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Catalytic activity of *Burkholderia (Pseudomonas) cepacia* encapsulated in silica aerogels in esterification and hydrolysis as a function of the gel and solvent hydrophobicities

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Abstract

Lipase from *Burkholderia* (formerly *Pseudomonas) cepacia* was encapsulated in silica aerogels dried by the supercritical method with CO_2. Aerogels with hydrophilic and hydrophobic functionalities were made by combining different proportions of tetramethoxysilane and methyltrimethosysilane as the silicon precursors. These aerogels were tested in the esterification of lauric acid by 1-octanol, as well as in the hydrolysis reaction of octyl laurate. These catalytic tests were carried out in two different solvents, namely water saturated isooctane or dioxane containing different initial water concentrations. The adsorption of the esterification and hydrolysis reactants, taken separately, on the most active aerogel in water saturated isooctane was studied.

The results showed that for esterification as well as for hydrolysis, the most active gels were the hydrophobic ones, independent of the solvent or water proportion used. Esterification reaction was much faster in water saturated isooctane, whereas hydrolysis was favored in dioxane, with the highest water concentration, as expected from the thermodynamics. As for the adsorption results, they showed that the reactant most taken by the gel is lauric acid. This study showed that the acid is not only adsorbed, but seems to undergo a reaction with the gel.

Keywords: Esterification; Hydrolysis; Adsorption; Encapsulation; Hydrophobic

1. Introduction

The synthesis of efficient biocatalysts by encapsulation of lipase from *Burkholderia cepacia* in silica gel materials has been well documented, in particular for the esterification of 1-octanol with lauric acid [1,2]. For this reaction, it was shown that hydrophobic groups in the gel were improving the activity of lipase which acts at water–oil interfaces. On the other hand, Secundo et al. [3] for instance studied the transesterification of vinyl butyrate by 1-octanol catalyzed by the same lipase, immobilized on supports of different natures, in different solvents including 1,4-dioxane, at different water activities. These authors showed that a concurrent hydrolysis reaction occurred and that the hydrolysis activity of the lipase increased with the thermodynamic activity of water in the solution, as expected from thermodynamics. Reetz et al. [4] studied the hydrolysis of an ester catalyzed by sol–gel entrapped lipases in aqueous medium. A

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small amount of acetone was added to the reaction mixture to make the ester soluble, relative activities (compared to free lipase) up to 110% were obtained *with Humicola lanuginosa*. In both the cases, the lipase catalytic activity also largely depended on the nature of the support material and the solvent. In this report, dioxane appeared to be a good solvent for hydrolysis. In the time scale of the experiments (a few days), the *Pseudomonas cepacia* (PC) lipase did not apparently seem to undergo deactivation, even though this solvent is hydrophilic, hence possibly harmful to lipases according to general statements [5].

The aim of the present work was to study the esterification of lauric acid with 1-octanol and the reverse hydrolysis carried out in two different solvents, namely water saturated isooctane ($a_w \approx 1$) [1] and dioxane at different initial water concentrations. Hydrolysis was also carried out in pure water. Water saturated isooctane was prepared by equilibrating isooctane with an excess of water in a sealed flask (two immiscible liquid layers), itself in equilibrium with saturated water vapor, that is to say in an atmosphere with relative humidity of 100% to a water thermodynamic activity $a_w \approx 1$ [12]. However, since isooctane is a solvent virtually immiscible with water, it has a very low water concentration (c_w) when saturated. The latter concentration is related to a_w by a very high thermodynamic activity coefficient $\gamma_w = a_w/c_w$. Using water saturated isooctane as the solvent is an easy way to work at a constant water activity.

In the case of hydrolysis, it was interesting to examine if a hydrophilic gel would help to favor the hydrolysis by taking the water from the solvent, that is to say nearby the active center, whereas hydrophobic gels could be expected to expulse the water from the gel's network. Encapsulation was also expected to offer a protection for the enzyme against deterioration by the hydrophilic solvent, provided that the proper gel was selected. Water activity has been shown to be an important parameter for the catalytic activity of biocatalysts. When a reaction is carried out in a hydrophobic solvent, it is common to first equilibrate the supported enzyme, the reactants and the solvent at a given water activity a_w, for instance against water saturated metal salts [6–9]. In the hydrolysis study, water was one of the substrates and had to be mixed with a water miscible solvent (dioxane). In order to simplify this procedure, a known amount of water was added directly to the reaction mixture, followed by the gel, assuming that the equilibration of the catalyst in situ proceeds fast enough not to influence the reaction kinetics. The amount of water contained in the gel, in the solvent (anhydrous dioxane) and in the reactants was negligible compared to the amount added and can, therefore, be ignored. Even though the water activity is unknown, the same water concentration was obtained in all experiments. Besides, according to Henry's law for very dilute solutions such as used in the present study, a_w is proportional to c_w, so that using c_w instead of a_w is just a question of scaling factor. At last, this technique made it possible to study the esterification as well as the inverse hydrolysis in the same solvent conditions, hence to cross-check the effect of the immobilization solid on both reactions.

