil

Hydrolytic activities of free and immobilised lipase were assayed by the olive oil emulsion method according to the modification proposed by Soares et al. [17]. 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 emulsion, 4 ml of 100 mM sodium phosphate buffer (pH 7.0) and either free (1 ml, 5 mg ml^{-1}) or immobilised (100–250 mg) lipase was incubated for 5 min at 37 °C. The reaction was stopped by the addition of 10 ml of acetone–ethanol solution (1:1). The liberated fatty acids were titrated with 25 mM potassium hydroxide solution in the presence of phenolphthalein as an indicator. 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^{-1}) under the assay conditions (37 °C, pH 7.0, 150 rpm). Analyses of hydrolytic activities carried out on the lipase loading solution and immobilised preparations were used to determine the coupling yield (η, %) according to

$$\eta\,(\%) = \frac{U_s}{U_0} \times 100 \quad (1)$$

in which U_s is the total enzyme activity recovered on the support, and U_0 is the enzyme units offered for immobilisation.

2.4. Activity of immobilised lipase in the esterification of butanol with butyric acid

Reaction systems consisted of heptane (20 ml), *n*-butanol (300 mM), butyric acid (300 mM) and immobilised lipase in sol–gel matrices (1.0 g, dry weight). The mixture was incubated at 37 °C for 24 h with continuous agitation at 150 rpm. The butanol consumed and product formed were determined by gas chromatography using a 6 ft 5% DEGS on Chromosorb WHP, 80/10 meshes column (Hewlett-Packard, Palo Alto, CA, USA) and hexanol was used as an internal standard. Esterification activity was expressed as micromole of butyl butyrate formed per minute per gram of dry support (μmol g^{-1} min^{-1}).

2.5. Surface area determinations

The surface area measurements were performed by adsorption, using nitrogen as the adsorbate. The samples were previously degassed to below 50 mmHg at room temperature and the analyses were performed at 77 K, using liquid nitrogen. The equilibrium interval was 5 s. The surface area was calculated using the Brunauer–Emmett–Teller (BET) method. Pore volume and superficial area distribu-

tions, based on the BJH calculations [18] were evaluated by the BET apparatus software (NOVA 1200-Quantachrome).

2.6. Thermogramivemetric analysis

The weight loss of the samples upon heating was determined in a TGA apparatus (TGA-50 Shimadzu–Thermogravimetric Analyzer) over the range 25–1000 °C, with a heating rate of 20 °C min^{-1}, using air as the purge gas.

2.7. Fourier transform infrared spectroscopy

The samples of free lipase (CRL), pure silica (PS), and immobilised derivatives were submitted to the FT-IR analysis (spectrophotometer FT-IR BOMEM MB-100). The spectra were obtained in the wavelength range from 400 to 4000 cm^{-1} for evaluation of the immobilisation procedures.

3. Results and discussion

As previous reported by Reetz et al. [9], the type of silane precursor used to prepare the hydrophobic matrices could affect the activity of the encapsulated lipase. In this study, lipase was encapsulated within sol–gel matrices derived from TEOS, MTMS and PDMS in the presence and absence of PEG or PVA. The derivatives were characterised with respect to their morphologic properties, i.e. mean pore diameter, surface area and pore size distribution, and used in the ester hydrolysis and synthesis. In control experiments it was demonstrated that the alkyl-substituted silanes themselves in the absence of lipase show essentially no activity. In the reining part of this, samples are designated by PS for pure silica, EN for encapsulated lipase in absence of additive, EN1 for encapsulated lipase in the presence of PEG, and EN2 for encapsulated lipase in the presence of PVA followed by the silane used (PS-TEOS, EN-TEOS, EN1-TEOS, PS-MTMS, EN-MTMS, EN1-MTMS, EN2-MTMS, PS-MTMS/PDMS, EN-MTMS/PDMS, EN1-MTMS/PDMS, EN2-MTMS/PDMS).

3.1. Surface area, pore volume and pore diameter

Table 1 shows the results with regard to the specific surface area (BET method) and mean pore diameter for sol–gel matrices and their derivatives. The BET surface areas were calculated from corresponding plots, giving correlation factors higher than 0.9995 and *C* values of 39–288, demonstrating the validity of this method.

Surface area and mean pore diameter results indicated a significant influence of the silane precursor and organic macromolecules additives (PEG or PVA) on the derivative properties. Larger pore sizes were obtained with TEOS as precursor (EN), and the addition of PEG resulted in an increase in the mean pore diameter for EN1-TEOS and EN1-MTMS derivatives. This can be associated mainly with the inhibition of gel contraction during the derivative synthesis. In contrast, encapsulated lipase in the presence of PVA gave a decrease in the mean pore size (EN2-MTMS). These results are in agreement with those described by Keeling-Tucker et al. [15], in which lipase from *Candida rugosa* was encapsulated into sol–gel-processed glasses derived from pure TEOS in the presence and absence of PEG or PVA. These authors showed that physical properties of encapsulated lipase samples improved with increasing PEG concentration, but deteriorated with addition of PVA.

