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Functionalization of mesoporous silica for lipase immobilization Characterization of the support and the catalysts

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Abstract

A support for enzyme immobilization was prepared by functionalization of mesoporous silica with octyltriethoxysilane. The features of the surface enables the adsorption of lipase from *Candida antarctica* B via strong hydrophobic interactions, enhancing the stability of the adsorbed enzyme molecules. Derivatives with a high enzyme loading (200 mg protein/g of silica) can be obtained due to the high porosity and surface properties of the support while the immobilization occurs in a monolayer fashion. The lack of inactive enzyme aggregates, together with the high enzyme loading, are responsible for the high catalytic activity achieved by these species. Derivatives were prepared with different lipase loading, and the activities were tested and compared to the commercial derivative Novozym 435. The stability of the catalyst and hence its industrial applicability were tested by performing subsequent reaction cycles of acylation of ethanolamine with lauric acid in acetonitrile. Conversion was quantitative even after 15 reaction cycles.
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1. Introduction

Immobilization of lipases is frequently performed via adsorption through hydrophobic interactions between the surfaces of supports and the lid of lipases. Non-covalent nature of the linkage is not usually a significant drawback for application of lipases in anhydrous media, since desorption of the lipase is not likely to occur in such conditions. This method [1] has proven very useful to achieve hyperactivation of most lipases: the hydrophobic surface of the matrix resembles the interface that induces the conformational change on lipases necessary to enable free access of substrates to their active centers. This mechanism is therefore quite interesting not only to provide high activity lipase derivatives, but it also suggests that such surfaces might be directing enzyme molecules towards a particular orientation [2] which seems to be the only possibility to "freeze" their active configuration.

However, simultaneous obtention of high enzyme loading and high catalytic efficiency is not easy, mainly due to the high tendency of lipases to form inactive enzyme aggregates. Indeed, some immobilized derivatives are prepared by precipitation of lipases using cold acetone on the supports (celite for instance). Precipitation of enzyme aggregates or even the immobilization in multilayers diminishes catalytic efficiency and as a result unnecessarily increases the cost of the final derivative. Al-Duri and Yong reported that the immobilization in multilayers might induce a non-favorable orientation of the lipase on the support [3]. The right distribution of enzyme molecules in a monolayer does not always happen, and lipases from different sources can be immobilized in monolayer or multilayer fashions on the same support [4]. Catalytic efficiency can also be seriously diminished because of diffusional restrictions, which are a common limitation of immobilized biocatalysts [5].

Immobilized lipases are generally used to perform biotransformations of most interesting industrial applications that quite often take place in non-aqueous media. The most frequently used supports are those of hydrophobic nature (e.g. resins like Accurel [6], Celite, Duolite [7], etc.). Also

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activation of silica (hydrophilic) or controlled pore glass have been reported using alkyldimethylchlorosilanes as the reagents to increase the surface hydrophobicity, so that adsorption of lipases can also be performed on hydrophobized silica [8,9].

The choice of supports is often limited by some other factors related to their structure: one is the pore shape and size. The widths of micropores range from 0.3 to 2.0 nm, mesoporous substances have pore sizes from 2 up to 50 nm and macropores range from widths of 50 up to 10^5 nm [10]. Silica and other inorganic materials with pore diameter of around 8 nm, allow only the immobilization of small enzymes within the pores. The larger ones can only be placed on the external surface of the support particles [11]. The use of meso- or macroporous supports decreases these unwanted effects and the derivatives usually retain higher activities and have higher enzyme loading [11]. According to some authors [8,12], a convenient pore size is around 100 nm. A disordered network of pores and channels, may only allow the smallest substrates to penetrate while the bigger substrates would clog the channels, slowing down the reactions. Thus, a heterogeneous pore size distribution interferes not only with the optimization of enzyme load on the internal surfaces, but also with the diffusion of substrates and/or products.

Another key factor is the surface area. In general, the wider the pore size is, the lower is the surface area and hence, the enzyme loading should be lower for an equivalent density of active groups available for the enzyme per area unit. Thus, catalytic efficiency depends on the equilibrium of these three parameters: monolayer enzyme arrangement, pore size (assuming homogeneous distribution) and surface area.

On these bases, we propose the use of a rational approach to obtain immobilized lipase catalysts with optimized features. The design includes every step of the process: the choice of the support (mesoporous, high surface area and narrow pore size distribution), the chemical treatment of its surface, the immobilization process, and the characterization of the biocatalyst (enzyme loading in monolayer fashion, activity and stability). This was approached using three test reactions: hydrolysis of two different substrates in aqueous medium and acylation of ethanolamine with lauric acid in organic medium.

2. Experimental

2.1. Materials

The support material used was silica MS-3030 (surface area 300–320 m^2/g, average pore diameter 30–40 nm, data from manufacturer), a generous gift from Silica PQ Corporation (Valley Forge, PA, USA). Reactions on silica were carried out under nitrogen using Schlenk techniques. Solvents (analytical grade) were dried and deoxygenated prior to use. Soluble lipase from *Candida antarctica* fraction B (Novozym 525) (CaLB), and Novozym 435 (immobilized CaLB) were kindly donated by Novo Nordisk (Denmark). Octyltriethoxysilane was purchased from Aldrich. Electrophoresis and Bradford reagents were from Sigma and Bio-Rad. Substrates: tributyrin (glycerol tributyrate), *p*-nitrophenyl propionate (*p*-NPP), ethanolamine and lauric acid and all other reagents were from Sigma. Solvents used in the mobile phase of HPLC: methanol and acetonitrile (from Scharlau, Barcelona, Spain) were HPLC grade, and water was Milli Q quality (Millipore, Massachusetts, USA).