Aerogels with different hydrophilic and hydrophobic characteristics and with or without polyvinyl alcohol (PVA) as an organic additive, were synthesized by hydrolysis of two silica precursors (one alkoxide and one alkyl alkoxide). The enzyme was introduced as an aqueous solution and the gels were dried by the supercritical CO_2 method. The catalytic tests results were analyzed in terms of initial reaction rates, and conversions at equilibrium for the esterification reactions was also examined.

Adsorption was studied for the most active type of aerogel, namely the hydrophobic one, containing or not PVA or enzyme, to check if these components modified the nature of the surface, thus influencing the adsorption capacity of the gel [10,11]. This was done for lauric acid, 1-octanol and octyl laurate separately in water saturated isooctane. The percentage of product adsorbed at equilibrium was determined. The recyclability of the doped aerogels in the esterification reaction was also studied, as well as the influence of the encapsulation of the lipase on the evolution of it's activity with storing time.

2. Experimental

2.1. Materials and methods

The reactants used in this study were PVA with an average molar mass of $M = 15{,}000$ (Fluka), methanol (R.P. Normapur-Prolabo), methyltrimethoxysilane (MTMS, 98%, Aldrich) and tetramethoxysilane

(TMOS, 98%, Aldrich), isooctane (Fluka, 99.5%) and dioxane (Fluka, 99.5%). The adsorption studies of substrates on the silica gel itself, hence which do not depend on the encapsulated enzyme were all carried out with *Pseudomonas cepacia* (Amano PS) kindly provided by Amano. This lipase was purified prior to use according to the method described by Secundo et al. [3]. All the catalytic studies (esterification and hydrolysis) were carried out with *P. cepacia* bought from Fluka.

2.2. Preparation of the lipase solutions

Two different preparation procedures of the lipase solutions were used for the enzyme coming from the two different suppliers.

For the hydrolysis and esterification studies, fresh enzyme solutions were prepared by dispersing about 37.5 mg of crude powder from Fluka, in 30 ml of deionized water, the insoluble particles were eliminated by centrifugation at 1700 rpm for 20 min. The supernatant was recovered and the protein concentration of the solution obtained was determined using the commercial test "BCA-200 Protein Assay Kit" from Pierce. Solutions with a concentration of about 3 mg/ml were obtained.

For studies on the adsorption of the esterification/hydrolysis reactants and products in the gels, fresh enzyme solutions were prepared by dispersing about 1.2 g of crude powder from Amano in 20 ml of pH 7–8 buffer (buffer A). The insoluble particles were eliminated by centrifugation at 1700 rpm for 15 min. Ammonium sulfate was added to the supernatant to 35% of saturation and the solution stirred at room temperature for 2 h. The precipitate obtained was collected by centrifugation at 3500 rpm for 30 min, and the pellet dissolved in 10 ml of buffer A and dialyzed against water for 48 h at 4 °C. The protein concentration of the solution was determined using the commercial test "BCA-200 Protein Assay Kit" from Pierce. Solutions with a concentration of about 0.5 mg/ml were obtained. It can be assumed that the difference in concentration between the two solutions prepared with different methods comes from the fact that small proteins are eliminated by the dialysis process but not by simple centrifugation. As the "BCA-200 Protein Assay Kit" is a mean to determine the concentration in proteins, the concentration in lipase can be overestimated with Fluka enzyme. For both lipase solutions, the aqueous preparations were stored at 5 °C.

2.3. Synthesis of the gels

All the aerogels were prepared according to the same procedure. The method used was the one described by Schwertfeger et al. [11], it consisted in dissolving MTMS and TMOS in methanol in a quantity such that the molar ratio of Si provided by MTMS, to the total Si provided by MTMS + TMOS, was $r_M \approx 0.2$. The molar ratio of methanol to total Si was $r_{meth} \approx 2$. This solution was hydrolyzed in part (56% of the water by mass) with the aqueous solution of lipase and in part with deionized water or with the aqueous solution of PVA. The mass of encapsulated enzyme was deduced from the amount of enzyme solution introduced in the gel. In practice, about 0.6 mg of enzyme was introduced in the gels made for the hydrolysis and esterification studies and 0.1 mg in the gels made for the adsorption studies. After gelation, the gels were aged in a sealed container at room temperature during 24 h. The wet gels were dried in supercritical CO_2 after exchanging the gel liquid for acetone by dialysis during 24 h. The supercritical drying technique used a "Supercritical Point Drier" from Polaron and was performed according to a method described in previous papers [2].

