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Immobilization in quartz fiber felt reinforced silica aerogel improves the activity of *Candida rugosa* lipase in organic solvents

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Abstract

Candida rugosa lipase is a very useful catalyst, but its rapid inactivation by simple alcohols is a drawback. The present study was focussed on the encapsulation of this enzyme in silica aerogels reinforced with quartz fiber felt. The activity of the immobilized lipase in an organic solvent could be significantly improved over that of the free enzyme and of previous immobilization techniques, by evaporating the alcohol formed during a pre-hydrolysis of the silica precursor, before adding the aqueous enzyme solution. The alcohol evaporation technique was previously used by other authors to immobilized enzymes, but applied to xerogels dried by evaporation, while in the present case the wet gels obtained were dried by the CO_2 supercritical method to obtain aerogels. Besides, such silica aerogels were also reinforced by impregnating a commercial ceramic quartz fiber felt of *St. Gobain* with the silica sol containing the enzyme, before gelation. The ceramic composites heterogeneous biocatalysts obtained could be used for a large number of times without any apparent deterioration.

Keywords: Lipase from *Candida rugosa*; Esterification; Aerogels; Ceramic fiber felt reinforcement

1. Introduction

Some enzymes are very resistant to solvents such as simple alcohols (methanol, ethanol). This is the case of the lipase from *Burkholderia cepacia* which was the focus of our previous research [1–3]. Hence, this lipase could very easily be incorporated inside solid silica porous gels, by mixing the enzyme with the liquid solution containing the silica precursors and reactants used to make a silica gel, before gelation. Such enzymes resist rather well to the alcohol produced by hydrolysis and condensation of the silica precursors, during the chemical process where the silica gel network is built. This is one of the key points which made it possible to easily incorporate a number of lipases in sol–gels where their specific activity could be magnified. Sol–gel materials comprise xerogels, which are gels dried by evaporation with important shrinkage [4], ambigels which are dried by evaporation without shrinkage because they contain a high concentration of hydrophobic groups and they are poorly wetted by polar liquids [5], and aerogels which are dried without (or with limited) shrinkage by the CO_2 supercritical method [1].

On the other hand many other enzymes, including the lipase from *Candida rugosa* [6], are inactivated by simple alcohols. In order to incorporate these enzymes with an acceptable activity in inorganic gels, it is then necessary to adjust the gelation chemistry. An elaborate technique consists in performing a transesterification of the silica precursors with a higher order alcohol such as glycerol, to obtain a new silica precursor which does not produce a harmful alcohol during the gelation process [7]. Or it is possible to use sodium silicates instead of alkoxides as the precursors of silica [8]. Nevertheless, one of the most simple technique is to prehydrolyze the silica precursors in conditions where hydrolysis is fast and condensation slow (acidic catalysis) and to evaporate the alcohol produced during this step before adding the enzyme [9]. This was the method used in the present study with the lipase from *Candida rugosa*. This lipase is a bulky enzyme with molar mass $\approx$60,000 g mol^{-1} [10], useful in the resolution of chiral esters [11]. Its inactivation in organic solvent has been studied by fluorescence [12]. Previous studies on the encapsulation of this lipase from *Candida rugosa* in silica aerogels without alcohol evaporation, ended in an activity at best comparable with the free enzyme [2,13]. Other recent immobilization techniques by adsorption, including on silica gel, resulted in a loss of activity [14,15]. In the present technique, methanol evaporation

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was combined with aerogel encapsulation by CO_2 supercritical drying.

Moreover, some of the silica aerogels were mechanically reinforced by impregnating a quartz ceramic fiber felt with the silica sol containing the enzyme, before gelation. The ceramic composites biocatalysts obtained were applied in a standard esterification reaction of lauric acid by 1-octanol, in isooctane.

2. Experimental part

2.1. Materials and methods

The chemicals used in this work were tetramethoxysilane (TMOS) and isooctane from Fluka, methyltrimethoxysilane (MTMS), lauric acid and 1-octanol from Aldrich. The lipase AYS (lot LAYY055046S) from the *Candida rugosa* (termed CRL) was purchased from Amano. Quartzel® silica fiber felt mat (80 g m^{-2}, dry mat uncompressed thickness ≈11 mm) from the St.-Gobain company was used to reinforce the aerogel monoliths.