It should be pointed out however that the very small pore volume obtained for the MTMS and MTMS/PDMS is conductive to incorrect mean pore diameter determinations, consequently Table 1 results for the mean pore diameter for MTMS and MTMS/PDMS pure silica and encapsulated derivatives (EN, EN1, EN2) are to be taken cautiously. In spite of that BET is an accepted methodology in the literature to determine porous structure parameters with sol–gel materials [16]. To reduce uncertainty in the mean pore diameter determinations in MTMS and MTMS/PDMS materials it was decided to use for future work either mercury porosimetry or thermoporometry.

A typical isotherm is shown in Fig. 1 (EN-TEOS). The stepped intrusion curve clearly denotes different volume size ranges. Furthermore, it can be observed that a considerable volume was intruded at relative low pressures, which is most certainly related to the filling of the small interparticle voids. This volume was thus excluded from the calculations of the surface area.

Values shown in Table 1 are in the same range as several published data for similar systems, although much higher or much lower values have been reported [16,18–20]. These discrepancies can be explained by the nature of the carrier surface (TEOS, MTMS and MTMS/PDMS). BET surface area measurements are based on the adsorption of N_2 molecules on the carrier surface, the bond length of an N_2 molecule being 1.1 Å, whereas lipase molecules have diameter one or two orders of magnitude lager than this. Consequently, there may be larger regions of the surface located in cavities in which protein cannot penetrate and thereby the binding available to the enzyme is reduced [19,20]. However, protein adsorption is not necessarily restricted to a monolayer cover on the carrier. Adsorption of secondary protein layers has been reported. This can be observed as a kink in the adsorption isotherm after an apparent saturation of the carrier (MTMS and MTMS/PDMS).

The gel pore size distributions reported in Fig. 1(a) and (b) were determined by the BJH method using the nitrogen adsorption isotherms, which appears to be favoured by workers, both because it corresponds to a more stable adsorbents condition and it correlates better with the enzyme immobilisation data [18,19].

Fig. 2 shows the pore size distribution of the pure silica samples (PS-TEOS, PS-MTMS, PS-MTMS/PDMS) and the immobilised derivatives in silica (EN-TEOS, EN1-TEOS, EN-MTMS, EN1-MTMS, EN2-MTMS, EN-MTMS/PDMS,

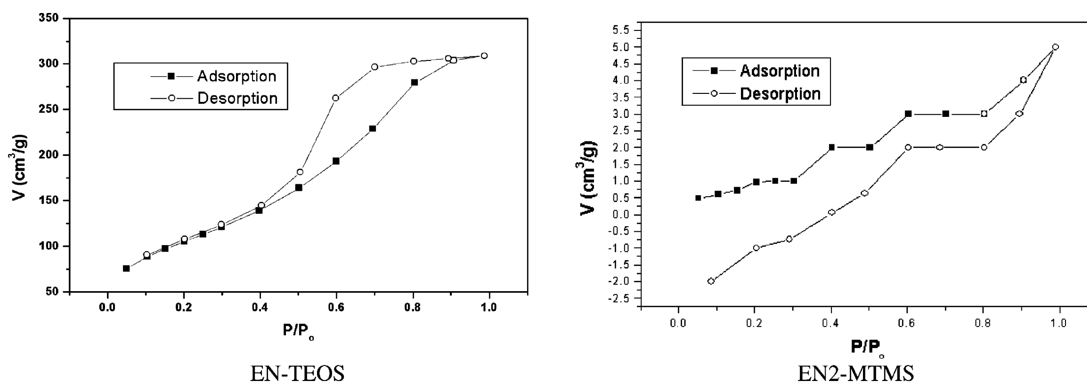


Fig. 1. Gas adsorption/desorption isotherm obtained for EN-TEOS and EN2-MTMS: adsorption (■); desorption (○).

PS-TEOS

EN-TEOS

EN1-TEOS

PS-MTMS

(a) EN-MTMS

EN1-MTMS

Fig. 2. Pore size distributions ($dV/d\log D$) of the pure silica gel (PS), and the immobilised derivatives in silica gels (EN: silica with entrapped lipase; EN1: silica with entrapped lipase in the presence of PEG; EN2: silica with entrapped lipase in the presence of PVA).