In the hydrolysis and esterification studies, the composition of the gels differed in the introduction or not of PVA or MTMS, the gels containing no MTMS were termed hydrophilic. The adsorption study was only carried out with the most efficient gels. The composition of the gels differed in the introduction or not of enzyme or PVA. They always contained MTMS and TMOS, giving them a hydrophobic character. The synthesis parameters of all the samples are presented in Table 1 (hydrolysis study) and in Table 2 (adsorption study).

2.4. Esterification and hydrolysis study

The catalytic activity of the encapsulated enzyme was tested in the esterification of lauric acid with 1-octanol as well as in the reverse hydrolysis of octyl laurate, also studied by Reetz et al. [1].

For the esterification tests, each gel of ≈200 mg was grounded to a powder with an average particle

Table 1
List of samples made for the esterification and hydrolysis study

Material	Methanol (mmol)	MTMS (mmol)	TMOS (mmol)	Water (mmol)[a]	Lipase (1) (mg)[b]	PVA (2) (mg)[c]
AWEP	5.8	0.56	2.2	18.9	0.58	6
ASPV	5.8	0.56	2.2	18.9	0.58	0
AHYD	5.8	0	2.8	18.9	0.58	6
AHSP	5.8	0	2.8	18.9	0.58	0

[a] Total water provided in part by the lipase solution (1) or the PVA aqueous solution (2) and in part by deionized water.
[b] Provided by a 3 mg/ml aqueous lipase solution.
[c] Provided by a 4% (in mass) aqueous PVA solution.

size of less than 200 μm, for which the reaction was not limited by diffusion of the substrate and products. This powder was introduced in a flask containing 100 mg (0.5 mmol) of lauric acid, 130 mg (1 mmol) of 1-octanol and 10 ml of water saturated isooctane or dioxane containing different initial molar ratio of water to lauric acid, namely 4, 8 and 32. The catalytic reaction was carried out at 30 °C in a shaking water bath at a speed of 180 rpm. This way, the gels were not deteriorated as they would be by magnetic stirring. In all the cases, at determined times, 50 μl aliquots were taken from the reaction solution and analyzed by gas chromatography (polar capillary column: BP21, 12 m, 0.22 mm, i.d.); temperature program: 5 min at 100 °C, up to 200 °C at 10 °C/min, 4 min at 200 °C; injector: 220 °C; detector FID: 220 °C; carrier gas: nitrogen.

The same types of gels were tested in the hydrolysis reaction. As the ester was not commercially available, it was retrieved from reaction media obtained after esterification reactions and purified by preparative chromatography on a silica column, elution was done with dichloromethane.

The gel powder was added to ≈10 ml of 1,4-dioxane or water saturated isooctane in a 30 ml closed flask containing ≈0.5 mmol of octyl laurate. The catalytic tests were then carried out exactly in the same experimental conditions than the esterification reactions. Different initial molar ratio of the hydrolysis water to ester (termed r_H), namely $r_H = 4$, 8 and 32, were examined. In the case of hydrolysis in water, extraction of the organic products from the aqueous reaction medium was done with isooctane and the fraction extracted was analyzed with GC (same conditions than stated previously).

Recycling of the biocatalyst has been tested only for the best gels (hydrophobic with PVA). The tests were carried out in the same conditions than classical esterification tests, except that the gel was first introduced in a Teflon capsule with small pores before introduction in the reaction medium. This suppressed the filtration step and enabled an easy recovery and washing of the catalyst. Washing of the gel was done with isooctane, repeated until no trace of products or reactants was detected in the filtrate (washing was usually repeated five times).

The storage of the enzyme was also studied either in aqueous solution or encapsulated in the gel. Two different procedures were used. In the first one, aerogels were synthesized with fresh enzyme solution, then stored at room temperature for different periods of

Table 2
List of samples made for the adsorption study

Material	Methanol (mmol)	MTMS (mmol)	TMOS (mmol)	Water (mmol)[a]	Lipase (1) (mg)[b]	PVA (2) (mg)[c]
AWEP	5.8	0.56	2.2	18.9	0.1	6
AWOP	5.8	0.56	2.2	18.9	0.1	0
AWOE	5.8	0.56	2.2	18.9	0	6
AWOEP	5.8	0.56	2.2	18.9	0	0

[a] Total water provided in part by the lipase solution (1) or the PVA aqueous solution (2) and in part by deionized water.
[b] Provided by a 0.5 mg/ml aqueous lipase solution.
[c] Provided by a 4% (in mass) aqueous PVA solution.

time, before being tested in esterification. The second procedure consisted in preparing a fresh aqueous enzyme solution, then storing it at 5 °C. Aerogels were made from this solution after increasing periods of storing time, and tested just after drying.