The lipase, either free or encapsulated in fiber felt reinforced or in un-reinforced aerogel, as described in next section, was applied in the esterification of lauric acid (HOLau) with 1-octanol (octOH), at 30 °C, in 10 ml isooctane, under constant agitation, in a shaking bath at 180 rpm. This reaction was used as a model in previous studies with the lipase from *Burkholderia cepacia* (BCL) [1,3,16]. In all these tests, including for encapsulation, the lipase was used in the as purchased powder state from Amano. Nevertheless before a test, all reactants and enzyme (free or encapsulated) were equilibrated during ≈48 h at a water thermodynamic activity $a_w = 0.81$, with water saturated ammonium sulphate salt. The formation kinetics of the reaction products was followed by analysis in a gas chromatograph (GC). The catalytic activity was determined as the specific initial ester formation rate per mg of enzyme powder v_0 in U mg^{-1}, where 1U = 1 μmol(ester formed) min^{-1}. The chromatograph was a Shimadzu GC-14B, equipped with a capillary polar column from SGE (ref: 12QC2/BP21 0.25), with the following characteristics: length 12 m, internal diameter 0.22 mm, external diameter 0.33 mm. The heating program was as follows: hold 5 min at 100 °C, followed by heating at 10 °C min^{-1} up to 190 °C, ending with 4 min hold at 190 °C. The carrier gas was nitrogen, the injector temperature 220 °C, the detector temperature 220 °C and the detector a flame ionisation detector (FID).

In real industrial applications, a solid biocatalyst is intended to be extensively recycled, and possibly to be used successively in different conditions. Hence, for a given type of reinforced aerogel made from a given MTMS content, the reaction kinetics were studied with a set of only 3 reinforced samples. In detail, as an example for aerogels synthesized from 40% MTMS, 3 samples termed 40A, 40B and 40C were synthesized. Sample 40A was tested for lauric acid concentrations [HOLau] = 0.05 mol l^{-1} and successive 1-octanol concentrations [octOH] = 0.01; 0.02; 0.03; 0.04; 0.05; 0.06; 0.07 mol l^{-1}. Then it was tested at [HOLau] = 0.2 mol l^{-1} for the same series of 1-octanol concentrations, and finally for [HOLau] = 0.4 mol l^{-1} and again the same series of 1-octanol concentrations. Moreover, each couple of lauric acid and 1-octanol concentrations was successively repeated, before passing to the next set of substrate concentrations. Hence, overall, sample 40A was submitted to 42 successive tests. Also, in between two tests, each sample was abundantly washed in clean isooctane, until the isooctane rinse showed no significant concentration of either substrates or product. With sample 40B, a similar pattern of tests was followed regarding lauric acid, except that the succession of 1-octanol concentrations was 0.07; 0.08; 0.09; 0.1; 0.15; 0.2; 0.25 mol l^{-1}. At last, with sample 40C, the succession of 1-octanol concentrations were 0.3; 0.35; 0.4; 0.45; 0.5; 0.01 mol l^{-1}. Similarly, 3 reinforced aerogel samples termed 60A, 60B and 60C were synthesized from 60% MTMS and tested in the same set of conditions as those studied with aerogels synthesized from 40% MTMS. The aim of this experimental plan was to obtain a realistic view of the kinetic behaviour of catalysts in repeated industrial tests.

2.2. Aerogel encapsulation technique

Two different shaping of aerogels encapsulated lipase were studied. They only differed by the size of an aerogel sample and the presence of a quartz fiber felt reinforcement inside this aerogel. A first shaping consisted of un-reinforced relatively large aerogel monoliths (diameter ≈11 mm and dry height 4.5–5 mm). In a second shaping, small cylindrical pieces of quartz felt (diameter ≈4 mm, length ≈11 mm) were placed in Teflon® tubes of inside diameter ≈4.8 mm, before casting the silica sol which completely impregnated the felt. A same total volume of silica sol as previously used for unreinforced monoliths made it possible to impregnate 5 such felt samples. These reinforced samples were extruded out of the Teflon tubes after gelation, to give small cylindrical reinforced aerogel particles of length ≈11 mm and diameter ≈4.8 mm. The un-reinforced samples were very useful to conveniently study the gelation kinetics and gel characteristics, independently from the fiber felt. On the other hand, reinforced samples were applied to study the kinetics and recycling behaviour, because it was previously found such composites could be used repeatedly without any significant mass loss or apparent integrity deterioration [17].