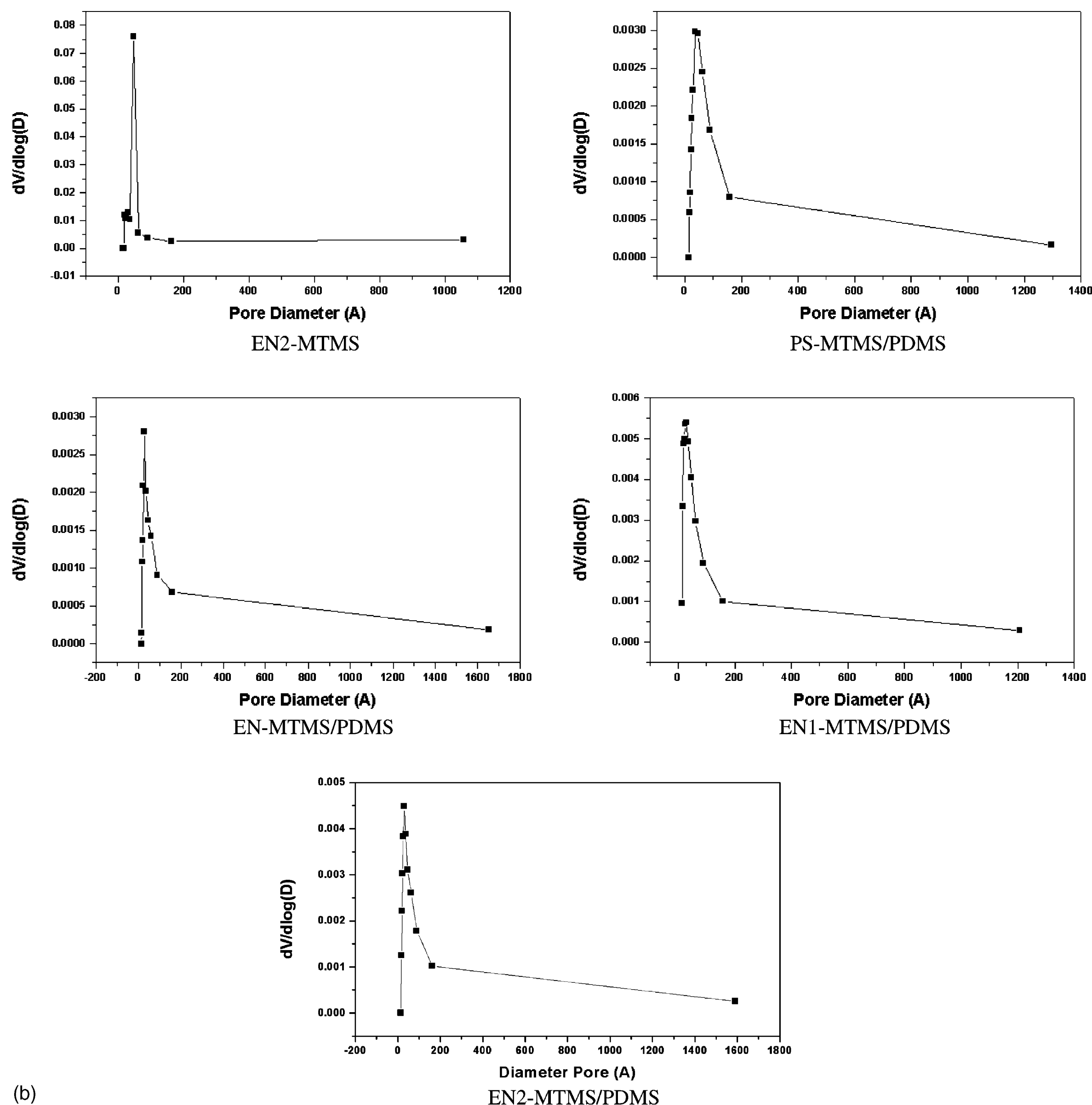


Fig. 2. (*Continued*).

EN1-MTMS/PDMS, EN2-MTMS/PDMS) on an incremental (derivative) basis, $dV/d\log D$, to highlight the differences between the samples. From these plots, the following conclusions can be withdrawn:

(1) Samples PS-TEOS, EN-TEOS, EN1-TEOS, PS-MTMS/PDMS and EN-MTMS/PDMS exhibit an unimodal distributions of pores, whose sizes are well whiting the size range measured by gas adsorption but are near the lower limit of detection of the porosimeter.
(2) With regard to samples EN1-MTMS, EN2-MTMS, EN1-MTMS/PDMS, EN2-MTMS/PDMS, it was noted that of the area measured by this technique probably was not accurate, because of the small pore size or small surface area of these samples. This may cause unexpected problems such as, for instance, pressure drops higher than those estimated assuming monomodal and narrowly distributed pore distribution samples.
(3) With regard to samples PS-MTMS, EN-MTMS and EN1-MTMS, a bimodal distribution outline of pore sizes was detected by gas adsorption technique. In this case, samples show two peaks, probably due to the methodology of preparation of the support and the immobilised derivatives (Fig. 2b).