2.2. Methods

2.2.1. Determination of protein in the commercial enzyme solution

The protein content was determined according to the Bradford method. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of the enzyme extract was performed as described by Laemmhi [13].

2.2.2. Immobilization

2.2.2.1. Functionalization of support. Silica MS 3030 was supplied with high purity, so further purification was not necessary. Activation was performed using a modification of the method described by Weetall [14]: namely 1 g of silica previously degassed at 80 °C under vacuum for 12 h, was suspended in a 10 mL solution of octyltriethoxysilane in toluene (1:4 v/v). The suspension was gently stirred for 72 h at room temperature. The suspension was filtered and washed twice with dry toluene, and three times with hexane and acetone, and finally exhaustively vacuum dried. This support is referred to below as octyl silica.

2.2.2.2. Thermal gravimetric analysis. Thermal analyses of silica and octyl silica were performed on a Mettler Toledo TGA/SDTA 851^e apparatus. Typically, 5 mg of the sample was heated from 25 to 1100 °C at a rate of 5 °C/min under N_2 at a flow rate of 200 mL/min.

2.2.2.3. Immobilization procedure. The immobilization was performed according to the following procedure: different amounts of the commercial extract of CaLB ranging from 0.04 to 2.4 mL were dissolved in 25 mM phosphate buffer, pH 7.0, up to a total volume of 4 mL. After assaying the esterasic activity of these solutions, 100 mg octyl silica was added and maintained in suspension with a helical stirrer. Aliquots from suspension and supernatant were withdrawn at 10 and 30 min to assay their hydrolytic activities. Final time is determined by the lack of activity, or low constant activity of the supernatant. After that, suspensions were filtered and washed three times with 10 mL volumes of 50 mM phosphate buffer. Control samples with the corresponding enzyme dilutions were prepared and assayed at the same intervals as the suspension samples. The supernatants

of the immobilization mixtures, as well as the liquids from these washings, were tested for protein desorption. This was performed by assays of their catalytic activities (esterasic activity) or by SDS-PAGE to detect the lipase band.

After the last washing with 50 mM phosphate buffer, pH 7.0, in a sintered glass funnel, the derivatives were washed twice with 10 mL dry acetone, filtered out and vacuum dried for at least 30 min to ensure a complete drying of the catalyst. Also, derivatives were dehydrated by incubation in a desiccator in the presence of different saturated salt solutions at different water activity values. Alternatively, derivatives were also prepared in the presence of polyethylene glycol (PEG) and the effect of these polymers to preserve catalytic activity was studied. PEG with molecular weight 6000 and 20,000 were added in a proportion of 10 mg PEG/g of silica.

In order to detect and to eliminate diffusional restrictions, 15 mg of the derivatives were suspended in 3 mL of 50 mM phosphate buffer, pH 7.0. The suspensions were placed in an ice bath and kept under vigorous magnetic stirring in order to break support particles. Aliquots of these suspensions were withdrawn every 10 min and their esterasic activities were assayed as explained below until a constant value that was considered the final activity value.

2.2.3. Determination of enzyme activity

2.2.3.1. Esterasic activity. Despite an assay for ester hydrolysis activity is not a specific test for lipase activity, this assay was selected for use as a routine assay because it is easy to conduct via spectrophotometric measurements and it provides a rapid assessment of relevant enzymatic activity. Hydrolysis of *p*-NPP was followed at 348 nm in a Kontron Instruments (Watford, Herts, UK) spectrophotometer equipped with stirring device and constant temperature capability. The cell contained 1.9 mL of substrate solution at 25 °C (0.4 mM *p*-NPP in 50 mM sodium phosphate buffer, pH 7.0). Aliquots of the suspension were diluted in different proportions in 50 mM phosphate buffer (pH 7.0) prior to being added to the cell (50 μL) to facilitate the analysis. Aliquots from the supernatant were not diluted: 50 μL were added directly to 1.9 mL substrate solution. One esterase unit corresponds to consumption of 1 μmol *p*-NPP/min ($\varepsilon_{p\text{-NPP}} = 5150\,\text{M}^{-1}\,\text{cm}^{-1}$).

2.2.3.2. Tributyrin activity. The hydrolysis of tributyrin measures lipase activity by the liberation of butyric acid according to the assay method described in the Roche catalogue for their Chirazyme lipases. Hydrolysis of tributyrin was monitored titrimetrically in a Radiometer Copenhagen pH-stat, using 100 mM sodium hydroxide. The 48.5 mL potassium phosphate buffer (10 mM, pH 7.0) were incubated in a thermostated vessel at 25 °C and stirred sufficiently. Then, 1.47 mL tributyrin were added and the pH-stat was started to keep the pH at 7.0. When the pH stabilizes, 5 mg catalyst were added and the consumption of NaOH was determined. One lipase unit corresponds to consumption of 1 μmol NaOH/min.

2.2.3.3. N-Acylation of ethanolamine with lauric acid. Vials containing 12.2 mg ethanolamine, 40 mg lauric acid and 1 mL acetonitrile were incubated at 40 °C. The 500 tributyrin units of Novozym 435 or CaLB-octyl silica derivative were added and the suspension was stirred at 200 rpm in a thermostated orbital shaker. The reaction course was followed by HPLC according to the method described by Fernández-Pérez and Otero [15]. Equilibrium was reached within 1 h with both catalysts.