With both types of aerogel shaping described above, identical series of silica sols containing the lipase were used. These sols were made from mixtures of MTMS and TMOS, with a series of MTMS molar ratio (0, 20, 40, 60, 80 and 90%). For instance with 20% MTMS, 11 silica sol samples with volume ≈500 μl per sol were made by mixing TMOS (4.5040 g), MTMS (1.0504 g) neutral H_2O (5.4360 g) and H_2O acidified with HCl at pH 2.8 (0.6946 g) for 10 min. During this first step, the silica precursors were mostly hydrolyzed, while very limited silica condensation occurred. Hydrolysis was an exothermal reaction which produced a homogeneous transparent silica sol. In a second step, the methanol liberated during the previous hydrolysis step was evaporated in a rotating evaporator, at 30 °C and 100 rpm, during 10 min. It produced a translucent and viscous homogeneous liquid. Because not only the alcohol but also some water evaporated during this process, distilled water was added after cooling, to approximately recover the total initial solution volume. The sil-

ica sols obtained are presently termed pre-hydrolyzed silica sols. Separately, a CRL solution was prepared by dissolving a given mass of CRL powder from Amano in Tris–HCl buffer at pH 6.5. Each gel sample was prepared by mixing identical volumes (typically ≈500 μl) of an enzyme solution and the pre-hydrolyzed silica sol.

Moreover, for the un-reinforced aerogels, two types of enzyme solutions were made by dissolving an increasing mass of the CRL powder (from 0 to 150 mg), either in 500 μl phosphate buffer at pH 6.5 (series 1), or in 450 μl deionized water H_2O to which 50 μl of a 0.2% NaF solution (series 2). This step was studied to select an appropriate gelation protocol of MTMS, a silica precursor otherwise difficult to transform to a gel. Other similar gels were made by changing the proportions of MTMS to 0, 40, 60, 80 and 90%. At last, for each type of sols, a range of increasing enzyme loads were encapsulated, by using lipase solutions of increasing concentration.

With the reinforced samples, only the sol protocol using NaF was retained, and a silica sol containing a total of ≈20 mg of enzyme was always used to synthesize one catalytic sample. With this enzyme load, one catalytic test sample actually comprised 5 small reinforced aerogel cylindrical particles, for a total dry mass of ≈300 mg, including a total quartz felt mass ≈39 mg (≈7.8 mg per particle).

Before CO_2 supercritical drying, all samples were covered with 2.5 ml pH 6.5 buffer during ≈36 h, to condition the enzyme in an appropriate pH. This operation was followed by dialysis in acetone for ≈24 h and drying by the CO_2 supercritical method, according to a procedure previously described [1].

3. Results and discussion

Regarding the gelation kinetics, Fig. 1 shows that addition of the enzyme powder and of NaF both decreased the gelation time of unreinforced samples, while the addition of MTMS increased this gelation time. NaF brings F^- anions which are good Lewis base catalysts of silica condensation from MTMS. Hence this gelation catalyst was retained to synthesize the reinforced samples. The wet unreinforced gels were transparent at low enzyme and MTMS loading. They turned more opaque with an increasing enzyme and/or MTMS loading. In any way they all turned white opaque after supercritical drying. The dry reinforced samples were themselves small white cylindrical particles, with excellent elastic properties. They could be repeatedly and reversibly compressed between fingers without any apparent deterioration. Determination of the protein concentration in the buffer used for aging the gels according to the BCA reagent test® from Pierce, showed that ≤10% of the proteins in the 20 mg enzyme powder was washed out of the gel.

For each type of aerogel made with a given MTMS content, the total initial formation rate of the ester octyl laurate (octOLau), was found to increase linearly with the initial enzyme load in the lipase solution used to make the gel, in a range up to more than 50 mg. As a result of this study, further catalytic kinetics investigations with reinforced aerogels were carried out with a total enzyme load of 20 mg for each aerogel sample (itself comprising 5 small reinforced particles as previously stated).