The isotherms of the pure silica and immobilised derivatives samples are similar to those corresponding to the PS-MTMS, PS-MTMS/PDMS, EN-MTMS, EN1-MTMS, EN2-MTMS, EN-MTMS/PDMS, EN1-MTMS/PDMS, EN2-MTMS/PDMS (data not shown). This is confirmed by the lower values reported in Table 1 for both surface area and mean pore diameter of the samples

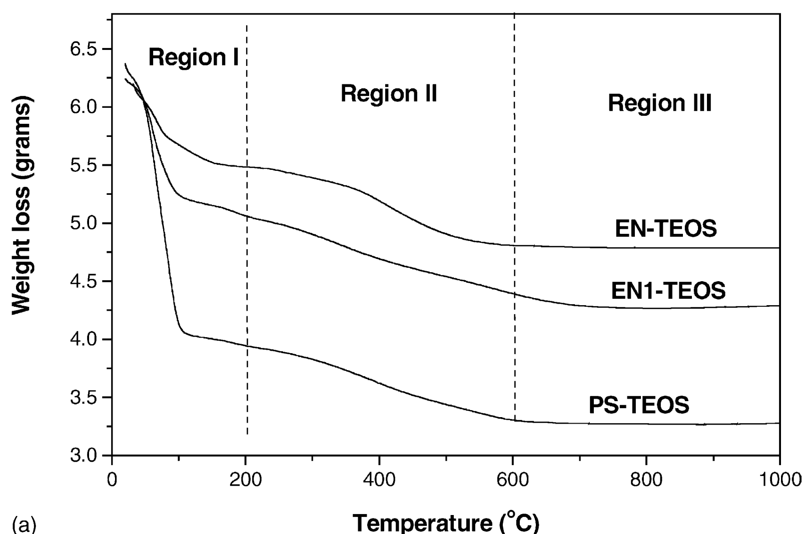
(PS-MTMS, PS-MTMS/PDMS, EN-MTMS, EN1-MTMS, EN2-MTMS, EN-MTMS/PDMS, EN1-MTMS/PDMS, EN2-MTMS/PDMS).

3.2. TGA results

Thermogravimetric analysis provides an important tool for thermal stability studies of macromolecules. This technique allows to determining the temperature range at which a heated sample undergoes a major conformational change by means of monitoring the thermal weight loss profile. To simplify the discussion about the thermogravimetric analysis, the weight loss curves were divided into three regions as shown in Fig. 3(a)–(c).

In Region I, TEOS derivatives exhibited a sharp weight loss (Fig. 3a) while MTMS (Fig. 3b) and MTMS/PDMS (Fig. 3c) derivatives showed a small weight loss, and this difference is attributed to TEOS leading to matrix structures that facilitates the loss molecular bound water. It is expected that up to end of Region I (200 °C) residual reactants from the preparation of pure silica and immobilised derivatives, and the adsorbed water molecules were eliminated.

In Region II, which extends from 200 to 600 °C, the weight loss was substantial, and it is attributed primarily to the removal of water by dehydroxylation and some loss of organic constituents (C, H, O and N) in the form of volatiles either present or formed by the beginning of organics decomposition, including lipase. According to Brinker and Scherer [21], in this region dehydroxylation reactions occur leading to structural relaxation, a process that takes place by diffusive motion of the network and removes excess free volume.



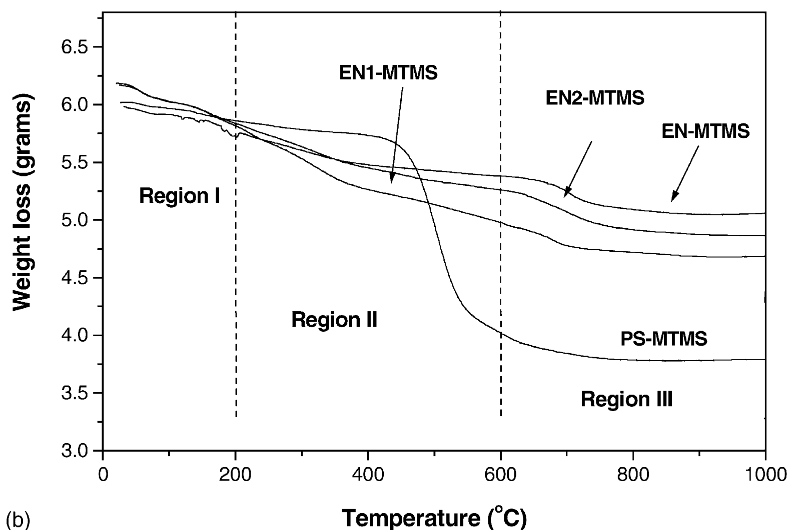


Fig. 3. Thermogravimetric curves for silica gels and immobilised derivatives. Three regions are indicated corresponding to (I) primarily weight loss, (II) shrinkage proportional to weight loss, and (III) primarily shrinkage for the immobilised derivatives in silica gels (EN-TEOS, EN1-TEOS, EN-MTMS, EN1-MTMS, EN2-MTMS, EN-MTMS/PDMS, EN1-MTMS/PDMS) and pure silica gels (PS-TEOS, PS-MTMS, PS-MTMS/PDMS).

