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Ethanol improves lipase immobilization on a hydrophobic support

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Abstract

A mesoporous silica functionalized with octyl groups had been used as the support for lipase (from *Candida antarctica*) immobilization. The hydrophobicity provided by the hydrocarbon chains together with the excellent morphologic characteristics of the solid leads to high enzyme loadings in monolayer fashion. However, in this kind of systems the aqueous enzyme solution cannot easily access all the inner surface of the pores because of the highly hydrophobic nature of these surfaces. Thus, the presence of low ethanol concentration decreases the hydrophobicity of the channels and the access of enzyme seems to be significantly improved. The monolayer capacity (400–500 mg protein/g of octyl silica) increases twice compared to the corresponding immobilization in the absence of ethanol (200 mg protein/g of octyl silica). The activity of the derivatives prepared in the presence of ethanol was also significantly improved: 33,000 tributyrin units/g of catalyst at the monolayer limit, which is four- to five-fold higher than the activities of the corresponding derivatives prepared in the absence of ethanol.

Keywords: Enzyme immobilization; Lipase; Hydrophobic supports; Hydrophobicity; Mesoporous silica; Monolayer

1. Introduction

Enzymes are macromolecules of proteic nature, with molecular weights above 20,000 Da, and molecular sizes above 4 nm diameter roughly. Therefore, the textural properties of the materials to be used as support for enzyme immobilization are strongly affected by these dimensions. However, a high surface area and high porosity are necessary but not sufficient to achieve high enzyme loading and high catalytic efficiency: the chemistry of the surface and the environment is the other key factor to be considered.

Regarding morphologic characteristics, the requirements are mainly related to surface area and porosity, and (to a minor extent) also to particle size [1]. These parameters are related to each other. Thus, a small particle size involves a high surface area. However, an inappropriate porosity may prevent the use of the internal surface of the support material. Actually, the internal surface available depends on the (mean) pore diameter and the pore size distribution. Bosley and Clayton [2] studied the adsorption of lipase from *Mucor miehei* on controlled pore glass of eight different pore sizes, functionalized with methyl groups. They concluded that the larger the pore diameter, the faster the adsorption rates. According to these authors the pore diameter should be four- to five-fold the protein diameter in order to prevent restrictions to the access of the enzyme. In the case of the lipase from *Candida antarctica* B (CaLB), the longest diameter is about 7 nm, so the minimum pore diameter should be about 28 nm. But only with pore sizes over 100 nm (in the range of macropores), catalytic efficiency is independent of the pore size (defined as the ratio between catalytic activity and enzyme loading) [2].

As pointed out above, chemical interactions play a main role in the immobilization process and yield. For instance, the internal surface may not be fully used to adsorb enzyme molecules, even when pore sizes are wide enough. Two different research groups have studied the distribution of the enzyme throughout the particles of polymethyl metacrilate (macroporous) in the commercial derivative Novozym 435 (immobilized lipase from *C. antarctica* B). By direct [3] or indirect [4] methods, both concluded that the enzyme was only present in the external bark of the support beads. In these cases the authors claim that protein–protein interactions are the forces preventing

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the whole covering of the surface rather than the interactions protein–macroporous matrix.

One should also consider the possibility that the presence (or the absence) of this kind of interactions can prevent the total access of protein to the internal surface of the pores. The most extended method to perform lipase immobilization is to attach the enzymes to the surface of a support through hydrophobic interactions [5]. This matrix may be a hydrophobic material [6] or a hydrophilic one whose surface has been functionalized with hydrophobic groups [2,7–9]. Sometimes also sol–gel entrapment of lipases is performed with silane precursors with [10] or without [11] additives, yielding catalysts with different properties according to the hydrophobic/hydrophilic nature of the systems. Particularly, hydrophobic alkyl-trimethoxysilane co-precursors have been used to obtain organically modified xerogels, the best results being obtained with the highest hydrophobic systems [11]. However, in systems where the enzyme is linked to a pre-existing support, the result may depend on the structure of the particular enzyme: lipases containing hydrophobic lid (*Rhizomucor miehei*, *Humicola lanuginosa*) behaved differently than those without lid (*C. antarctica* B) [9]. But a high hydrophobicity is not always the best choice [8]; the buffered aqueous solution containing the enzyme should attain the whole surface of the support: not only the external one but also the inner surface of the pore channels. The access to the internal structure can be limited according to the wettability of the support. With a very hydrophobic surface, the contact angle with water is usually higher than 90° [11] and consequently the aqueous phase either cannot enter the pore, or it can only reach the very external part of the channel corresponding to the meniscus. In this case the catalyst particle would only contain enzyme molecules in the outer shell of the bead, whilst the inner core would remain void. This is an undesirable situation since most of the matrix surface would remain unexploited.

