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Journal of Molecular Catalysis B: Enzymatic 32 (2005) 193–203
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Compared esterification kinetics of the lipase from *Burkholderia cepacia* either free or encapsulated in a silica aerogel

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Received 7 July 2004; received in revised form 7 December 2004; accepted 8 December 2004
Available online 8 January 2005

Abstract

This paper presents an experimental comparison of the kinetics of esterification catalyzed with the lipase from *Burkholderia cepacia*, either free, or encapsulated in a silica aerogel dried by the supercritical CO_2 method. The operational characteristics, in terms of pre-equilibration at given water thermodynamic activity a_w, mass of enzyme in the gel, size of aerogel particles, are presented. The kinetic model known as Bi–Bi Ping Pong with inhibition by both substrates has been found to fit relatively well with the experimental results, except when both substrate concentrations were high with the encapsulated enzyme. All kinetics constants were found to be increased by aerogel encapsulation. In particular V_{max} was increased by a factor of the order of 10 per mg of enzyme.

Keywords: Lipase; Aerogel; Esterification; Kinetics

1. Introduction

The sol–gel encapsulation of enzymes is a field at the crossing of materials sciences and biotechnology, which receives a significant attention in both fields [1–4]. On the other hand, regarding catalysis by lipases at large, the kinetics of reactions have been extensively reported in the literature, and a large variety of possible kinetics mechanisms have been gathered in a book by Segel [5].

In the present paper, the aim is to grasp some insight on how encapsulation in an aerogel modifies the kinetics mechanism. In a recent previous publication [6] we reported on the compared kinetic mechanisms of free and aerogel encapsulated lipase from *Burkholderia cepacia*, in a transesterification reaction. The latter paper was also largely focussed on comparing aerogels made with a different proportion of hydrophobic groups, and catalysis in mixtures of hydrophilic and hydrophobic solvents. The present paper is addressing a simple esterification reaction, in a hydrophobic solvent (isooctane) with the same lipase as previously and in only one type of aerogel made from 20% of a silicon precursor carrying a hydrophobic group (methyltrimethosysilane). The precise aim was first to study in details the influence of the catalytic test experimental conditions on the activity, in particular the water thermodynamic activity a_w, the aerogel particle size, the mass of enzyme in the gel, the temperature, and recycling. Secondly, a comparison of the kinetic models for the free and for the encapsulated enzyme had to be made, because this can also bring some information on how the aerogel may influence the enzyme activity.

2. Experimental part

2.1. Materials and methods

The lipase from *B. cepacia* (previously known as *Pseudomonas cepacia*) was kindly provided by Amano as a lyophilized powder (lipase PS 40 U/mg noted BCL). This lipase was either tested as such to determine the catalytic activity of the so-called free enzyme, or previously purified and

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doi:10.1016/j.molcatb.2004.12.006

used in aqueous solution, as described further on, to determine its activity after encapsulation in a silica aerogel.

The chemical reactants used for the sol–gel synthesis, in this study, were polyvinyl alcohol, termed PVA (average molar mass 15000 g/mol from Fluka); methanol (R.P. Normapur-Prolabo), acetone (Riedel de Haën, 99.8%), and the silicon precursors methyltrimethoxysilane (MTMS, 98%, Aldrich), and tetramethoxysilane (TMOS, 98%, Fluka). Other reactants comprise the metallic salts $(NH_4)_2SO_4$, LiBr, $MgCl_2{\cdot}6H_2O$, $SrCl_2{\cdot}6H_2O$, KNO_3 (Fluka, ≥99%) and $(NH_4)_2SO_4$ (Fluka, 99.8%) to study the effect of water activity, in particular ammonium sulfate, ultra pure water prepared by an ELGA PURELAB UHQ water purification system, hydrochloric acid (Prolabo, 37%), tris(hydroxymethyl)aminomethane (Fluka, 99.8%) noted Tris and technical grade acetone (Prolabo).

The compounds used to test the catalytic activity of the enzyme were isooctane (Fluka, 99.5%) as the solvent, lauric acid (200.32 g/mmol, Aldrich, 99.5%) and 1-octanol (130.23 g/mol, Fluka, 98%) as the substrates.

2.2. Enzyme purification process

For aerogel encapsulation, the commercial enzyme powder was first dispersed in water and centrifuged to eliminate the insoluble part, as described by Reetz et al. [7]. Then the protein was precipitated from the soluble part, recovered and further dispersed in water and finally dialized against pure water, according to a technique adapted from works by Pencreac'h et al. [8–11] and Secundo et al. [12].