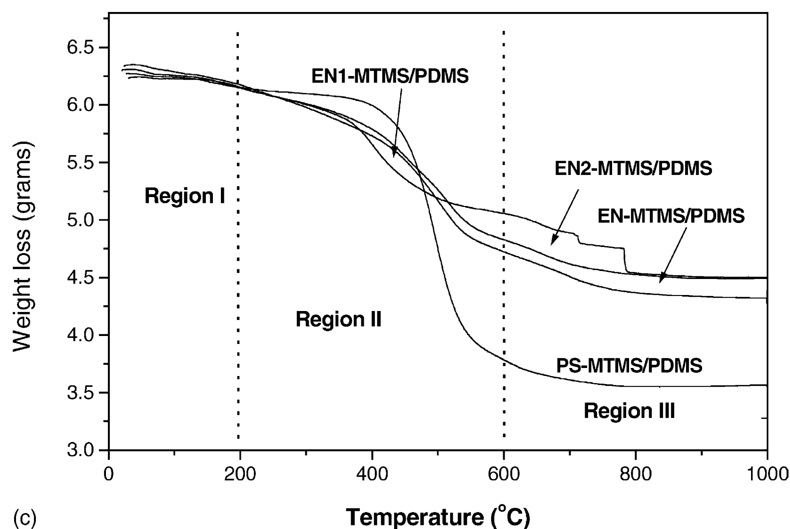


Fig. 3. (*Continued*).

Table 2
Coupling yield of different gel types immobilised *Candida rugosa* lipase

Experiment	Enzyme content in sample[a] (g)	Hydrolytic activities (μmol mg^{-1} min^{-1})		Coupling yield (η, %)
		Free lipase	Immobilised lipase[a]	
EN-TEOS	0.29	476.81	66.40	13.98
EN1-TEOS	0.25	403.17	128.90	31.98
EN-MTMS	0.21	339.63	1.00	0.29
EN1-MTMS	0.17	282.46	2.30	0.81
EN2-MTMS	0.19	308.68	3.70	1.20
EN-MTMS/PDMS	0.22	366.61	9.20	2.51
EN1-MTMS/PDMS	0.26	430.54	13.00	3.02
EN2-MTMS/PDMS	0.25	404.59	6.10	0.50

[a] Activities were corrected with the coupling yield.

In Region III, which commences in the vicinity of T_g, little weight loss occurred both for the immobilised derivatives, and for the samples containing pure silica. The weight loss in this region is associated with final dehydroxylation reactions [21] and definitive carbonisation of organic compounds, including the lipase.

We propose that the lower values obtained for the weight loss associated with the lipase encapsulated derivatives is the result of an increased matrix thermal stability resulting from interactions among silane precursors and organic components (lipase and additives).

3.3. Hydrolytic activity

The hydrolytic activities and coupling yields (η, %) for encapsulated CRL in different gel types are listed in Table 2. Among the precursors tested, the highest hydrolytic activity (128.9 U mg^{-1}) and coupling yield (31.98) were obtained with matrices prepared with TEOS as alkyl-substituted silanes.

Encapsulated lipase within matrices prepared with MTMS or MTMS/PDMS as precursors gave very poor hydrolytic

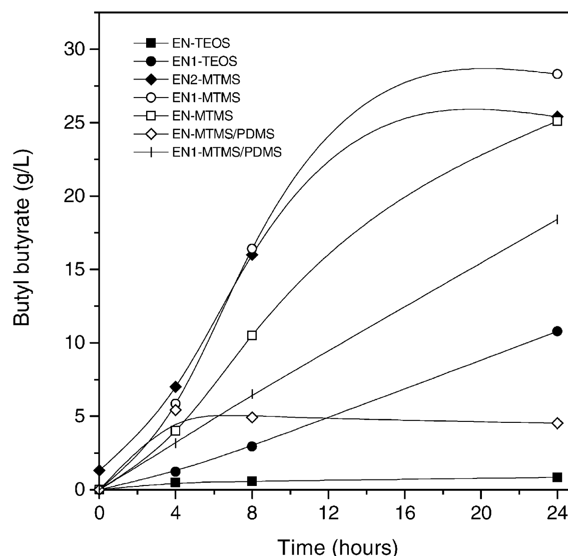


Fig. 4. Synthesis of butyl butyrate using immobilised derivatives in silica gels.

activities (1–13 U mg^{-1}) corresponding to coupling yields from 0.29 to 3.02%.