Coating of silica surface with octyl groups as a support to immobilize lipase from *C. antarctica* B was described elsewhere [12]. This functionalization transforms a hydrophilic material into a highly hydrophobic one, providing excellent properties to interact with the lipase and enabling high enzyme loading in a monolayer fashion. Nevertheless, such high hydrophobicity might not be a suitable environment for the enzyme dissolved in an aqueous medium to penetrate the pore channels. This leads to wonder how homogeneous the enzyme distribution is in this system. The use of ethanol to pre-wet hydrophobic supports prior to their use for lipase immobilization has been frequently reported [5], and usually mentioned only in experimental sections to facilitate the contact between the support and the buffered enzyme solution. However, comparative studies about the effect of the presence or absence of ethanol on the immobilization process and yields or on the features of the catalysts have not been found. Ethanol induces changes in secondary structure of enzymes [13–15], although a null or even positive effect of low concentrations on the activity of some enzymes has been reported [13,14]. The decrease of hydrophobicity of the microenvironment by the presence of the cosolvent and the effect on the enzyme distribution and loading on the catalysts have been studied in this work.

2. Materials and methods

2.1. Materials

Soluble lipase from *C. antarctica* fraction B (CaLB; Novozym 525) was generously donated by Novozymes Spain S.A. The silica MS3030 was a kind gift from Silica PQ Corporation (Valley Forge, PA, United States of America). Octyltriethoxysilane was from Aldrich. *p*-Nitrophenil propionate (pNPP) and glycidyl tributyrate (tributyrin) were from Sigma. Solvents (analytical grade) were all dried and deoxygenated prior to use.

2.2. Methods

2.2.1. Functionalization of the support

Silica MS 3030 was activated as described elsewhere [11] according to the method described by Weetall [16]: to 1 g of degassed silica, 75 mL of 10% octyltriethoxysilane in toluene were added. After gently stirring for 72 h, the suspension was filtered and washed and finally exhaustively vacuum dried. This support is named thereafter as octyl silica.

2.2.2. Immobilization procedure

The scheme of immobilization of lipase from *C. antarctica* on octyl silica via non-covalent hydrophobic interactions consists of several steps:

1. Addition of ethanol on the octyl silica.
2. Suspension of ethanol-silica on the enzyme solution.
3. Mild stirring of suspension. Checking catalytic activities of suspension and supernatant until loss of activity in the supernatant.
4. Filtration, washing and drying of the solids.

Detailed explanations on the procedure are given below.

One milliliter of ethanol was added to 100 mg octyl silica and was left to equilibrate in a closed vial for at least 10 min. This will be called “pre-humectation” process. Different amounts of the commercial extract of CaLB ranging from 0.04 to 2.4 mL containing from 2.5 to 60 mg protein were dissolved in 25 mM phosphate buffer pH 7.0, up to a total volume of 10 mL. The protein content of the commercial lipase extract had been previously determined by Bradford method.

After assaying the esterasic activity, the enzyme solution was added on the vial containing the silica in ethanol and kept in suspension with a helical stirrer. Aliquots from suspension and supernatant were withdrawn at 15 min intervals to assay their respective esterasic activities. Final time was determined by the lack of activity, or low constant activity of the supernatant. The suspensions were then filtered and washed three times with 10 mL volumes of 200 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 by assays of their catalytic activities (esterasic activity). After

the last washing with phosphate in a sintered glass funnel, the derivatives were suspended in 10 mL dry acetone, filtered out and vacuum dried for at least 30 min to ensure a complete drying of the catalyst.