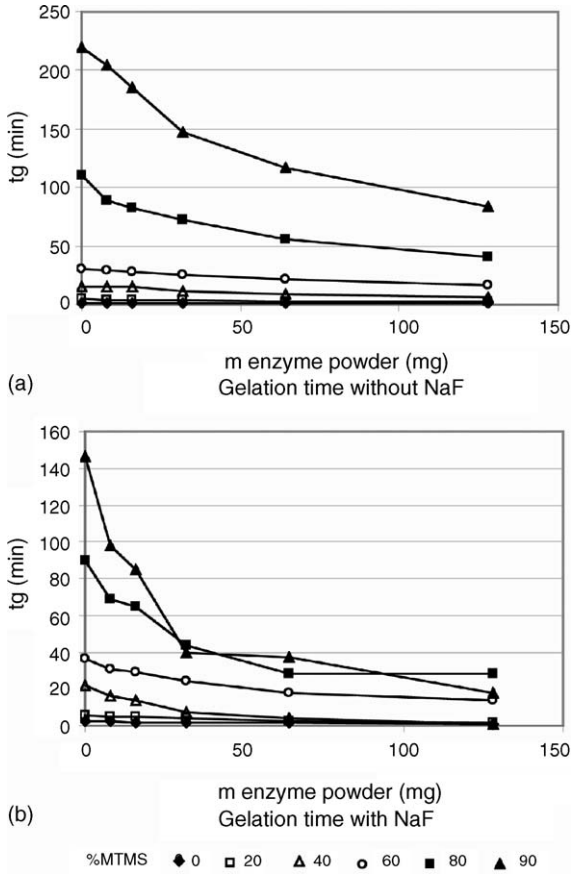

Fig. 1. Gelation time of gels made with increasing enzyme load and MTMS content: (a) without NaF and (b) with NaF.

The specific initial rate per mg of enzyme powder encapsulated, determined from these initial linear data, was found to be higher with the encapsulated enzyme than with the free enzyme, with all aerogels except the aerogel made from 0% MTMS (Fig. 2). Amongst aerogels where no NaF was added as a condensation catalyst of silica, the aerogels made from 40% MTMS showed the highest specific activity, while the most active aerogels were made from 60% MTMS when NaF was used to condense silica (Fig. 2). The data in Fig. 2 suggest that for the aerogels made

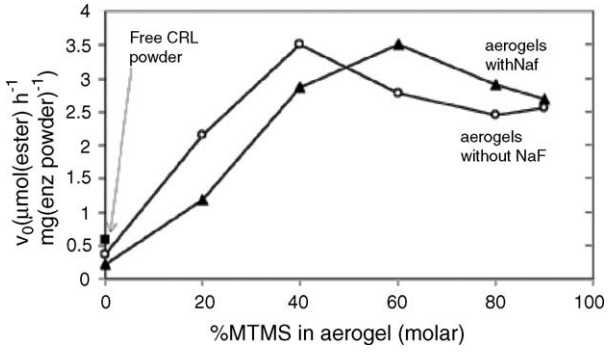

Fig. 2. Specific initial esterification rate as a function of the %MTMS used to synthesize the aero gels, per mg of enzyme powder originally added to the preparation. Comparison of gels made without NaF or with NaF and the free enzyme (reaction of 0.5 mmol HOLau + 1 mmol octOH at 30 °C, in 10 ml isooctane, 20 mg enzyme powder).

with up to 40% MTMS, the F^- anions may possibly have a minor inhibition effect on the enzyme. Nevertheless NaF was not soluble in isooctane so that it was not possible to directly test such an inhibition with the free enzyme. On the other hand, in the aerogels made from 60% MTMS, this possible inhibition was well compensated for by a more extensive condensation of this precursor.

Transmission electron micrographs (TEM) of aerogels synthesized from 40% MTMS, without enzyme or with 128 mg enzyme powder, at a magnification of 250,000, showed the very open porous texture of the aerogels. The aerogel containing the enzyme had a slightly coarser structure (Fig. 3). No enzyme or enzyme agglomerates could be observed by this technique, which was to be expected given the low atomic mass of atoms composing the protein. Nevertheless, the texture of the aerogel which contained the enzyme appeared uniform, and the size of the largest mesopores (i.e. a few nm) fitted the size of an individual enzyme molecule. Hence, the TEM observations supported the view that the enzyme was well dispersed inside the gel.