It is worth noticing that the lipase activity profile determined titrimetrically against oil-emulsions (as described in materials and methods) is subject to many interference factors, since the lipase activity is dependent on the way the substrate is presented to the enzyme [22–24]. A small percentage of surface-active contaminants or hydrolysis products could markedly alter the properties of the surface phase.

Therefore, the activity values are apparent activities and are determined by a combination of factors, including protein accessibility, the fraction of active protein, mass transfer of the substrate to the glass, diffusion of substrate and product within the glass (which is known to be slow for intact monoliths). Hence, the results for either MTMS or MTMS/PDMS direct reflect the low effectiveness of lipase encapsulated within these matrices in the hydrolysis reactions.

On the other hand, the improvement in activity for the PEG samples as compared to samples without PEG is likely due to a combination of possible factors, including: (1) improved protein accessibility or function; (2) enhanced partitioning of the hydrophobic substrate into the less polar matrices; (3) larger pore sizes (polymer-doped monoliths did not shrink as much as the undoped samples). The low apparent activity compared to solution suggests that the rate of diffusion of materials in and out of the glass is restricted

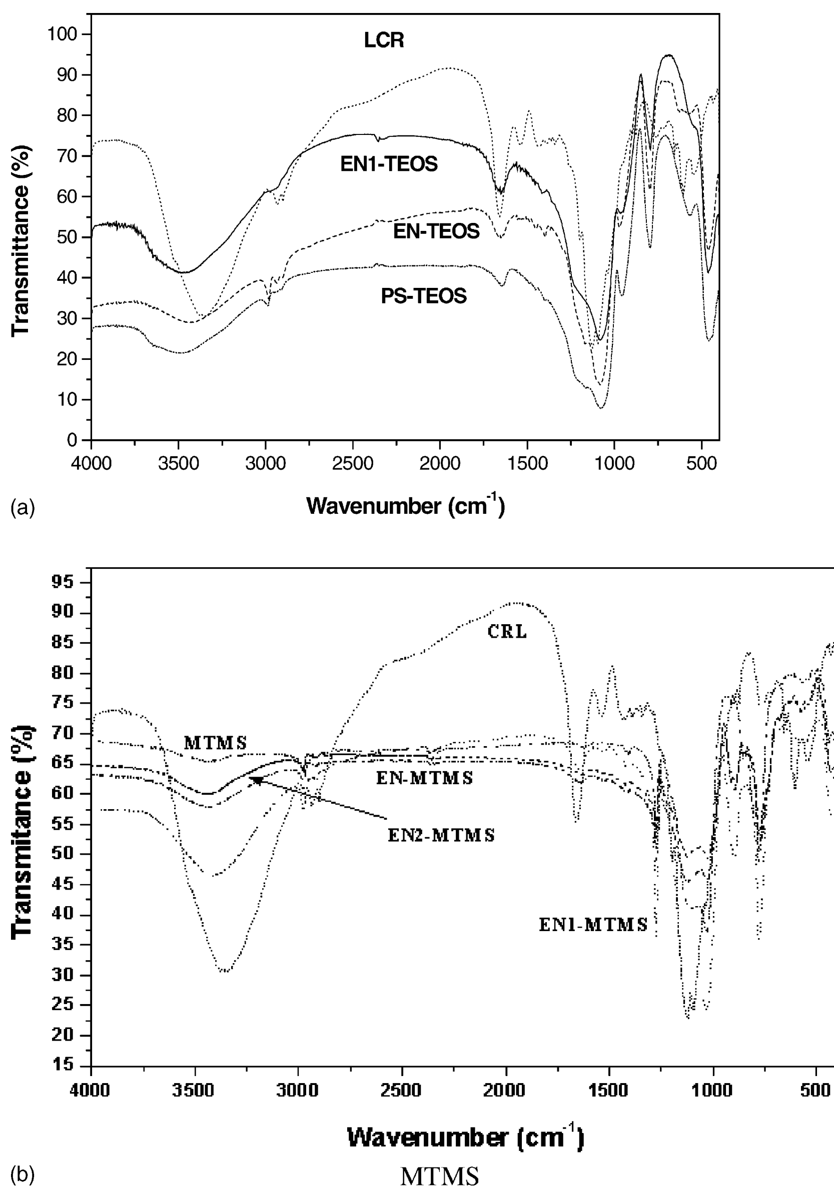
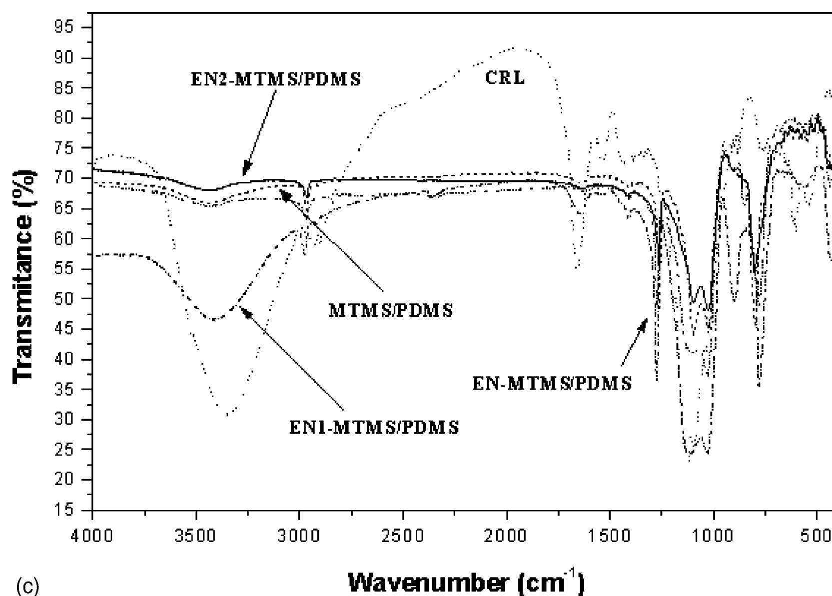