2.2.4. Subsequent reaction cycles

After 1 h reaction, acetone was added in order to dissolve completely the product (*N*-hydroxyethyl amide of lauric acid) [15]. The derivative was washed with acetone three times and then filtered out and vacuum dried. Then the dry derivative was weighted to correct the amounts of substrates and the volume of acetonitrile. A new reaction was then started, and this was repeated up to 15 reaction cycles.

2.2.5. HPLC analyses

HPLC analyses of the reaction mixtures of ethanolamine were performed as previously described [15]. The mobile phase was 90:10 v/v methanol–water to separate the amide, 95:5 v/v methanol–water to separate the lauric acid, and 100% methanol for the amide-ester. All the mobile phases contained 0.1% acetic acid. The corresponding retention times were 6.4, 6.2 min (both eluted at 1 mL/min flow rate) and 4.1 min, respectively (eluted at 2 mL/min flow rate). Identification of products and substrates was performed with a refraction index detector.

2.2.6. Nitrogen adsorption measurements

Nitrogen isotherms were measured at the temperature of liquid N_2 with a Micromeritics ASAP 2000 apparatus. Samples were previously degassed at 120 °C for 20 h. Nitrogen isotherms were obtained in both adsorption and desorption modes. The surface areas of supports and catalyst were determined by the BET method. The total pore volume was calculated from the amount of vapor adsorbed at a relative pressure (P/P_0) close to unity, where P and P_0 are the measured and equilibrium pressures, respectively. Pore size distribution curves were established from the desorption branches of the isotherms using the BJH model [16].

3. Results and discussion

3.1. Protein contents

The initial enzyme extract of CaLB contained 25 mg protein/mL of solution, according to the Bradford assay. SDS-polyacrylamide gel electrophoresis of CaLB extract was performed and as shown in Fig. 1, there is one major

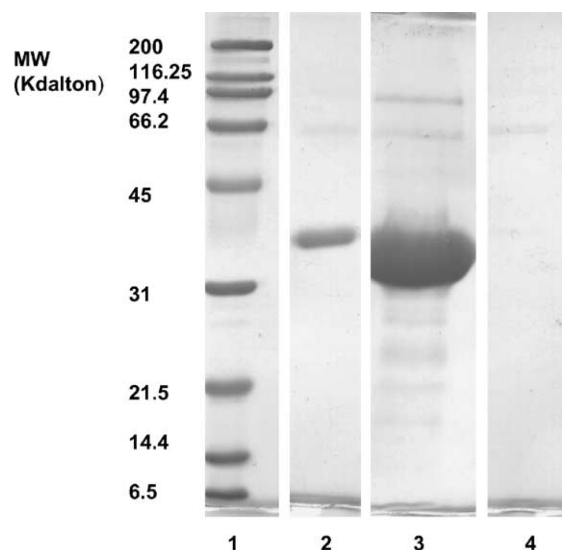

Fig. 1. SDS-polyacrylamide gel electrophoresis of the commercial extract of lipase from *C. antarctica* fraction B (Novozym 525, Novo Nordisk). Samples were electrophoresed in a 12% (w/v) polyacrylamide gel. The crude extract was dissolved according to the protein content determined by the Bradford's method, to get a solution of 0.8 mg/mL. Lane 1: 5 μL of protein molecular weight standard; lane 2: 20 μL of crude extract solution; lane 3: 6 mg of immobilized lipase were suspended in 60 μg of water and boiled in loading electrophoresis buffer; 20 μL of the suspension; lane 4: 20 μL of supernatant withdrawn after 1 h contact-time for immobilization.

band corresponding to the molecular weight described in the literature for CaLB: 35.517 kDa [17].

3.2. Immobilization

The linkage lipase–support herein studied is driven by hydrophobic interactions as it has been pointed out in the Introduction. The presence of octyl groups on the surface of the pores should provide very hydrophobic properties to the silica, and there are some indirect clues of a high degree of hydrophobization of the silica surface. The best evidence is that a high enzyme load can be achieved (enzyme adsorption did not occur significantly on non-functionalized silica).

The experimental confirmation was obtained from thermal analysis of modified and unmodified silica. Two main weigh loss regions were observed (not shown). The first loss (common to both silicas) occurred at a temperature range between 25 and 90 °C and was followed by a moderate slope. It was assigned to water and solvent losses. The second one was only observed in the octyl silica between 450 and 650 °C, and was a sharp 6% weigh loss mainly due to the organic SiO_2 modifiers. It corresponded to a loss of 67 mg per gram of silica and was the straightforward experimental confirmation of a high degree of hydrophobization of the support.

According to the protein determination performed previously, amounts of CaLB extract between 10 and 600 mg protein/g of silica were added to prepare derivatives with growing enzyme loads (Table 1).

Assays of esterasic activity were performed during the immobilization process, and compared to the activity of a control enzyme solution. The activity of the control solution remained constant, which made unnecessary to determine the amount of protein prior and after the immobilization in order to quantify the amount of immobilized enzyme. But the activity of the suspension decreased as the enzyme was being adsorbed.