In details, typically 1.2 g of lyophilized enzyme from Amano was dispersed in 20 ml of a 0.1 M Tris–HCl buffer solution at pH 7.5. After stirring for about 20 min, the suspension was centrifuged at 2700 rpm for 10 min to eliminate the insoluble part, which represented about 90% of the initial weight of commercial powder. The supernatant solution was isolated and the proteins were precipitated from this solution by adding ≈6 g of ammonium sulfate $(NH4)_2SO_4$, (≈35% of a saturated solution) [12]. The latter mixture was stirred for 3 h at 4 °C, then centrifuged at 3700 rpm during 30 min to isolate the proteins, which were then recovered, dissolved in 10 ml of Tris–HCl buffer, and dialyzed 48 h at 4 °C against HPLC grade water. The dialysis membranes were in cellulose ester of the type float-A-lyzer® from Biovalley and had a MWCO of 10 kDa. This made it possible to eliminate all the proteins with a mass significantly smaller than that of the full enzyme molecules, as well as electrolytes such as salts. Dialysis was performed in 1.8 l water, at ≈4 °C and the dialysis water was changed every 5 h. According to the concentration in proteins determined by using the method of Pierce®, the purification yield was found to be slightly less than 1% (by mass), which is lower than that achieved by Pencreac'h et al. [9–11], and slightly lower than that achieved by Secundo et al. [12]. In some cases, more concentrated purified enzyme solutions were needed.

The concentration in proteins of aqueous solutions was determined according to a protocol described in the commercial test "BCA-200 Protein Assay Kit" from ®Pierce, by UV–visible spectrophotometry. Typically the protein concentrations were ≈20 mg/ml after centrifugation, ≈3.9 mg/ml after precipitation redissolution before dialysis and <1 mg/ml after dialysis. It must also be mentioned that for a given commercial enzyme batch, a large scatter in the final protein concentration (from ≈0.3 to 1 mg/ml) was obtained after purification. To understand these variations, two purified enzyme solutions were analyzed by electrophoresis on polyacrylamide gels, according to the technique of Laemmli [13], with a Mini PII equipment from Biorad. In both solutions, the polyacrylamide gels showed a blot due to proteins with a mass of 33 kDa, corresponding to the lipase of *B. cepacia*. On the other hand in one solution, another unidentified protein of mass 27 kDa also showed, and was associated with a higher protein concentration as experimentally determined.

2.3. Enzyme encapsulation in silica aerogels

The aerogels studied were made from a solution of MTMS and TMOS in methanol, such that the molar ratio of Si coming from TMOS to total Si was $r_{Si,T} = 0.8$. The presence of MTMS introduces hydrophobic functionalities in the gel, which are important to achieve a good lipase activity, as shown by Reetz [7,14]. The aqueous enzyme solution (pH7.5 Tris–HCl solution dialyzed against HPLC grade water) was introduced before silica gelation, as previously described. Typically, the two silicon precursors (82 μl MTMS and 328 μl of TMOS) were dissolved in methanol and added to a mixture of 192 μl of aqueous enzyme solution in 150 μl of a PVA solution (PVA concentration: 4%, w/w). Stirring was maintained during ≈2 h, until a clear homogeneous solution was achieved. Gelation generally occurred in ≈4 h after agitation was stopped. Reetz also showed that the addition of PVA improves the catalytic activity of encapsulated lipases [7,14], and leads to stiffer gels, which shrink less during drying. The total molar ratio of water used for hydrolysis of the Si precursors, per Si atom, was r_w ≈6.8.

In the present study, the gels were dried by the CO_2 supercritical method. This technique was used for the first time by Kistler [15]. For this purpose, a wet gel must be heated in an autoclave to a temperature above the critical temperature T_c and a pressure above the critical pressure P_c of the liquid inside the gel's pores. When a gel contains fragile biomaterials, which do not withstand heat, such as enzymes, it is therefore necessary to use a liquid with a low T_c, such as CO_2 ($T_c = 31\,^{\circ}C$, $P_c = 7.37$ MPa). Silica gels dried by this technique usually have a high specific surface area (e.g. 800 m^2/g or higher) and mainly a very high pore volume (up to 98% porosity) [16,17]. They contain a high proportion of mesopores (pore size between 2 and 50 nm) and are termed aerogels. A major problem with the CO_2 supercritical drying is that liquid CO_2 is generally poorly miscible with the liquid inside the wet gel pores. Hence it is necessary to proceed to