Fig. 5. FT-IR spectra for the free lipase (CRL), pure silica gel matrices (PS-TEOS, PS-MTMS, PS-MTMS/PDMS), and the immobilised derivatives in silica gels (EN-TEOS, EN1-TEOS, EN-MTMS, EN1-MTMS, EN2-MTMS, EN-MTMS/PDMS, EN1-MTMS/PDMS, EN2-MTMS/PDMS).



MTMS/PDMS

Fig. 5. (*Continued*).

and may produce activity values that do not directly reflect protein behaviour.

3.4. Esterification activity

It is common to compare the esterification activity for an immobilised enzyme, to that for the hydrolytic activity. In the present study, addition information on the catalytic activity was obtained by testing the derivatives prepared according to our method in synthetic applications, that is, in esterification reactions with *n*-butanol and butyric acid. This reaction was selected since it gave measurable results with the greatest accuracy in a short span of time and with minimum amount of the lipase. In addition this reaction system has been used by our group as a standard reaction system for lipases immobilised on several supports [22,23].

On the esterification reaction different activity dependence was found (Fig. 4). Better performance was achieved by derivatives resulting from the encapsulation of lipase in gels prepared with MTMS as precursor in the presence of PEG. This preparation exhibits an increased esterification activity (155 μmol g^{-1} min) that is three times greater than that prepared with TEOS (52 μmol g^{-1} min), and 1.74 times greater than that prepared with MTMS/PDMS (89 μmol g^{-1} min) as precursor.

The effect of pore size was also noted on the esterification activity that significantly increased for samples having larger pores (EN1-MTMS, see Table 1 and Fig. 4). This is in agreement with Bosley and Clayton [25], whose data showed that for maximum efficiency in a typical esterification reaction a pore diameter greater than 1000 Å is required.

However, in spite of the many indications that can be found in literature, there are not reports that correlated the "particular behaviour" of lipases in the adsorption on hydrophobic solids to the interfacial activation of lipases.

3.5. Chemical characterisation of the free lipase, pure silica and immobilised derivatives

Lipase encapsulated samples (EN1-TEOS, EN-MTMS, EN1-MTMS, EN2-MTMS, EN-MTMS/PDMS, EN1-MTMS/PDMS, EN2-MTMS/PDMS), pure silica (PS) and free lipase (CRL) were characterised by FT-IR. The FT-IR spectrum (cm^{-1}) is the same silica as the characteristic 810 cm^{-1} (Si–O–Si silica), 110 cm^{-1} (Si–O–Si silica) and 3400 cm^{-1} (O–H silica) bands (Fig. 5(a)–(c)).

As described in the literature [18], the enzyme lipase has two characteristic bands at 1650 and 3380 cm^{-1} (primary and secondary amino groups) as exhibited in Fig. 5(a)–(c). Those bands are also displayed in the spectra for the immobilised derivatives, revealing the presence of primary and secondary amino groups (lipase), particularly in the samples EN1-TEOS, EN1-MTMS and EN1-MTMS/PDMS probably due to the positive effect of PEG.

FT-IR investigation of single-step acid and base-catalyzed silica by Brinker and Sherer [21] showed that acid-catalyzed conditions resulted in larger concentration of internal hydroxyl groups. Fig. 5(a)–(c) sequences of infrared spectra show a progressive reduction in the ∼3400 cm^{-1} band assigned to internal and mutually hydrogen-bonded surface hydroxyls, for samples EN1-TEOS, EN2-MTMS, and EN2-MTMS/PDMS, associated with Region II in the TGA curves (Fig. 3(a)–(c)). Similar behaviour has been documented [21] in numerous silicate and multicomponent silicate systems, confirming the relationship between shrinkage

and condensation reactions of different types precursors in Region II.