Suspensions were filtered when the absence of activities of the supernatants indicated that 100% of the initial enzyme had been immobilized in the derivatives loaded up to 200 mg protein/g. This is in agreement with the lack of protein band in the supernatant in the electrophoresis shown in Fig. 1. Alternatively, the suspensions of the top loaded derivatives were filtered when the activity of the supernatant remained constant. In these cases there was no full adsorption of the enzyme presumably because the amount of protein exceeded the capability of the support area. (This point is further studied and discussed.) After the washing and drying processes, the derivatives were resuspended in order to assay their final activities.

A number of different effects seemed to have an influence on the activities measured of the catalysts, as can be seen in Table 1, depending on the type of assay, the diffusional limitations and the enzyme loading. The *p*-NPP

Table 1
Activities of the final derivatives obtained with different CaLB loading

Enzyme offered (mg protein/g)	Tributyrin activity			*p*-NPP activity		
	Initial (units/g)[a]	Recovered (units/g)[b]	Recovered activity (%)[c]	Initial (units/g)[a]	Recovered (units/g)[b]	Recovered activity (%)[c]
10	1056	455	43	65	16.4	25
50	5280	1000	19	320	80	25
100	10560	2000	19	620	208	33
200	21120	4000	19	1200	584	48.6
400	42240	4600	11	2700	727	27
600	63360	6700	10.5	4000	650	16.3
Novozym 435		5000				

[a] Total units of soluble lipase initially offered to 1 g of support.
[b] Lipase units recovered at the end of the immobilization process in 1 g of catalyst.
[c] Percent final activity: $b/a \times 100$.

assay is very fast (1–2 min), so derivatives were severely subjected to internal diffusion limitations. On the opposite, a much slower assay (hydrolysis of tributyrin takes about 25 min) was not as much hampered by internal diffusion, but it was not appropriate to control the immobilization process. Internal diffusional limitations in *p*-NPP assay could be eliminated as the particle size of the catalysts was reduced (suspended in an aqueous solution) as described in Section 2.2. The activities remained roughly unchanged up to 100 mg protein/g. The reduction of particle size of the catalysts could not be performed in tributyrin assay because the enzyme derivative has to be added in solid form, so diffusional restrictions could not be avoided. Therefore the activity decreased as the enzyme loading was higher. Enzyme–support interactions are very intense in low loaded derivatives (*p*-NPP assay) and this causes activity losses. At 200 mg protein/g the loading is high enough to avoid this situation (maximum activity is achieved in these conditions). But higher loadings led to lower activities, which could not be recovered by reducing the particle size. This might be due to the formation of inactive enzyme aggregates in these conditions, as it is further discussed. Spectrophotometric assay of Novozym 435 in *p*-NPP hydrolysis is not shown because the nature and the large particle size of the beads, made very difficult to obtain clear and reproducible results.

In all cases derivatives underwent additional activity losses after all the process (Table 1). In a previous work, the activity of lipase from *Thermomyces lanuginosus* was reported to increase by a 10-fold factor upon immobilization on a similar support [18]. This is consistent with the conformational change ("open lid") induced by the hydrophobic interface. Nevertheless, interfacial hyperactivation by contact with the support was not observed with CaLB, which is in agreement with the data reported in the literature about the lack of lid in this enzyme [19]. In the present study, the activity diminished with regard to the initial one in all CaLB derivatives (see Table 1). The shape of the binding site of this lipase is a not-deep cleft [20], where the inner walls of the pocket are covered with hydrophobic amino acids and the available space is very limited [21]. The dimensions of the binding site seem to enable only small changes. These small changes in tertiary structure, induced by the hydrophobic interactions with the support might alter the tetrahedral intermediate and negatively affect catalytic activity.

Several possibilities were considered in order to search the causes for the decrease found in final activity: (a) enzyme leakage during the washings with phosphate buffer after the immobilization; (b) structural damage of the protein caused by the desiccation process; (c) formation of enzyme aggregates on the surface of the support; (d) distortion of tertiary structure due to strong interaction with support.

3.2.1. Protein desorption

Leakage of enzyme might occur because the protein is non-covalently linked to the matrix, especially in aqueous medium. Octylsiloxane groups are susceptible of cleavage, which would also mean desorption of the lipase from the matrix. Therefore the possibility that the activity was lost because of enzyme desorption during the washings was considered and studied. The activity of the supernatant indicated the end point of the immobilization process: a constant value in highly loaded derivatives or the lack of activity in the low loading ones. But once immobilization process is finished, the washings with buffer might shift the non-covalently linked enzyme molecules.

Therefore supernatants of these subsequent washings were tested for esterase activity. They were all fully inactive (data not shown) which indicates that there is no significant desorption. This seems to suggest a high strength of the adsorption of the enzyme on the support, favored by the very hydrophobic octyl groups. This feature is most relevant since it provides a certain irreversible character to the immobilization through hydrophobic interactions.

3.2.2. Desiccation process

CaLB underwent activity decreases when suspended with silica, and then a steeper drop after drying with acetone. Activities during the immobilization course are shown in Fig. 2.

Acetone has been used to precipitate proteins on supports with significant losses in enzyme activity [7]. Although we have only used small amounts of acetone for very short times (just enough to vacuum dry the derivatives) a certain loss of activity was detected. Wehtje et al. [22] also found that most of the activity was lost when almost all the water was evaporated. These authors used albumin, polyethylene glycol and other polymers to protect the enzyme from deactivation in different steps of the process of immobilization and evaporation. We tried to preserve enzyme activity by adding polyethylene glycol to the soluble enzyme prior to suspend it with the support. Fig. 2 shows that the use of PEG did not significantly improve the recovered activity of our catalysts. The reason of the different effect of the polymer could be related to other parameters, since these authors used a different support, and different enzymes.