an intermediate exchange with a liquid miscible with both the fluid in the wet gel (mostly methanol and water), and liquid CO_2. In the present study, acetone was used. The exchange was done by simply dipping the wet gels in acetone for 20 h. Next, the gels were placed in a "Supercritical Point Drier" autoclave of the society Polaron®, and liquid CO_2 was introduced to progressively replace the acetone inside the wet gel pores. For this purpose, the gels were left for 30 min in liquid CO_2, new CO_2 was then injected and the mixture CO_2/acetone flushed out of the autoclave, an operation which was repeated three times to fully exchange acetone for liquid CO_2 inside the gels. Next, the temperature and pressure were raised to 35 °C and 8.5 MPa, respectively, hence beyond the critical point values of CO_2 and, maintained in these conditions for 45 min. Finally, the supercritical CO_2 was evacuated as a gas by slowly releasing the pressure, e.g. from 8.5 to ≈6.5 MPa in ≈30 min, so as to maintain the temperature at a constant value of ≈35 °C, higher than the critical point temperature of CO_2. The latter procedure is necessary to avoid cutting the liquid-gas coexistence line of CO_2 by adiabatic cooling. Below ≈6.5 MPa, the CO_2 evacuation can be accelerated without any danger for the aerogel. The dried aerogels obtained were stored at $T = 4$ °C before being used.

Typically, the mass of a dry aerogel sample was ≈200 mg and contained from 0.05 to 0.1 mg added purified enzyme. It was not easy to determine the exact amount of enzyme in a dry aerogel, by direct analysis of this aerogel. However determination of the amount of protein lost during the various stages of the encapsulation process was attempted, by measuring the concentration of proteins in the various fluids in contact with the gel, by means of the Pierce method. Actually, no free enzyme was found in the acetone, after the intermediate liquid exchange, before placing the gel in the autoclave. On the other hand, analysis of the acetone extracted from the autoclave by CO_2 exchange, showed that about 20% of the protein initially introduced in the gel was evacuated with the CO_2 and therefore lost from the gel's network.

Because of variations in the activity and concentration of different enzyme solutions, all aerogels used to study a given catalysis characteristic in this paper, for instance to determine the kinetic mechanism, were made from the same purified enzyme solution.

2.4. Catalytic activity

The catalytic test used to determine the enzymatic activity was the esterification reaction of lauric acid with 1-octanol which produces octyl laurate and water.

The effect of pre-equilibration of the reaction medium on free enzyme or encapsulated enzyme, was studied for a range of water thermodynamic activity a_w achieved with water saturated solutions of the following metal salts $(NH_4)_2SO_4$, LiBr, $MgCl_2{\cdot}6H_2O$, $SrCl_2{\cdot}6H_2O$ and KNO_3. The study on the catalysis kinetic mechanism was carried out after pre-equilibration of reaction media and catalyst at the optimum a_w, for the encapsulated lipase as well as the free one. In the case of the recycling study of an aerogel, the water thermodynamic activity a_w was not optimum, but the solvent used was water saturated isooctane as done by Reetz et al. [18].

In all investigations, except when one parameter was changed and mentioned, the reaction was carried out with mole numbers of lauric acid $n_{lau} = 0.5$ mmol, and 1-octanol $n_{oct} = 1$mmol, in a solvent volume (isooctane) = 10 ml, placed in a 30 ml flask. The catalyst was either added as free enzyme (powder), or as 200 mg aerogel encapsulated enzyme. A diffusion limitation study described further on, was carried out in order to select the size of the gel particles to avoid limitation by diffusion of the substrates and product. As a result of the latter study, most catalytic tests were performed with aerogels pieces of ≈4 mm.

The reaction temperature was maintained at 30 °C in a shaking water bath agitated at 180 rpm after checking that no mass transfer limitation external to a gel occurred at this agitation speed. This method of mixing was also chosen in order to avoid mechanical grinding of the gel, which occurred with magnetic stirring.