2.2.3. Determination of enzyme activity

2.2.3.1. Esterasic 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 pNPP was followed at 348 nm in a Kontron Instruments (Watford, Herts, UK) spectrophotometer equipped with stirring devices and constant temperature capability. The cuvette contained 1.9 mL of substrate solution at 25 °C (0.4 mM pNPP 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 in the cuvette. One unit of esterase activity corresponds to consumption of 1 μmol of pNPP per minute ($\varepsilon_{pNPP} = 5150\ M^{-1}\ cm^{-1}$).

2.2.3.2. Tributyrin activity. The hydrolysis of tributyrin measures lipase activity by the liberation of butyric acid and it was monitored titrimetrically in a Mettler Toledo DL-50 pH-stat, using 100 mM sodium hydroxide. 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 unit of lipase activity corresponds to consumption of 1 μmol of NaOH per minute.

2.2.3.3. Activity loss after repeated cycles. A derivative with an enzyme loading of 200 mg g^{-1} was incubated for 1 h in acetonitrile at 40 °C under shaking at 200 rpm (10 mL acetonitrile/g of catalyst). Incubation conditions selected were the ones corresponding to a condensation reaction previously studied with an analogue derivative prepared in the absence of ethanol [12]. After this time, it was filtered and washed with dry acetone three times. Five milligrams were weighted and assayed for tributyrin hydrolytic activity, and the rest of the solid was resuspended in acetonitrile for a subsequent cycle.

2.2.4. Minimization of diffusional limitations

External diffusion may be neglected in the sufficiently stirred batch reactor. In order to diminish internal diffusion restrictions, particle sizes were reduced by abrasion: 15 mg of the immobilized preparations were suspended in 3 mL of 50 mM sodium phosphate pH 7.0 in a glass vial and submitted to vigorous magnetic stirring. The vial was kept in an ice bath in order to preserve unaltered enzyme activity. Aliquots of this suspension were withdrawn at different times and catalytic activities were determined (tributyrin assay). The activity increased with time, indicating that internal diffusional restriction decreased. The abrasion was maintained until the activity did not increase anymore, which led to consider that no restriction due to internal diffusion was left.

2.2.5. Determination of ethanol concentration

The concentration of ethanol necessary to obtain the desired effect of decreasing the hydrophobicity of the pore channels with no damage of the enzyme activity has to be determined. The activity of CaLB in solutions containing different ethanol concentrations was tested. Besides, CaLB was immobilized on octyl silica in the presence of different ethanol concentrations (with constant enzyme loading of 200 mg g^{-1}). The activities of both, soluble and immobilized enzyme preparations are shown in Fig. 1. A concentration of 10% in volume promoted the maximum activity in the immobilized derivative, whereas soluble enzyme was not affected by the cosolvent within the range of concentrations studied. Therefore 10% in volume of ethanol was set to perform the immobilization of CaLB on octyl silica in this work.

The experimental procedure to determine catalytic activities of enzyme solutions containing ethanol was as follows: 0.4 mL soluble lipase crude extract containing 10 mg protein were dissolved up to a total volume of 5 mL in sodium phosphate 50 mM containing the corresponding amount of ethanol, from 0.5 to 2 mL. Catalytic activities were assayed according to the protocols described above (hydrolysis of pNPP and tributyrin).

2.2.6. Thermal gravimetric analysis

Thermal analyses of octyl silica and a derivative with an enzyme loading of 200 mg g^{-1} were performed on a Mettler Toledo TGA/SDTA 851^e apparatus. Typically, 5 mg of the sample were heated from 25 to 1100 °C at a rate of 5 °C min^{-1} under N_2 at a flow rate of 200 mL min^{-1}.

2.2.7. Nitrogen adsorption isotherms

Nitrogen adsorption–desorption isotherms of supports and catalysts were recorded at −196 °C with a Micromeritics ASAP 2000 apparatus. Samples were previously degassed at 120 °C for 20 h. BET surface area, total pore volume and pore size distribution were obtained from data supplied.