Different methods were also tested in order to dry the catalysts with minor loses of activity, namely: vacuum drying overnight, and desiccating up to particular water activity values in the presence of saturated salt solutions: LiCl ($a_w = 0.115$), $Mg(NO_3)_2$ ($a_w = 0.536$), NaCl ($a_w = 0.7$). As shown in Fig. 3, the effect of salts did not improve the recovered activity of the catalyst. The use of acetone was the least aggressive method to the enzyme activity. Vacuum drying overnight (not shown) also yielded activity values lower than the ones in acetone.

3.2.3. Enzyme aggregates

The formation of enzyme aggregates on the surface of the support can be discarded in derivatives having 200 mg protein/g or less, according to the Langmuir isotherm behavior discussed below.

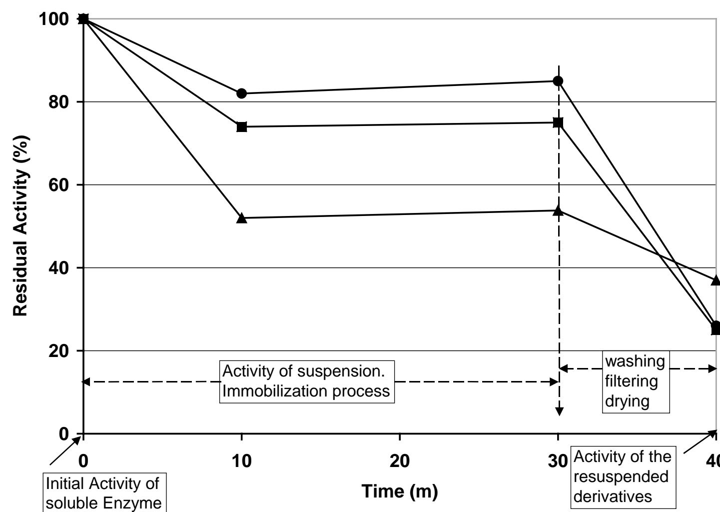

Fig. 2. Effect of the absence (squares) or presence of polyethylene glycol during immobilization on the activity of a 100 mg protein/g derivative. Molecular weight of PEG: 6000 (circles) or 20,000 (triangles). Initial activity corresponds to the one prior to the suspension with the support.

3.2.4. Interaction with the support

The most probable reason for the activity loses in the immobilization of CaLB is an intense interaction of this protein with the support surface. Salis et al. [23] also described activity loses of CaLB immobilized on EP-100. The authors attributed the inactivation to strong interactions with the support in derivatives with low loading, and to internal diffusion in high loading derivatives. In our case diffusional restrictions can be neglected because the final activities were measured after breaking the particles of support. Therefore either with low or high loading derivatives, the enzyme distortion due to strong interactions is held responsible for the activity losses.

The assumption should be sustained by an increase of the enzyme stability as a consequence of an intense enzyme–support multi-interaction. In order to verify this possibility, soluble *C. antarctica* lipase B and our octyl silica derivative were incubated in aqueous medium at 60 °C. Fig. 4 shows an eight-fold thermal stabilization of the lipase by immobilization. The decreased activity and increased stability can therefore be ascribed to a strong interaction enzyme–support, which is also consistent with the negligible leakage of the enzyme even in aqueous media.

3.3. Surface area and pore size distribution

As shown in Table 2, the surface area (S_{BET}) decreases from 305 to 254 m^2/g when the silica is functionalized. Anchoring the enzyme produces a further decrease in the surface area to values of 207 m^2/g. The hysteresis loops of the N_2 adsorption–desorption isotherms of silica, octyl silica and lipase–octyl silica are of type IV according to the Brunauer et al. notation [10], which corresponds to a mesoporous material (see Fig. 5).

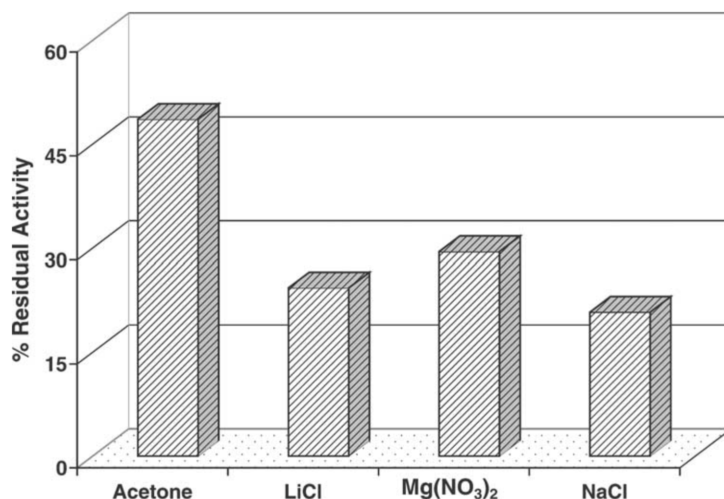

Fig. 3. Effect of the presence of acetone or saturated salt solutions in the drying step on the activity of the biocatalyst. Water activity values defined by the salts are given in the text. Derivative: 200 mg protein/g.