2.5. Adsorption study

The adsorption tests were carried out with each component of the equilibrium reaction (lauric acid, octyle laurate and 1-octanol) taken separately, except with water, for the most active type of gel (namely hydrophobic with PVA). In practice, a gel sample of ≈200 mg with an apparent volume of 0.57 cm^3 was grounded to a powder. The powder was added to ≈10 ml of water saturated isooctane (in order to be in the same conditions than in the reactions) in a 30 ml flask containing either 1 mmol of alcohol, 0.5 mmol of acid or 0.5 mmol of ester. These values were chosen in order to reproduce the initial conditions used in the esterification/hydrolysis reactions carried out in isooctane. The adsorption tests were carried out at 30 °C in a shaking water bath at 180 rpm until an equilibrium was reached, and 50 μl aliquots were taken at determined times and analyzed by GC.

3. Results and discussion

3.1. Esterification study

As shown on Fig. 1, the initial esterification rate is much higher in water saturated isooctane than in dioxane, no matter what the initial water content in the dioxane is. Indeed, for the most active aerogel termed hydrophobic aerogel with enzyme and PVA (AWEP), the initial relative rate is 40–50 times higher in isooctane than in water-containing dioxane. The second best biocatalyst is the hydrophobic gel containing no PVA (ASPV), whereas the two hydrophilic gels (containing PVA or not, and respectively termed AHYD and AHSP) behave similarly and are five times less active than aerogel AWEP and four times less than ASPV in isooctane. As it has been shown before, hydrophobicity is a very important characteristic for an aerogel to encapsulate the lipase and improve its catalytic activity. It can be pointed out that, for any given solvent or water content, the best gels are the hydrophobic ones, the hydrophilic ones being slightly less active in dioxane and much less in water saturated isooctane.

For all the different initial water amounts in dioxane, similar initial rates were obtained. However, an apparent optimum rate was generally observed for a ratio $r_H = 8$, with all the gels and with free enzyme but not with ASPV. As we know, hydrophilic solvents (i.e. dioxane) tend to take some of the enzyme's water hydration molecules. This can prevent an optimum functioning of the enzyme which needs hydration to be more flexible. It is also known that an excess of water can have a deteriorating effect on the enzyme, by a denaturation process [5]. Therefore, the better rates observed for $r_H = 8$ could be explained by an optimum amount of water, enough for a correct hydration of the enzyme, but not too much, so it is not denaturated. In water saturated isooctane, the denaturation cannot occur because only a negligible amount of water is present which is probably completely used for hydration of the enzyme. With all the gels, the initial esterification rate was much better than with free lipase. Indeed, the rate was up to 10 times higher with AWEP than with free lipase in isooctane. The catalytic activity of the free lipase was itself higher in isooctane (8 μmol h^{-1} mg^{-1}) than in dioxane (0.4 μmol h^{-1} mg^{-1}) no matter the initial water amount present in the reaction medium. Therefore, the encapsulation improves the catalytic activity of the lipase, most probably by acting on its conformation and dispersing it more in the gel than directly in the solvent of the reaction. Indeed, when the lipase powder is loaded directly in the organic medium, agglomerates form, which could also explain the low activity of the free enzyme.

If one now takes into account the influence of r_H on the conversion in esterification reaction reached at equilibrium in dioxane (Fig. 2), it is obvious that at higher water contents, thermodynamics are not favorable to the formation of the ester. Therefore, the higher the initial water amount, the lower the conversion at equilibrium. It can be pointed out that, at a fixed r_H the same conversion at equilibrium is obtained, independent of the gel used, which is in agreement with thermodynamics. The gel only influences the kinetics. When working in water saturated isooctane, the reaction is almost complete with all the gels. It can be assumed that the hydrophobic solvent favors the esterification by expulsing the water from the gel and