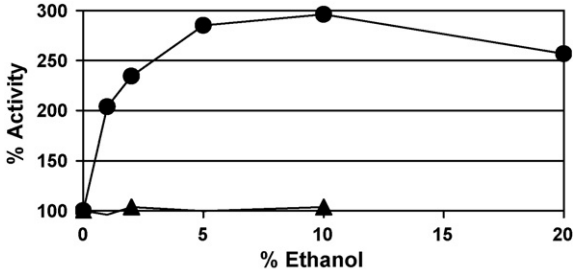


Fig. 1. Effect of ethanol concentration on the activity of CaLB. Triangles: percent activity of a CaLB solution in phosphate containing different ethanol concentrations. One hundred percent was the activity of soluble enzyme in the absence of ethanol. Circles: percent activity of 200 mg g^{-1} derivatives prepared in the presence of different ethanol concentrations. One hundred percent was the activity of the derivative prepared in the absence of ethanol. Activity was determined in tributyrin hydrolysis.

2.2.8. 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 [5,6], 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:

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

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

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

$$\ln Q_e = \ln K_F + \frac{1}{n} \ln C_e \quad (2)$$

where K_F (mL g^{-1}) 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.

3. Results and discussion

3.1. Immobilization of lipase and effects of ethanol

The presence of activity in the suspension during and after the immobilization process and the absence of activity in the supernatant after this process indicate that the lipase has been linked to the octyl silica. The linearity between initial enzyme loadings and catalysts activity until 400–500 mg/g proofs sufficiently that the enzyme has been successfully immobilized on the support. Furthermore, the presence of enzyme within the silica matrix was also proved by the different behavior of the octyl silica compared to a lipase derivative in thermogravimetric analysis. Both samples underwent a weight loss between 400 and 600 °C, corresponding to the octyl groups. But only the one containing lipase displayed a weight loss of 14% between 200 and 400 °C, which correlates to its enzyme loading (200 mg g^{-1}).

We had previously observed how octyl silica was very difficult to suspend in an aqueous enzyme solution. On the contrary, when the loading capacity of the same solid pre-humected with ethanol was studied, it was perfectly suspended in the enzyme solution. The activity of lipases and their ability to be non-covalently immobilized on hydrophobic supports is known to be closely related to the hydrophobicity of the environment. Likely, the presence of ethanol may contribute to a rearrangement of the secondary structure involving a higher accessibility of some hydrophobic side chains of amino acids, which in such less polar medium become more exposed to the surface of the protein. This may be deduced from the changes in the fluorescence spectrum reported for other enzymes in different ethanol concentrations [13,15]. Consequently the immobilization on an octyl-functionalized surface should be improved. On the other hand, catalytic activity of soluble lipase in the presence of low ethanol concentrations (up to 10%) was not negatively affected by these changes, as seen in Fig. 1. As mentioned in the Methods section (2.2.5), the derivatives with the same enzyme loading (200 mg g^{-1}) prepared in the presence of different ethanol concentrations underwent increases in their final activities. This suggests a positive effect of ethanol, which is maintained within all this range. More interestingly, when these derivatives were compared to the previous ones prepared in the absence of ethanol at different enzyme loadings [12], the activities were significantly higher (Fig. 2). The maximal activity achieved without ethanol was 6700 tributyrin units/g (enzyme loading 600 mg g^{-1}), whereas in the presence of ethanol derivatives with 37,000 tributyrin units/g were obtained (enzyme loading 500 mg g^{-1}).

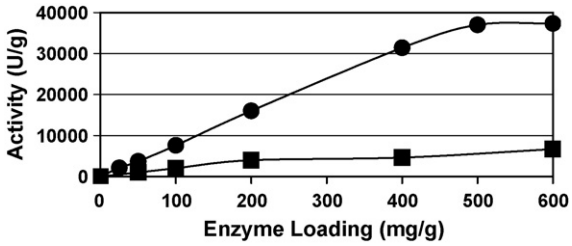


Fig. 2. Effect of the presence of ethanol on the activity of the derivatives prepared with increasing enzyme loading. Enzyme loading is given in mg protein per gram of silica. Catalytic activity is given in tributyrin units per gram of catalyst. Squares: immobilization in the absence of ethanol. Circles: immobilization in the presence of ethanol.