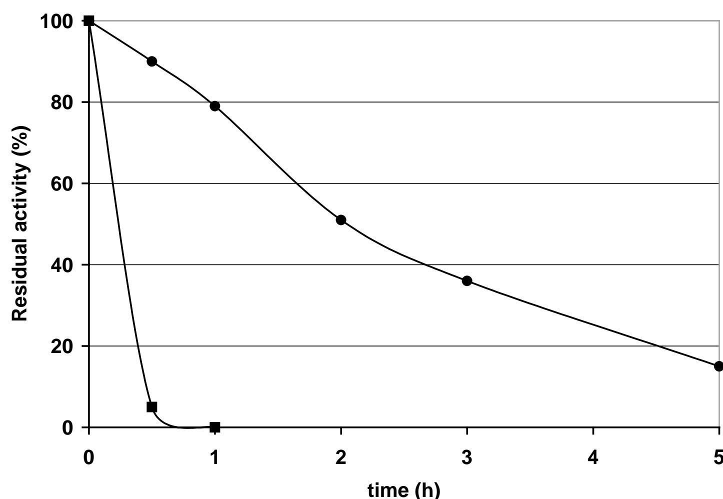

Fig. 4. Thermal stabilization of immobilized CaLB. Residual activity of soluble lipase (squares) and immobilized in octyl silica (circles) incubated in 50 mM sodium phosphate, pH 7.0, 60 °C. Catalytic activity was tested in hydrolysis of *p*-NPP assays. Derivative 200 mg protein/g.

Table 2
Textural data obtained from nitrogen adsorption isotherms

	Silica	Octyl silica	Enzyme–octyl silica[a]
BET surface area (m^2/g)	305	254	207
BJH desorption cumulative pore volume (pores 0.1–300 nm diameter) (cm^3/g)	2.9	2.2	1.7
BJH desorption average pore diameter (nm)	27.9	25.0	22.4

[a] Enzyme loading: 400 mg/g.

Fig. 6 shows that the pore size distribution of this support calculated using the BJH method [16] is narrow. There is no significant contribution of the micropore region, so most of the support surface is available to the molecules of enzyme. The pore volume and mean pore diameter undergo only a very moderate decrease from silica to octyl silica (Table 2). Larger differences were not expected because of the small size of the octyl chains compared to the pore diameter. Nevertheless, the difference between pore sizes (either before or after functionalization with octyl chains) without and with enzyme is hardly significant, and it does not justify the presence of the protein inside the pore channels. According to structure explorer [24] the dimensions of the lipase from *C. antarctica* B are: 6.92 nm × 5.05 nm × 8.67 nm, and this

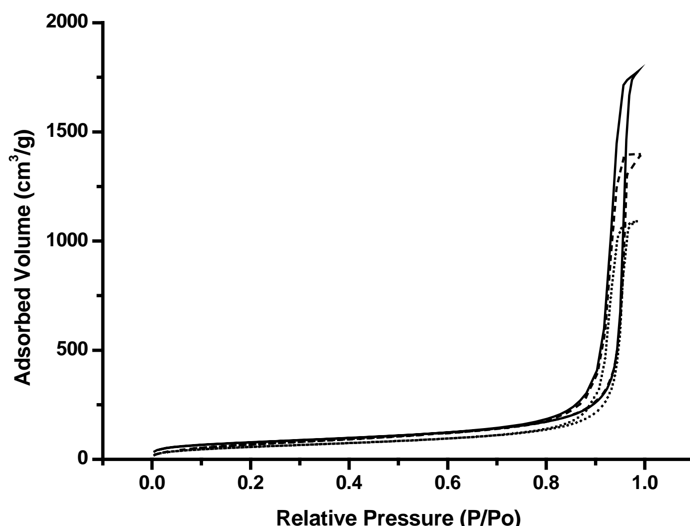

Fig. 5. Nitrogen adsorption isotherms. Unmodified silica (continuous line), octyl silica (dashed line) and lipase–octyl silica (dotted line). Enzyme loading: 400 mg/g.

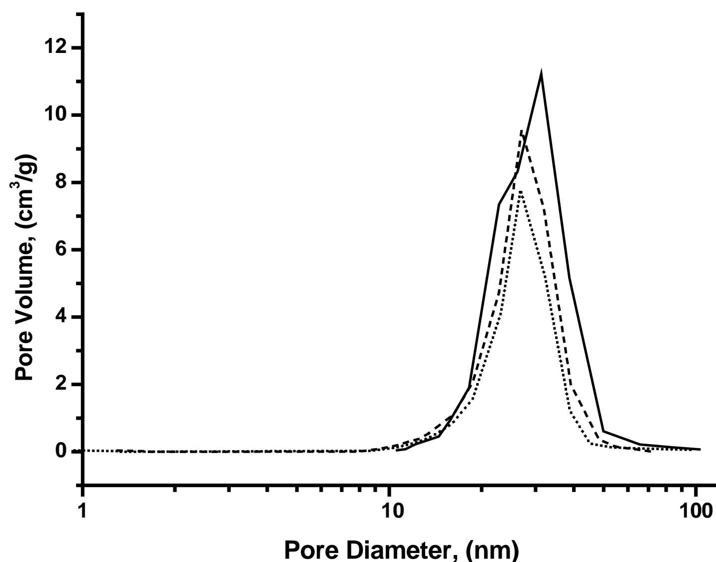

Fig. 6. Pore size distribution of unmodified silica (continuous line), octyl silica (dashed line) and lipase–octyl silica (dotted line). Enzyme loading: 400 mg/g.

molecular size would make the pore size to decrease very noticeably. Adsorption isotherms were performed in denaturing conditions for enzymes (120 °C for 20 h), so most probably the tertiary structure of the protein was unfolded during the process in such conditions, therefore the data would not correspond to the real volume of the folded native protein.