The specific surface area and pore volume of reinforced aerogels synthesized from 40 and 60% MTMS are reported in Table 1. They were determined by the Brunauer, Emmett and Teller method [18]. The pore size distributions were determined by the Roberts method [19] from the desorption branch of the adsorption isotherms. They are reported in Fig. 4. Aerogel synthesized from 60% MTMS resulted in a higher pore volume and larger mesopore radius, than aerogel synthesized from 40% MTMS.

Table 1
Specific surface area A_{sp} and pore volume V_{sp} of reinforced aerogels determined from nitrogen adsorption isotherms

MTMS (%)	A_{sp} ($m^2\ g^{-1}$)	V_{sp} ($cm^3\ g^{-1}$)
40	810	1.49
60	690	1.77

Regarding the kinetics study on reinforced aerogels, each test conditions defined by one set of 1-octanol and lauric acid molar concentrations was repeated twice successively with the same sample, before recycling this sample to the next test conditions. Generally, the difference between the initial esterification rate of the 2 repeated tests was $\leq$2%. The average of these 2 values was reported in Fig. 5. The successive ranking of the test conditions in which a sample was recycled is well illustrated in Fig. 5a, which gathers all data obtained successively with sample 40B synthesized from 40% MTMS. This figure shows that the

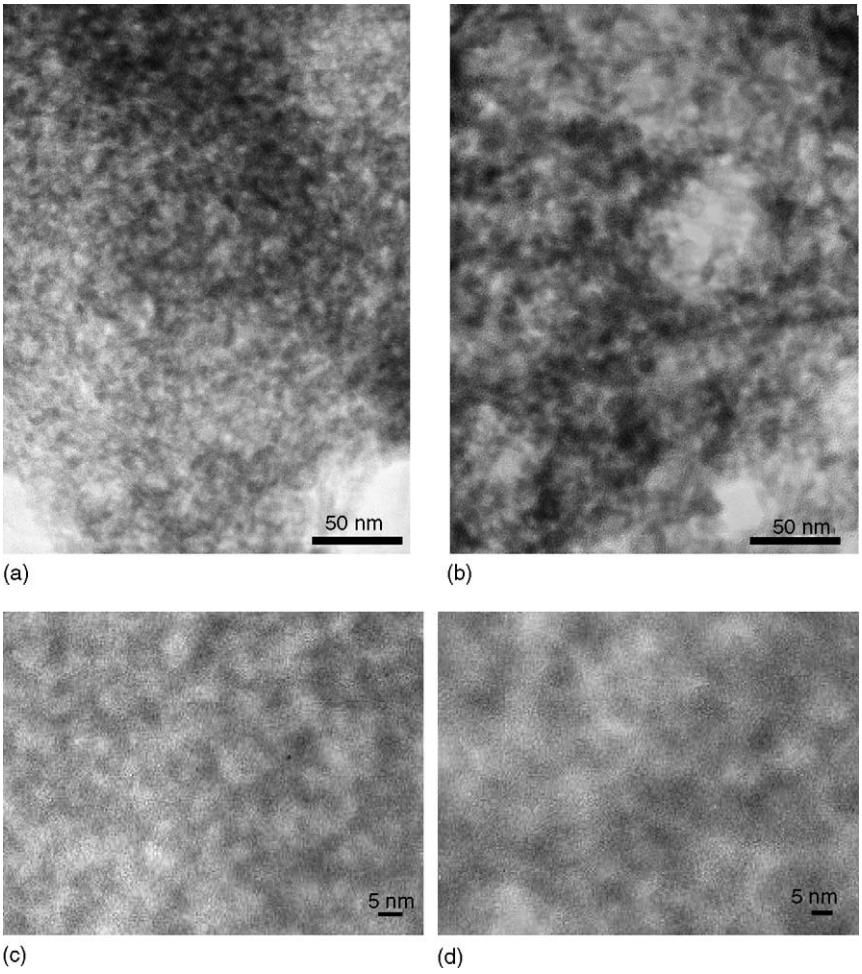

Fig. 3. TEM micrographs of silica aerogels made from 40% MTMS: (a and c) without enzyme, (b and d) with 128 mg CrL powder.