The possibility of enzyme losses due to desorption from the support is very likely to occur in aqueous reaction media, provided the non-covalent nature of the enzyme–support linkage. However, lipases are generally used as catalysts in organic and nearly anhydrous media, where leakage is not expected to occur. Therefore the stability of our derivatives was tested through incubation in organic medium simulating the conditions of a reaction during subsequent cycles. Residual activities between 100% and 80% were kept during at least nine subsequent incubation/reaction cycles. The stability shown enables these derivatives to be considered for industrial applications.

3.2. Immobilization: mono or multilayer

The relationship between the activity of derivatives and their respective enzyme loadings is linear until high loading values. Catalytic efficiency is defined as the ratio between the activity and the load of an immobilized enzyme; this relationship is kept constant for the loading values where the linearity is maintained, and it is worth to be determined. Fig. 3 shows the catalytic efficiency for the series of immobilized CaLB derivatives prepared on octyl silica in the presence of EtOH. The value remains constant until approximately 400–500 mg g^{-1} and decreases for higher enzyme loadings.

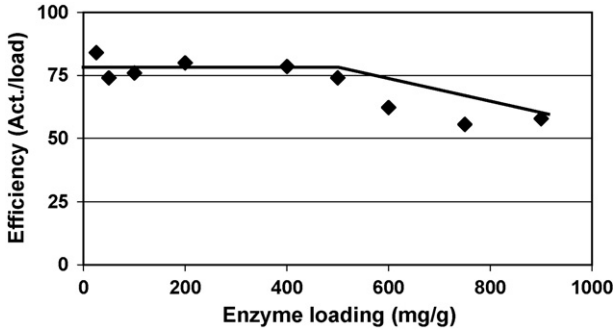


Fig. 3. Catalytic efficiency of the derivatives prepared with increasing enzyme loading in the presence of ethanol. Enzyme loading is given in mg protein per gram of support. Efficiency (in tributyrin units per mg of enzyme) is given as the ratio between the activity (in tributyrin units per gram) and the enzyme loading of each catalyst.

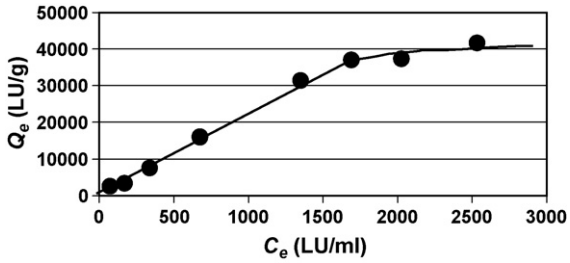


Fig. 4. Adsorption isotherm of CaLB on octyl silica. Lipase immobilized on octyl silica in the presence of ethanol vs. lipase in the corresponding enzyme solutions. Q_e: enzyme loading in lipase units per gram of support (tributyrin assay). C_e: lipase in the liquid phase in lipase units per mL of solution (tributyrin assay). See Eq. (1).

Table 1
Values of Langmuir and Freundlich constants for *Candida antarctica* B lipase immobilized on octyl silica pre-wet with ethanol

Langmuir constants			Freundlich constants	
K_L (mL g^{-1})	a_L (mL mg^{-1})	K_L/a_L (LU g^{-1})	K_F (mL g^{-1})	1/n
34.48	10.45×10^{-4}	33,000	55.43	0.857

Adsorption isotherms, i.e., enzyme in solution versus immobilized enzyme helps to clarify the more plausible scenario [6].

The pattern of immobilization of aggregates is represented by Freundlich isotherm (Eq. (2)), represented in Scheme 1, situations a and b. The aggregates may enter the pore and become immobilized in this form (a). Also, dispersed enzyme may enter the pore once the surface of the channels is fully covered by a monolayer of enzymes. The new molecules may interact with the former giving rise to a multilayer (b). In both cases the activity of derivatives would increase with growing enzyme concentration, but not in a linear fashion.

Langmuir isotherm (Eq. (1)) represents a system where no aggregation occurs and immobilization takes place in a monolayer. Activity remains constant despite enzyme concentration in solution increases over the monolayer limit because these additional molecules are not linked, so no more active centers are incorporated to the derivatives. This situation is represented in Scheme 1(c).