3.4. Enzyme loading/activity

Theoretically, the highly porous nature of the silica used in this work should enable to achieve high enzyme loading. As seen in Table 1, the proportionality between enzyme loading and catalytic activity is only kept up to 200 mg/g. With higher loading values the activity is lower than the corresponding to the enzyme contained.

3.4.1. Langmuir/Freundlich isotherms

In order to evaluate the support capacity, adsorption isotherms were experimentally determined. Isotherms of Langmuir and Freundlich were applied to describe adsorption isotherms [1,4], where Langmuir isotherm corresponds to the immobilization in a monolayer, and Freundlich isotherm corresponds to multilayer immobilization.

By linearization of the Langmuir representation according to the expression

$$\frac{1}{Q_e} = \frac{1}{K_L C_e} + \frac{a_L}{K_L}$$

and plotting $1/Q_e$ versus $1/C_e$, Langmuir constants may be calculated. Q_e is the lipase loading (lipase units/g support), C_e the lipase in the liquid phase (lipase units/mL solution). K_L (mL/g) and a_L (mL/mg) are the Langmuir constants. K_L is related to the support capacity, a_L related to the energy of adsorption, and K_L/a_L (lipase units/g support) the monolayer capacity of the support.

Freundlich constants can also be determined by the linearization of the Freundlich equation:

$$\ln Q_e = \ln K_F + \frac{1}{n} \ln C_e$$

where K_F (mL/g), and $1/n$ (unitless) are the Freundlich constants. K_F indicates the support capacity, and $1/n$ is the heterogeneity factor: the support surface is less homogeneous as $1/n$ is closer to 0. Table 3 shows Langmuir and Freundlich constants.

The monolayer capacity K_L/a_L is close to 4000 lipase units/g, which is the value where linearity is lost, and corresponds to 200 mg protein/g of support (see Table 1). The support surface can be considered homogeneous according to the $1/n$ value of 0.675. All these experimental data seem to confirm our assumption that the immobilization of *C. antarctica* B lipase on our octyl silica support occurs in a monolayer up to this loading. Lipase efficiency (defined as the activity divided by the loading) is constant for Langmuir pattern, whilst diminishes for Freundlich one. The plots of activity and efficiency versus enzyme loading are shown in Fig. 7 and it also confirms that the efficiency remains constant also up to 200 mg lipase/g of octyl silica and then diminishes at higher lipase loadings.

Despite the lipase from *C. antarctica* lacks the lid that enables an hyperactivation, the immobilization must be driven through an hydrophobic area of the enzyme. Otherwise it

Table 3
Values of Langmuir and Freundlich constants for *C. antarctica* B lipase immobilized on octyl silica

Langmuir constants			Freundlich constants	
K_L (mL/g)	a_L (mL/mg)	K_L/a_L (LU/g)	K_F (mL/g)	$1/n$
18.85	47.74×10^{-4}	3948	46.06	0.675

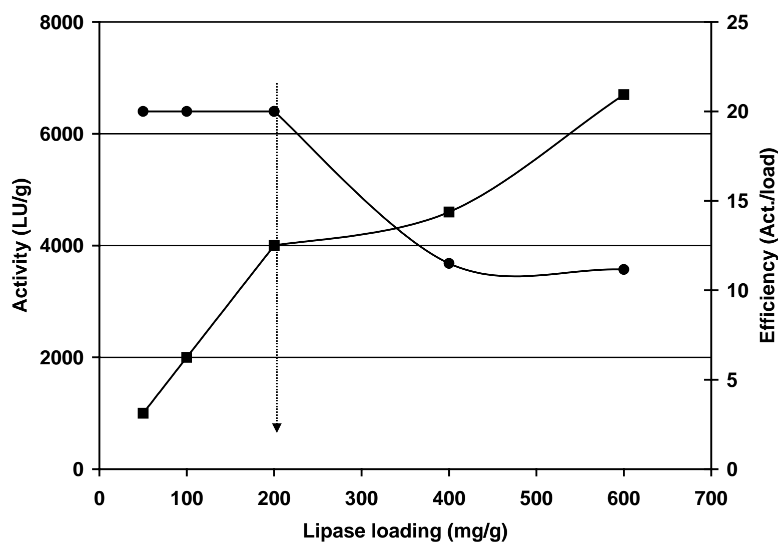

Fig. 7. Activity (hydrolysis of tributyrin) and efficiency vs. lipase loading. Activity: squares; efficiency: circles.

would be very difficult to explain why all protein offered is so rapidly linked to a hydrophobic support in a medium with rather low ionic strength. The interaction between both hydrophobic surfaces (from enzyme and support) ruling the immobilization process, seems to indicate that this is the orientation acquired by the enzyme. On the other hand, lipases tend to form aggregates by interaction between hydrophobic areas of different molecules. But if these areas are busy in the enzyme–support linkage, it seems reasonable to assume that the probabilities to aggregate are diminished. Therefore, this orientation would also enable high loading in a monolayer, and immobilization in multilayer would only occur with larger amounts of lipase: over 200 mg/g.