4. Conclusions

The behaviour of the sol–gel encapsulated lipase systems depend on the physical and structural properties of the support, and the physical and chemical properties of the lipase. The conformation of encapsulated lipase in a gel can largely be improved by grafting appropriate functionality, such as alkyl-substituted silanes and additives, on the gel network, which surrounds the enzyme. The encapsulation of CRL in sol–gel prepared by the hydrolysis of alkyl-substituted silanes like TEOS, in presence of PEG (EN1-TEOS), showed considerable hydrolytic activity. This can be explained by the increased mean pore size obtained with the use of an additive that inhibited gel contraction during support synthesis.

The encapsulation of CRL in sol–gel prepared by the hydrolysis of MTMS, in presence PEG of (EN1-MTMS), showed considerable esterification activity. This was related to the interactions inflicted by its hydrophobic-hydrophilic nature. The most efficient systems (EN1-TEOS and EN1-MTMS) appear to be worthy of further application in biocatalysis.

Acknowledgements

We acknowledge financial assistance from Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Araucária.

References

[1] F.X. Malcata, H.R. Reyes, H.S. Garcia, C.G. Hill Jr, C.H. Amundson, J. Am. Oil Chem. Soc. 67 (1990) 890.
[2] R.M. Kazlauskas, U.T. Bornscheur, in: H.J. Rehm, G. Reed (Eds.), Biotechnology—A Comprehensive Treatise, Wiley V.H.C. Verlag Gmbh, Germany, vol. 8, Berlin, 1998, p. 268.
[3] A.F. Hsu, T.A. Foglia, S. Shen, Biotechnol. Appl. Biochem. 31 (2000) 179.
[4] C.M.F. Soares, H.F. de Castro, M.H.A. Santana, G.M. Zanin, Appl. Biochem. Biotechnol. 91–93 (2001) 715.
[5] P. Buisson, C. Hernandez, M. Pierre, A.C. Pierre, J. Non-Cryst. Solids 285 (2001) 295.
[6] M.T. Reetz, A. Zonta, V. Vijayakrishnan, K. Schimossek, J. Mol. Catal. A: Chem. 134 (1998) 251.
[7] R. Fernandez-Lafuente, P. Sabuquilho, G. Fernandez-Lorente, J.M. Guisán, Chem. Phys. Lipids 93 (1998) 185.
[8] A. Wiseman, Handbook of Enzyme Biotechnology, Ellis Horwood, London, 1995, p. 385.
[9] M.T. Reetz, A. Zonta, J. Simpelkamp, Biotechnol. Bioeng. 49 (1996) 527.
[10] E.N. Kadnokova, N.M. Kostié, J. Mol. Catal. A: Chem. 18 (2002) 39.
[11] C. Kauffmann, R.T. Mandelbaum, J. Biotechnol. 62 (1998) 169.
[12] D.C.M. Dutoit, M. Schneider, P. Fabrizioli, A. Baiker, Chem. Mater. 8 (1996) 734.
[13] D. Avnir, S. Braun, O. Lev, M. Ottolenghi, Chem. Mater. 6 (1994) 1605.
[14] A. Alfaya, L.T. Kubota, Química Nova 25 (2002) 935.
[15] T. Keeling-Tucker, M. Rakic, C. Spong, J.D. Brennan, Chem. Mater. 12 (2000) 3695.
[16] G. Kunkova, J. Szilva, J. Hetflejs, S. Sabata, J. Sol–Gel Sci. Technol. 26 (2003) 1183.
[17] C.M.F. Soares, H.F. de Castro, F.F. de Moraes, G.M. Zanin, Appl. Biochem. Biotechnol. 77–79 (1999) 745.
[18] M.A. Ramos, M.H. Gil, E. Schact, G. Matthys, W. Mondelaers, M.M. Figueiredo, Powder Technol. 99 (1998) 79.
[19] K.K. Unger, Porous Silica its Properties and Use as Support in Column Liquid Chromatography, Elsevier Science, New York, 1979, p. 376.
[20] T. Gitlesen, M. Bauer, P. Adlercreutz, Biochim. Biophys. Acta 1345 (1997) 188.
[21] C.J. Brinker, G.W. Scherer, Sol–Gel Science: The Physics and Chemistry of Sol–Gel Processing, Academic Press, London, 1990, p. 549.
[22] C.M.F. Soares, H.F. de Castro, M.H.A. Santana, G.M. Zanin, Appl. Biochem. Biotechnol. 98–100 (2002) 863.
[23] E.B. Pereira, H.F. de Castro, F.F. de Moraes, G.M. Zanin, Appl. Biochem. Biotechnol. 98–100 (2002) 977.
[24] M.T. Reetz, Adv. Mater. 12 (1997) 943.
[25] J.A. Bosley, J.C. Clayton, Biotechnol. Bioeng. 43 (1994) 983.