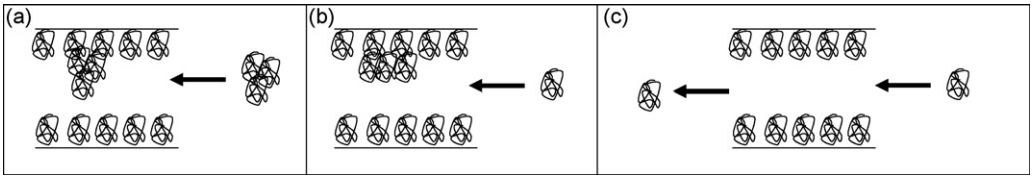
The relationship between the activity of the initial enzyme solution (lipase units per mL) and the final activity of the catalysts prepared (lipase units per gram of catalyst) shows a saturation shape as shown in Fig. 4, corresponding to Langmuir isotherm. By linearization of the Langmuir representation according to the expression (1) and the Freundlich isotherm according to the expression (2), the constants of Langmuir and Freundlich were determined. These values are shown in Table 1.

The results indicate that the monolayer capacity of ethanol pre-wet octyl silica is 33,000 lipase (tributyrin) units. By extrapolation from the data shown in Fig. 2, this activity corresponds to an enzyme loading between 400 and 500 mg protein/g of silica.

These figures contrast with the results previously reported [12], where the monolayer on octyl silica (without ethanol) was achieved with 200 mg g^{-1} enzyme loading. The presence of ethanol not only doubled the monolayer capacity of the support, but also had an effect on the heterogeneity factor of the surface: 1/n (0.675 in the absence of ethanol) increased to 0.857. In other words, ethanol also made the surface more homogenous. The activity of derivatives prepared in the presence of ethanol was higher than that of the corresponding ones with the same enzyme loading prepared in the absence of ethanol, as mentioned above (see Fig. 1).

3.3. Morphologic differences

Nitrogen adsorption isotherms were performed in order to explain the change in monolayer capacity (Fig. 5, outlet). Unmodified matrix (Si), octyl-functionalized silica (Si-oc), and different enzyme-linked octyl silica derivatives were compared. Despite monolayer capacity of derivatives prepared in the absence of ethanol was 200 mg g^{-1} (previously determined) derivative 400N was prepared with an initial enzyme loading of 400 mg g^{-1} in the starting aqueous enzyme solution with no ethanol. However, catalytic efficiency of this catalyst was low and the immobilization yields were poor, indicating that likely, less than 400 mg g^{-1} had been linked to the support (probably



Scheme 1. Immobilization of the enzyme in aggregate or dispersed form. (a) Aggregates formed in the external solution. (b) Aggregates formed within the pores. (c) No aggregation.

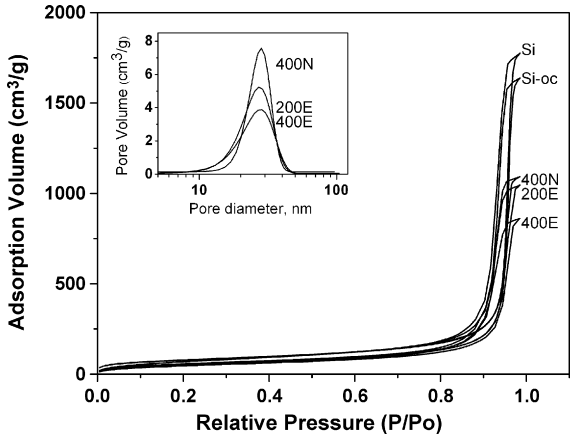


Fig. 5. Nitrogen adsorption isotherms. Unmodified silica (Si), octyl silica (Si-oc) and different lipase-octyl silica derivatives: 400N: derivative with an enzyme loading of 400 mg g^{-1} immobilized in the absence of ethanol. 200E and 400E: derivatives with enzyme loadings of 200 and 400 mg g^{-1}, respectively, immobilized in the presence of ethanol. Inset: pore size distribution of derivatives 400N, 200E and 400E.

no more than the loading corresponding to the monolayer, 200 mg g^{-1}). Two derivatives were prepared in the presence of ethanol: one with an enzyme loading lower than the limit capacity in these conditions (derivative 200E, with 200 mg g^{-1}) and another one with an enzyme loading corresponding to the limit capacity (derivative 400E, with 400 mg g^{-1}).