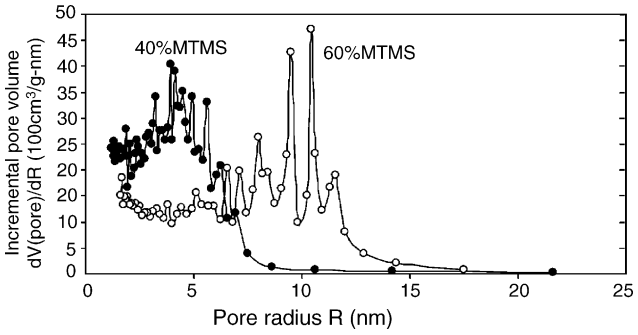

Fig. 4. Pore size distributions of fiber felt reinforced aerogels made from 40 and 60% MTMS.

activity of this sample increased drastically when it was recycled from test conditions 7 (molar concentrations of substrates [HOLAu] = 0.05 mol l^{-1}, [octOH] = 0.7 mol l^{-1}]) to test conditions 8 ([HOLAu] = 0.2 mol l^{-1}, [octOH] = 0.7 mol l^{-1}]). Such results indicated that the sample was not obviously inactivated after being recycled 14 times (conditions 1–7, each repeated twice). On the other hand, for 2 apparently identical samples tested in the same conditions, but with a different past history, the difference can be quite drastic. This is well illustrated in Fig. 5b, for sample 40B (test ranking #14) and sample 40C (test ranking #8), which both correspond to the same tests conditions ([HOLAu] = 0.2 mol l^{-1}, [octOH] = 0.7 mol l^{-1}]), but which had a quite different previous testing history. Actually, this dispersion in activity increased significantly with the molar proportion

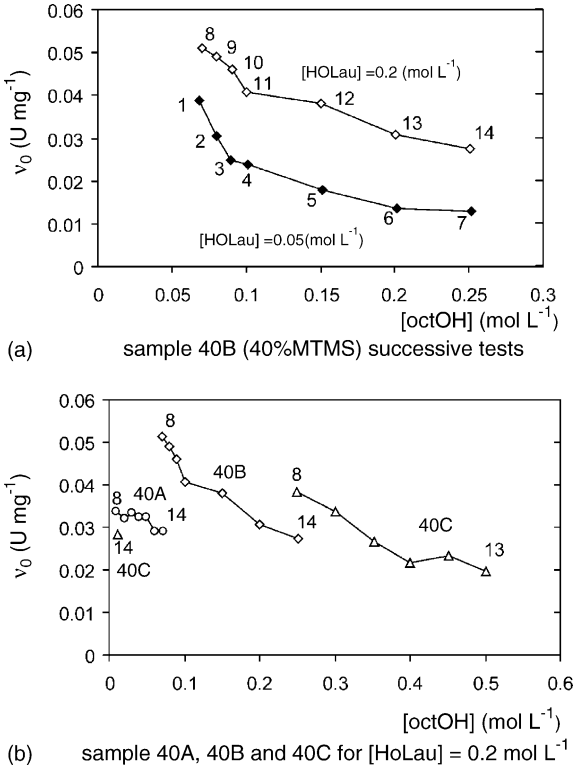

Fig. 5. Details of the tests made with each sample synthesized from 40% MTMS: (a) sample 40B. The numbers indicate the rank of each test condition; (b) samples 40A, 40B and 40C for a molar concentration of lauric acid [HOLau] = 0.2 mol l^{-1}.