The esterification was followed by gas chromatography and quantitative determination of the initial reaction rate was made by comparing the chromatograph signals intensity of the substrates and product with those of reference compounds, according to the method known as internal–external (standard added just prior to injection). For this purpose, 50 μl aliquots were taken from the reaction medium at regular intervals of time. Known concentrations of reference compounds were then added in the aliquots diluted by addition of 700 μl isooctane. The reference compounds were methylphenylacetate and capric acid (Fluka, 99%), which give GC signals respectively close to those of 1-octanol and lauric acid or octyl laurate. In the present paper, the focus was on the initial esterification rate, determined during the first 100 min of the reaction, where the linearity of ester formation as a function of time was checked. Actually, the conversion curves of the reactants were found to be linear up to ≈70% conversion. All tests were done in duplicate or triplicate. The enzymatic activity was measured as the initial formation rate of the ester octyl laurate in U, with 1 U = 1 μmol of octyl laurate formed in 1 h. Often, this activity was expressed per mg of enzyme. In the case of aerogel encapsulation, this amount was calculated from the volume and concentration of the enzyme solution introduced in the gel preparation before gelation. As explained previously, ≈20% of this enzyme was lost during supercritical drying. Nevertheless, this was not taken into account to present the data, because this value can only be considered as a rough estimation, and may vary from one gel to the other. For the test directly made with the commercial enzyme (termed free enzyme), the data are reported per mg of commercial powder.

The chromatograph was a Shimadzu GC-14B, equipped with a capillary polar column from SGE (Ref: 12QC2/BP210.25), 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, fol-

lowed by heating at 10 °C/min 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).

A number of parameters were investigated in the present kinetic study. These parameters are presented altogether with the corresponding results in the next sections. In each case, the aerogel samples and all catalytic tests were made at least in duplicate.

3. Results and discussion

3.1. Variation in the enzymatic activity

The effect of the purification process is well illustrated by the data gathered in Table 1, which shows the range of activities of enzyme powder lots and encapsulated enzyme after each step of the purification process. Clearly, the precipitation-dialysis technique was efficient. This table also shows that a large variation in the catalytic activity was reached for different solutions prepared otherwise by the same protocol, which was consistent with the results obtained by electrophoresis on polyacrylamide gels. As a consequence, each parameter studied later on (in particular the kinetic mechanism) was done with a single enzyme solution. For a given solution preparation, the activity was found to be reproducible with a precision ≈10% after encapsulation in similar aerogel samples prepared and tested with same experimental protocols.

A possible reason for the variation of the enzymatic activity could be its evolution during aging, either before encapsulation, or after. The loss of activity of purified enzyme when aged in the aqueous solution where it was purified, at +4 and −9 °C, was compared to the loss when aged after encapsulation inside a dry aerogel, at room temperature in closed containers. The results showed that for up to 2 months, the lipase did not lose its activity neither before encapsulation, nor after encapsulation. Hence the previous scatter cannot really be explained by different aging times of the purified solutions before use, for instance. On the other hand, the activity of two commercial enzyme lots were tested, first just after reception, secondly after about 6 or 5.5 months conservation at −9 °C in desiccators, in the un-purified as-received state. The results in Table 2 clearly show that an important loss of activity occurred during such a conservation procedure.

Table 2
Evolution of the commercial enzyme activity after long time storage at −9 °C in a dessicator

Enzyme lot	Just received enzyme U/(mg powder)	Aged enzyme U/(mg powder)	Activity loss (%)
3	3.5	1.5 (6 months)	60
4	2.0	1.3 (5.5 months)	36

3.2. Blank tests

Regarding the silica aerogels themselves, blank tests were performed with samples in which the enzyme solution was replaced by an equivalent volume of water at same pH. These tests showed no esterification activity at all.

3.3. Influence of pre-equilibration at a given water thermodynamic activity a_w

It is important to control the quantity of water available for the enzyme in the reaction medium. When a biocatalysis reaction is performed in organic solvents, many authors have reported that the addition of a small quantity of water was necessary to insure a proper activation of the enzyme, in particular to insure some conformation flexibility to the enzyme molecule [10,11,19]. It is generally admitted that a better control of the transformation kinetics can be achieved by working at a constant, optimal water thermodynamic activity a_w. Because water is produced in an esterification reaction, it is more simple to pre-equilibrate all components (aerogel, solvent and substrates) at a given water activity a_w, and to only determine the initial esterification rate. Pre-equilibration is generally done by achieving equilibrium, by the intermediate of the vapor phase, between these components and a water saturated aqueous metal salt solution [20].