Textural properties of all these samples calculated from the nitrogen adsorption isotherms are quantified and displayed in Table 2. Mean pore size of octyl silica is 24 nm, which is close to the 28 nm necessary to ensure the absence of restrictions to the access of the enzyme, as pointed out in Section 1. The available surface area diminishes as the surface is covered by octyl groups and furthermore by the enzyme molecules. The assumption that only 200 mg were likely linked in 400N is supported by the fact that surface areas of 400N and 200E show very close values. The available surface area of 400E is lower than the corresponding to 400N and 200E, as expected, because of the higher monolayer capacity previously determined. Accordingly, pore volume and pore size of 400N and 200E are also very close, and larger to the one of 400E. Thus, there are two systems (presence or absence of ethanol) where monolayer capacities are different, and where textural properties seem to be the same only when the same amount of enzyme is immobilized in each one.

Some questions arise from these results: how can the monolayer increase in a 100% value? Why are the textural properties

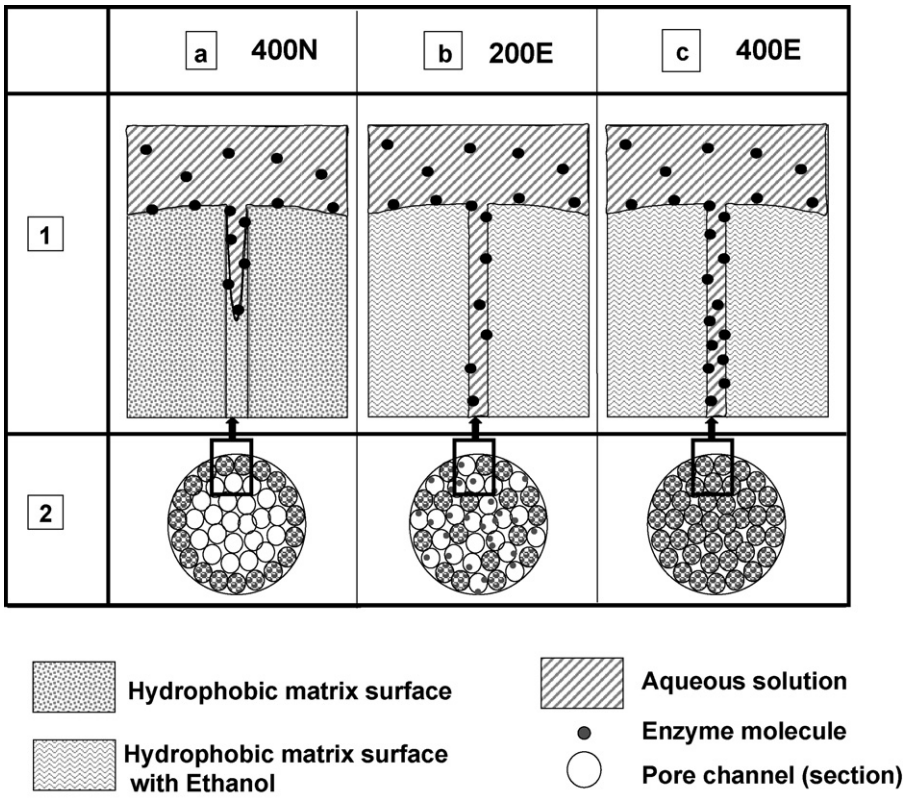
Table 2
Textural properties of the support and derivatives

	BET surface area (m^2 g^{-1})	Mean pore size (nm)	Pore volume (cm^3 g^{-1})
Si	275	27	2.72
Si-oc	264	24	2.34
400N	206	22	1.68
200E	205	21	1.61
400E	177	20	1.32

the same further monolayer limit conditions (400N) and far below monolayer (200E)? Or, in other words: how can the pore volume be the same with a monolayer and with half the molecules necessary for a monolayer? The pore size distribution curves (Fig. 5, inset) may help to answer these questions. Whereas the pore volume is the same for 400N and 200E (see Table 2), there is some difference in the shape of the pore size distribution peaks, which width are 11 and 16 nm, respectively. This seems to suggest that the enzyme might tend to be randomly spread on 200E (wider peak), because the monolayer is not yet achieved, so the total pore volume would correspond to pores with very different diameters. On the other hand, if the same amount of enzyme is distributed in a monolayer in derivative 400N (narrower peak), this probably means that there is a local high (or maximum) density area. But there must be some other areas mostly not attained by the enzyme, suggesting that there might be two major populations in the channels: one with a monolayer of enzyme (full) and another one with hardly any enzyme molecules (void). Thus, total pore volume would correspond to the mean value of both, full and void pores, which is compatible with a narrow distribution of pore sizes.