3.5. N-Acylation of ethanolamine with lauric acid

The lack of correlation between the hydrolytic and synthetic activity of lipases has been generally established. Furthermore, the applicability of these kind of catalysts (immobilized lipases) is mainly based on synthesis processes in industrial areas such as pharmaceuticals, food additives, pesticides, etc. Therefore a synthesis reaction was also necessary in order to check if they were suitable to catalyze condensation reactions in organic medium. The silica-based derivatives prepared had been already characterized as hydrolytic catalysts, so acylation reaction was only performed using the catalyst with the highest enzyme loading and the maximum activity per gram, and Novozym 435 for comparative purposes. The behavior of our derivative as synthesis catalyst as well as the corresponding operational stability was tested in organic medium. This parameter was defined as the stabilization achieved in the conditions of reaction after a number of subsequent reaction cycles.

The acylation of ethanolamine with lauric acid was performed at 40 °C in acetonitrile. *O*-Acylation of the ethanolamine can occur in the first place, but this compound cannot be isolated. The higher selectivity of the ethanolamine reaction toward *N*-acylation rather than *O*-acylation is attributed to the high reactivity of primary amino groups and to the ease of migration of the acyl residue between the amino and hydroxyl groups of the precursor ethanolamine molecule. It has been previously reported that $O \rightarrow N$ acyl migrations are spontaneous in *O*-monoacylated β- and γ-*n*-aminoalcohols with less than three-carbon atoms [25]. Besides, the insolubility of the amide (precipitation in the reaction medium) makes the process irreversible.

The reaction was catalyzed by both, Novozym 435 and our octyl silica-CaLB derivative 600 mg protein/g. The amounts of biocatalysts were calculated to be the minimum amount required by each of them to obtain quantitative conversion within an hour. In such conditions both catalysts displayed identical reaction courses (not shown). Therefore, any losses in enzyme activity would be immediately detected by lower conversion yields.

The product obtained was insoluble, so after each reaction cycle, the whole reaction mixture had to be washed with acetone in order to dissolve and separate the product. Nevertheless, the acetone seemed not to affect the catalytic activity since quantitative conversions were repeated at least for 15 cycles. Neither did the temperature, the catalyst remaining fully active for these 15 cycles at 40 °C, as shown in Fig. 8.

Our derivative displayed an excellent behavior as synthesis catalyst in both aspects: high levels of activity and an excellent operational stability in the conditions of the reaction. The excellent stability achieved was compared to that of the well-characterized commercial immobilized CaLB Novozym 435. The possibility of enzyme losses due to desorption from the support is not probably to happen in the anhydrous medium studied, and indeed it could be discarded by the quantitative conversion yield achieved.

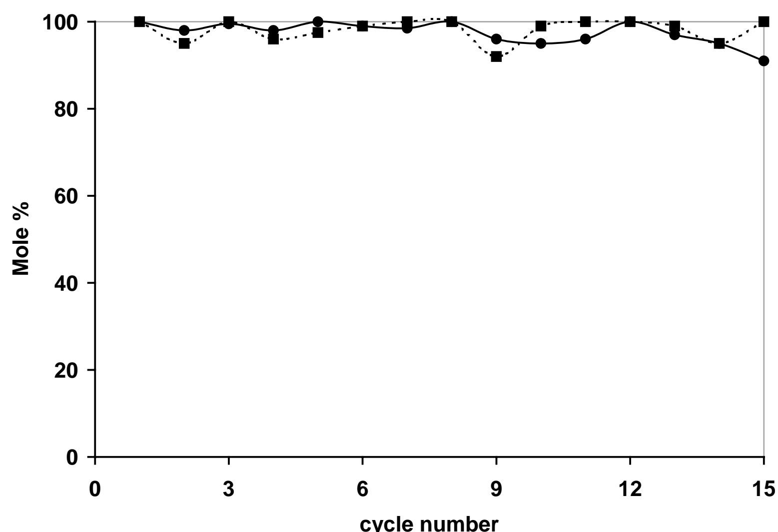

Fig. 8. Molar yield of acylation of ethanolamine with lauric acid in subsequent reaction cycles catalyzed by Novozym 435 (squares) and lipase–octyl silica (circles) 600 mg/g. Conditions and details of the reaction are described in Section 2.2.

4. Conclusions

Activation of a mesoporous silica having high surface area with octyltriethoxysilane has proven successful to cover the hydrophilic surface of silica with a hydrophobic layer of octyl groups. This has enabled to accomplish our objective: To achieve an efficient lipase immobilization in a monolayer, avoiding the formation of enzyme aggregates. Lipase from *C. antarctica* B was easily adsorbed onto this matrix by hydrophobic interactions between both macromolecules. The monolayer enzyme loading on this support was as high as 200 mg protein/g. These biocatalysts are suitable to work in non-aqueous media: homogeneously suspended in an organic medium in a stirred tank reactor. But it would also permit to design continuous column reactors, due to the mechanical resistance to pressure of silica. All these characteristics make the biocatalysts prepared to display highly efficient values of activity per weight unit.

The strong interaction of the enzyme with hydrophobic groups from the support contributed to decrease the mobility of the immobilized protein and thus to increase its stability. The derivatives achieved thermal stabilization of the lipase, and an excellent operational stability shown by the cycles of esterification reactions. These are the main properties that industrial biocatalysts should fulfill (including a support of low cost), which make the prospects of these new derivatives very promising.

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