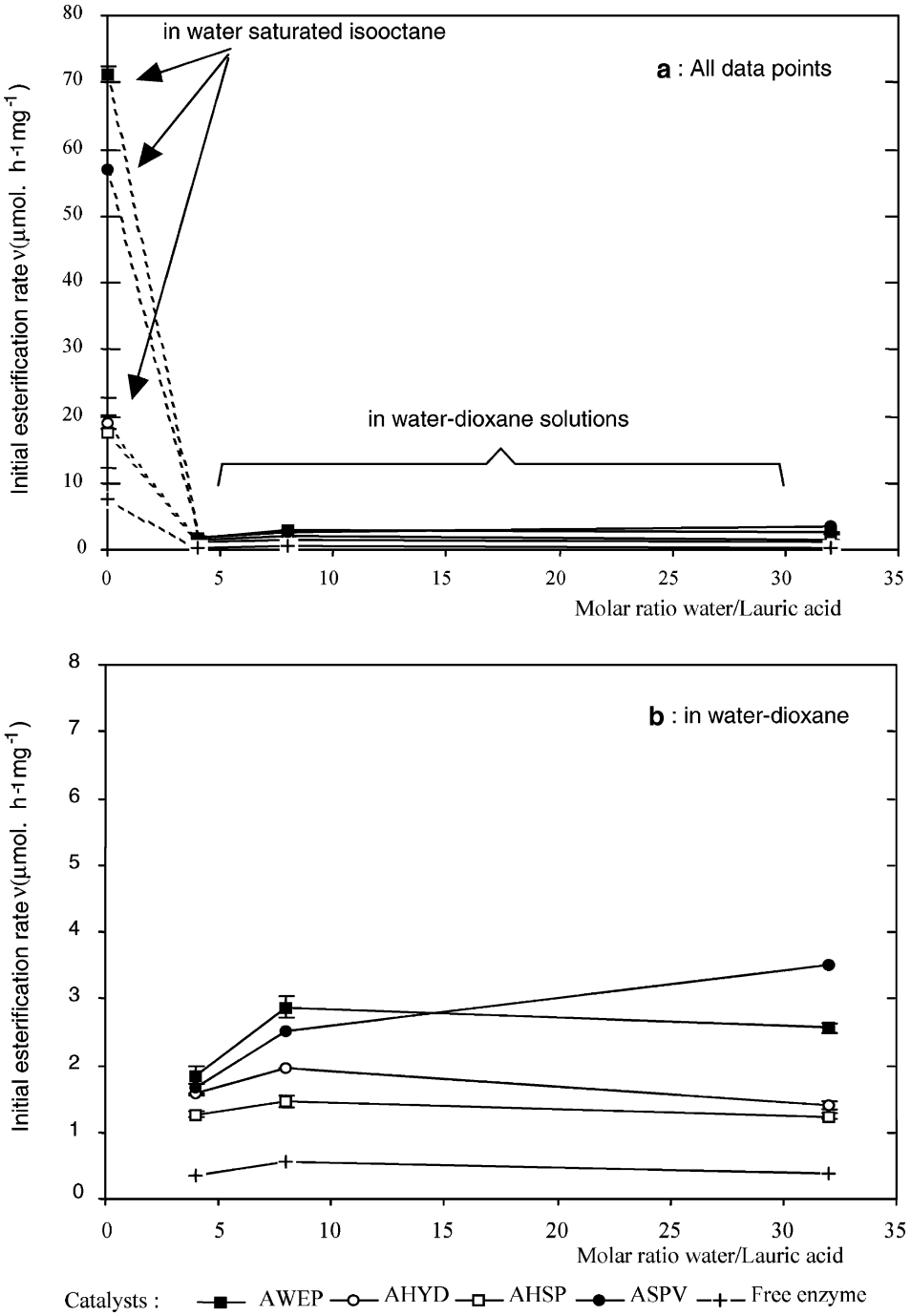

Fig. 1. Initial esterification rate of encapsulated enzymes, in water saturated isooctane and water–dioxane solutions with initial water/lauric acid molar ratio $r_H = 4$, 8 and 32 (reaction made with 0.5 mmol lauric acid, 1 mmol 1-octanol at 30 °C in 10 ml solvent).