This uneven enzyme distribution due to the presence or absence of ethanol can be understood by the difficulty of access of the aqueous enzyme solution to the pore channels with highly hydrophobic surfaces. This hypothesis is explained in Scheme 2(1). The presence of a miscible solvent (ethanol) (1b and 1c) even in a low concentration is enough to create a less hydrophobic environment that allows the aqueous solution to penetrate the pores. Therefore the whole internal surface of the pore channels is available to anchor enzyme molecules. If the amount of enzyme is lower than the monolayer capacity (derivative 200E), the molecules distribute randomly along all the internal surface of the pore channels (1b). Whereas if the enzyme loading rises to 400 mg g^{-1} (400E), the enzyme molecules uniformly cover the total surface and the monolayer is formed (1c). A transversal section of the catalysts beads is represented in Scheme 2(2). Fraction b would correspond to derivative 200E, where pores would still be ready to hold more enzyme molecules. Instead (2c) would represent the monolayer of enzyme molecules on the derivative 400E.

On the other hand, when the environment is highly hydrophobic (derivative 400N) the aqueous solution would form a meniscus at the entrance of the pore channels, so it could not penetrate any further (1a). The enzyme in this solution can only reach the external part of the pores, where it is very likely that a high local enzyme loading is achieved, having a similar density to the one in derivative 400E (for 400 mg g^{-1}). Then, the monolayer may be rapidly formed, limited to only this external part of the particle whereas the internal one would hardly contain any enzyme molecule. The total enzyme loading (200 mg g^{-1}) would be the average between the maximum density in the external bark and a very small or null density in the internal part of the silica bead. The transversal section of the catalyst particle may be represented in Scheme 2(2a).



Scheme 2. Distribution of enzyme in the pore channels. (a) Immobilization in the absence of ethanol. (1a) 400N: derivative prepared in the absence of ethanol with an enzyme loading and monolayer capacity of 200 mg g^{-1}. Partial access of enzyme in aqueous solution to the pores. (2a) Only the external core of silica beads can carry enzyme molecules. (b) and (c) Immobilization in the presence of ethanol. Total access of enzyme in aqueous solution to the pores. (1b) 200E: derivative prepared in the presence of ethanol with an enzyme loading of 200 mg g^{-1} (below monolayer). (2b) Enzyme molecules can be located anywhere inside the pore channels. (1c) 400E: derivative prepared in the presence of ethanol with an enzyme loading and monolayer capacity of 400 mg g^{-1}. (2c) Maximum density of enzyme molecules in a monolayer.

4. Conclusions

The decrease of the hydrophobicity in the internal pore surfaces by the cosolvent enables total access to the aqueous enzyme solution, so ethanol acts as an intermediate between the polar (enzyme solution) and the apolar (hydrophobic support) fractions of the system. The yields of immobilization of lipase through hydrophobic interactions on octyl silica in these conditions have been improved. This has been possible because the monolayer capacity for lipase immobilization of a highly hydrophobic support has been increased in a two-fold factor by the use of 10% ethanol to pre-wet the matrix.

Besides, the specific activity of the catalysts obtained in a monolayer may be as high as 33,000 units/g in trybutirin hydrolysis assay. This unusually high activity for immobilized CaLB derivatives provides nice expectancies to their industrial applications.

On the contrary, in the absence of ethanol the enzyme can only reach the external end of the highly hydrophobic pores. The available surface area becomes then significantly diminished and consequently the monolayer capacity is also lower. In these systems, the enzyme density would be very low in the internal core, but very high in the external bark, with a monolayer covering just this external fraction of surface area of the support.

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