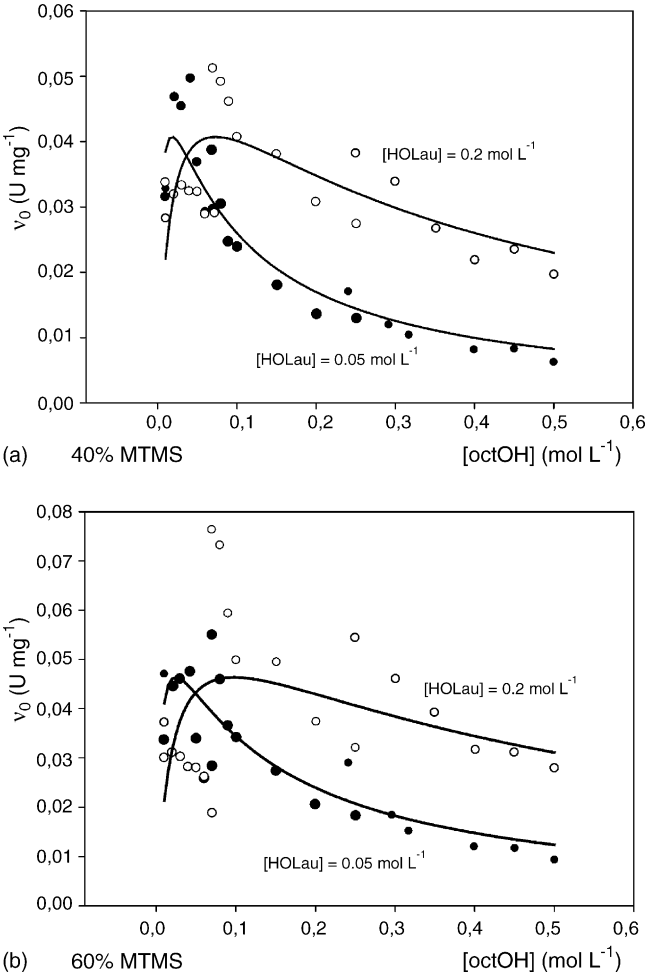

Fig. 6. Initial specific esterification rate of 1-octanol with lauric acid, as a function of the 1-octanol and lauric acid concentrations, in lipase encapsulated in aerogels made from (a) 40% MTMS and (b) 60% MTMS. The experimental data points and best theoretical curve fitting with a Bi–Bi Ping Pong law for the kinetics parameters given in Table 2, are reported.

of MTMS used to synthesize the aerogel and with the concentrations of lauric acid, as a comparison of Fig. 6a and b shows.

For a given type of aerogel (e.g. made from 40% MTMS, Fig. 6a), the dispersion in the complete series of experimental data points was such that an acceptable statistical fit with a simple kinetic model could not be found. Nevertheless, some significant range of values for the kinetics constants (Table 2), could be determined by applying a model previously applied to another lipase from *Burkholderia cepacia* [20]. In this model, applicable when inhibition by both substrates is strong, the specific initial

Table 2
Kinetics parameters determined by best fitting of the experimental data points in Fig. 6 with kinetics equation (2), as a function of the %MTMS from which the aerogels were synthesized

MTMS (%)	V_{max} (U mg^{-1})		k'_{al}		k'_{ac}	
	Average	Standard deviation	Average	Standard deviation	Average	Standard deviation
40	0.059	0.00742	0.0882	0.033	0.613	0.165
60	0.066	0.0134	0.103	0.0649	0.432	0.203

ester formation rate per mg of enzyme powder v_0 in U mg^{-1} is given by:

$$v_0 = \frac{V_{max}[ac][al]}{k'_{ac}[al][al] + k'_{al}[ac][ac] + [ac][al]} \quad (1)$$

where V_{max} is the maximum esterification rate (in U mg^{-1}); [ac] and [al] the concentrations (mol l^{-1}) of the acid (HOLau) and alcohol (octOH); k'_{ac} and k'_{al} are dimensionless kinetic constants.

Table 2 shows that the kinetics parameters determined for aerogels synthesized from 40 and 60% MTMS were not very different. Fig. 6 illustrates the theoretical curves computed with these kinetics constant as an indication, altogether with the full series of experimental data points.

4. Conclusions

The activity of the lipase from *Candida rugosa* in an organic solvent could be significantly improved, by comparison with that of the free enzyme and of other immobilization techniques, by encapsulation in quartz felt reinforced silica aerogels. This success could be achieved by evaporating the alcohol produced during hydrolysis of the silica precursors before adding an enzyme aqueous solution in the silica sol. Reinforcement of these aerogels with a quartz fiber felt and molding in Teflon® tubes, made it possible to synthesize heterogeneous biocatalysts samples with a uniform size, which could be used for up to 42 times without any significant deterioration, neither mechanically nor catalytically. A first comparison of immobilization in reinforced aerogels made from 40 and 60% MTMS did not seem to indicate a drastic difference between the kinetics behaviour of these 2 types of samples.

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