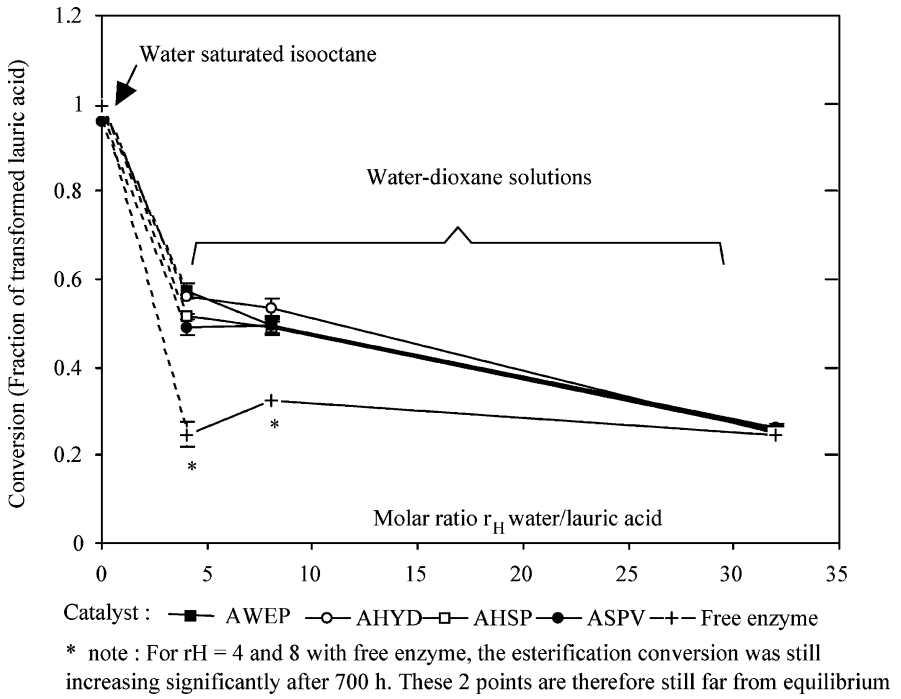

Fig. 2. Final lauric acid conversion of encapsulated enzymes, in water saturated isooctane and water–dioxane solutions with initial water/lauric acid molar ratio $r_H = 4$, 8 and 32 (reaction made with 0.5 mmol lauric acid, 1 mmol 1-octanol at 30 °C in 10 ml solvent).

by the very low content of initial water in the reacting media.

As for recyclability of the AWEP gels in esterification reaction, results are shown in Fig. 3. After four tests, the activity of the gels did not decrease. It even tended to slightly increase, maybe due to a better equilibration of the gel.

The results on the storage study are presented in terms of relative activity with respect to a gel loaded with fresh enzyme, in Fig. 4. They show that dry hydrophobic aerogel is a good means to keep the biocatalyst and protect it better against deactivation with time. The encapsulation probably protects the enzyme against denaturation by water which occurs in the aqueous solution.

3.2. Hydrolysis study

As shown on Fig. 5, in the case of hydrolysis also, the best activity was obtained with the hydrophobic gels, even though it had been thought that the hydrophilicity of the material would help attracting the water to the active site and favor the hydrolysis of the ester. Indeed, it seems that the gel only acts on the conformation of the enzyme and does not influence the displacement of the equilibrium. Hydrolysis is much faster in dioxane than in water saturated isooctane or in water, which can easily be explained by the fact that the organic substrate and the hydrolysis water are both soluble in dioxane, whereas water is insoluble in isooctane, and ester insoluble in water. In the case of hydrolysis, the hydrolysis ratio r_H has an influence on the initial rate of the reaction, the higher the initial water content, the higher the rate.

3.3. Adsorption study

The results of the adsorption study are analyzed in terms of percentage of alcohol, acid or ester adsorbed by the gel at equilibrium (Fig. 6). It has been noticed in GC analysis that in the case of the adsorption of lauric acid on gels containing enzyme and PVA (AWEP), an important amount of an impurity was formed, thus consuming the acid. This impurity has been identified

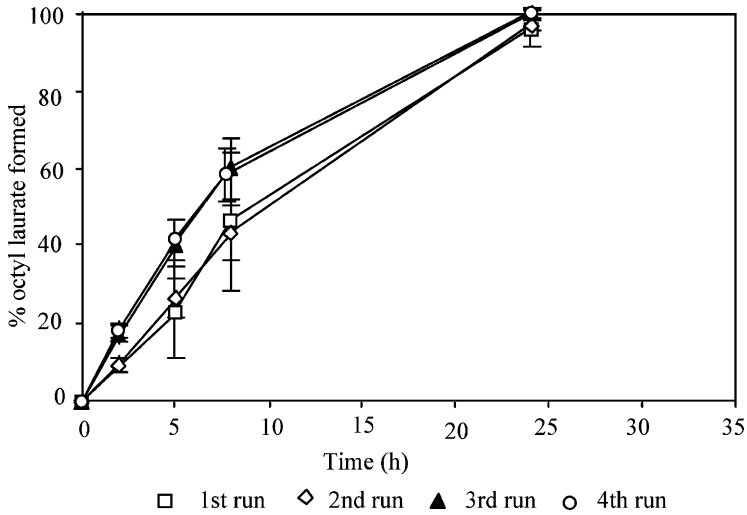

Fig. 3. Recycling behavior of an AWEP gel, in the esterification of 0.5 mmol lauric acid with 1 mmol 1-octanol in 10 ml water saturated isooctane. The aerogel was inside a Teflon capsule.

with GC–MS to be methyl laurate. One possible mechanism for the production of this ester is the reaction between lauric acid and residual methoxy groups from the gel which have not been hydrolyzed. Another possibility is that residual methanol (produced by the hydrolysis of methoxy groups during the polymerization of the gel) is present in the gel. Adsorption tests of lauric acid on a AWEP gel were done after

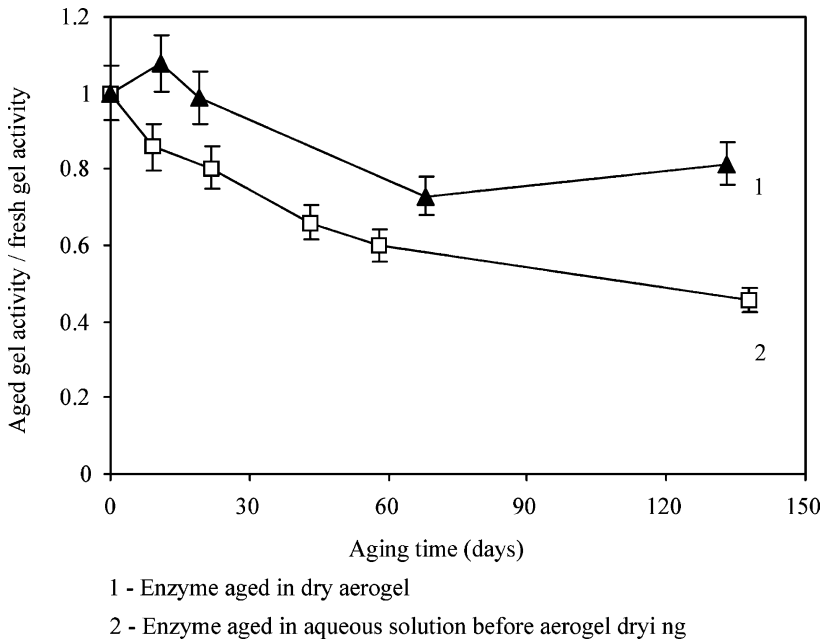

Fig. 4. Activity of AWEP aerogels, in the esterification of 0.5 mmol lauric acid with 1 mmol 1-octanol in 10 ml water saturated isooctane. (1) Enzyme aged in a aqueous solutions before encapsulation in an aerogel. (2) Enzyme aged inside a dry aerogel.

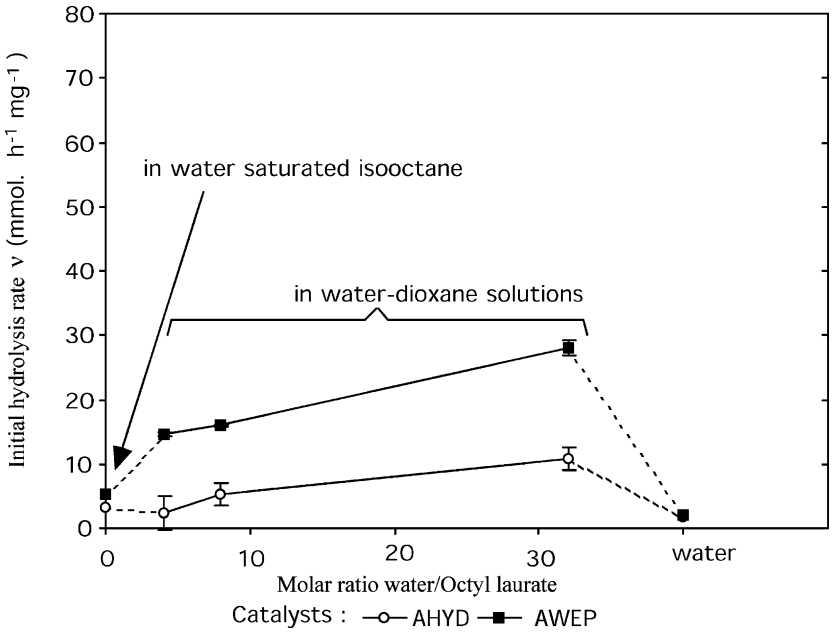

Fig. 5. Initial hydrolysis rate of encapsulated enzymes, in water saturated isooctane and water–dioxane solutions with initial water/octyl laurate molar ratio $r_H = 4$, 8 and 32 (reaction made with 0.5 mmol octyl laurate at 30 °C in 10 ml solvent).

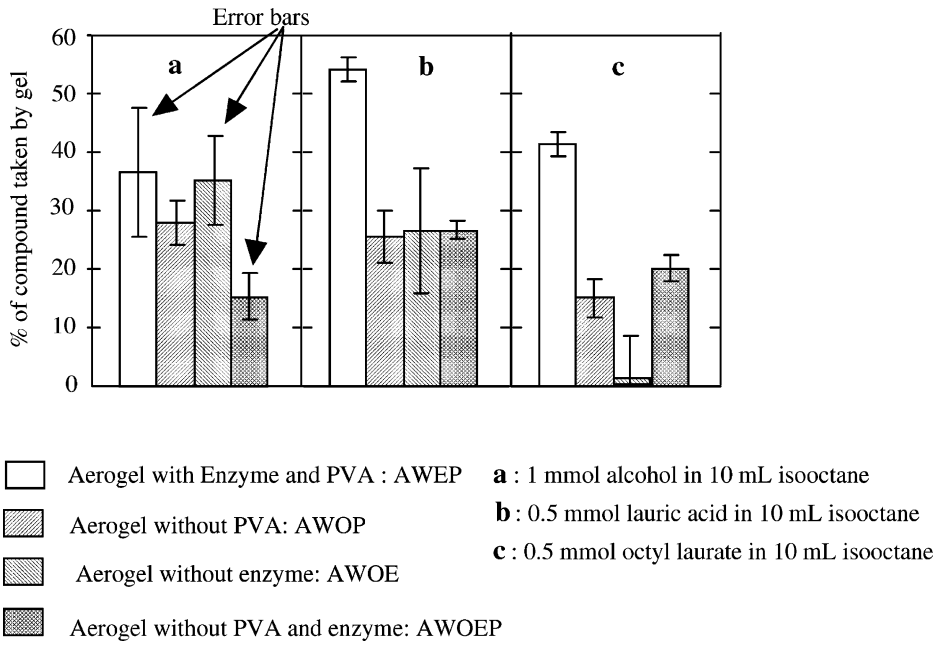

Fig. 6. Adsorption percent of reactants in 10 ml water saturated isooctane, on different aerogels: (a) 1 mmol 1-octanol; (b) 0.5 mmol lauric acid; (c) 0.5 mmol octyl laurate.

recycling the gel by filtration and washing. Almost no methyl laurate was formed this time. Therefore, it can be suggested that all the methoxy groups or the residual methanol were consumed to form methyl laurate during the first adsorption test. It is rather difficult to determine which of the two mechanisms occurs. If working with another solvent for the synthesis of the gel, methanol is still produced by the hydrolysis of the alkoxide precursors. On the other hand, if choosing another alkoxide precursor, both the alkoxy groups and the corresponding alcohol will be present in the gel. This impurity is also formed at high conversions but in much smaller quantities in the esterification reaction of lauric acid with 1-octanol. This can probably be explained by the fact that the kinetics of the latter esterification are much faster than those of the reaction between methoxy groups or methanol and lauric acid. The same phenomenon occurs when carrying out hydrolysis, where the formation of methyl laurate is observed. In that case, it is proposed that the mechanism of formation is a transesterification between octyl laurate and MeOH. As shown on Fig. 6, the adsorption of lauric acid is very important on AWEP gels. As explained before, this is not only due to adsorption, but also to a side reaction which seems to be catalyzed by AWEP gels and not by any other gels. For all the other gels (AWOP, AWOEP, AWOE) the percentages of acid adsorbed at equilibrium are of the order of 25%. At last, in the case of 1-octanol, no production of impurity is observed, the AWEP and the AWOE gels both favor the most the adsorption of the substrate. In fact, at equilibrium, 40% of the initial alcohol has been adsorbed. As for the two other gels they behave rather similarly, considering the precision of the GC results.

4. Conclusions

The lipase from *B. cepacia* encapsulated in silica aerogels can catalyze the esterification of lauric acid with 1-octanol, as well as the reverse hydrolysis of octyl laurate. The best material for encapsulation is a hydrophobic aerogel containing PVA, for both reactions and in all the solvents used. This can probably be explained by the fact that lipases act at a water–oil interface and the hydrophobic gel enables the enzyme to be in the right conformation for catalysis. For both reactions, the encapsulation improves dramatically the catalytic activity of the enzyme, and makes the biocatalyst easy to recover, to recycle (no loss of activity after four catalytic tests) and to store at room temperature without any significant loss of activity after 3 weeks and a loss of 20% after 4 months. The best solvent for esterification is water saturated isooctane, whereas for hydrolysis, dioxane is much better as it solubilises the reactants (water and ester). The adsorption study showed that there is a possible reaction between the non-hydrolyzed methoxy groups from the gel or residual methanol present in the gel and lauric acid to produce methyl laurate. This reaction is catalyzed by the enzyme itself. Washing the gel with isooctane before catalysis can be a way to check if residual methanol is washed away, in that case, no methyl laurate should be produced.

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