In the present study, the optimal a_w was determined both for the free (commercial) enzyme powder for a given enzyme lot, and for the aerogel encapsulated enzyme from another enzyme lot. For this purpose, free enzyme or aerogel encapsulated enzyme and, separately, the reaction medium (sol-

Table 1
Catalytic activity of lipase solutions after encapsulated in aerogels, depending on the original lot number from Amano, the solution and the purification stage

Lot number	Activity U/(mg $_{powder}$) free enzyme	Purification stage	Activity U/(mg$_{protein}$) encapsulated enzyme	Pre-equilibration
1	1.10	Centrifugation	≈94	Water saturated isooctane
		Precipitation/dialysis	≈550–1650[a]	
2	1.90	Precipitation	≈105	Water saturated isooctane
		Precipitation/dialysis	≈840 to ≈900[a]	
3	3.50	Precipitation/dialysis	≈1080	Water saturated isooctane
4	2	Precipitation/dialysis	≈1050	Water saturated isooctane
			≈1350–2650[a]	a_w = 0.81

The commercial powder activity was determined with 20 mg powder in the same conditions.
[a] Depending on the solution preparation from a given lot.

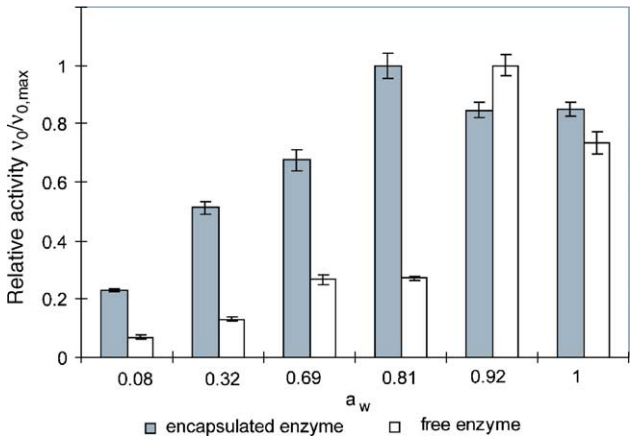

Fig. 1. Influence of the water thermodynamic activity a_w at which all components were pre-equilibrated, on the relative catalytic activity $v_0/v_{0,max}$ ($v_{0,max}$ maximum activity), for aerogel encapsulated enzyme and free enzyme powder. Each set of data was obtained on a single enzyme lot.

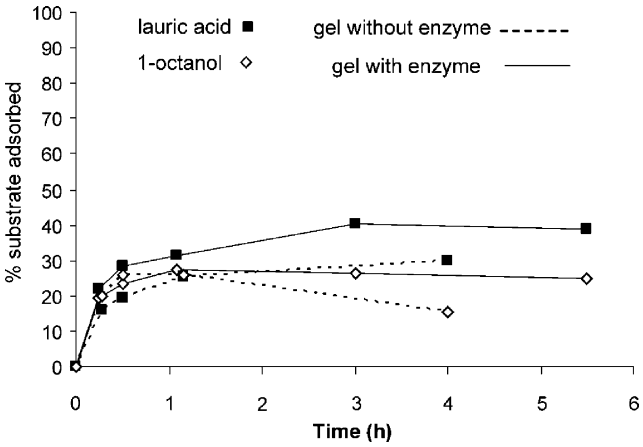

Fig. 2. Adsorption of 1-octanol or lauric acid in aerogels pre-equilibrated at $a_w = 0.81$.

vent + lauric acid + 1-octanol) were placed under controlled atmosphere, in the presence of water saturated metal salt solutions, for at least 40 h, at 20 °C. Six different a_w were used corresponding to equilibrium with either pure water, or the list of saturated salts previously mentioned [20]. Because two different enzyme lots were used, for the free enzyme and the encapsulated one, the relative catalytic activity $v_0/v_{0,max}$ ($v_{0,max}$: maximum activity) are reported in Fig. 1. The optimal a_w was found to be different for the free enzyme ($a_w = 0.92$) and the encapsulated one ($a_w = 0.81$). In further studies, each catalyst and reaction medium were then pre-equilibrated at the corresponding optimal a_w, except for the recycling study. It must also be noticed that the catalytic activity did not change much for a_w beyond this optimum values, in particular for the aerogel.

3.4. Influence of aerogel particle size

Three aerogel particles size, derived from similar aerogel monoliths, were tested: (1) single un-broken cylindrical aerogel monolith (height ≈0.5 cm, diameter ≈1.2 cm); (2) similar cylindrical monolith broken to pieces with a size of a few mm; (3) similar cylindrical monolith crushed to a powder of size ≤200 μm. The activity with these three different particles size, were respectively, $v_0 \approx 1.37 \pm 0.01$; 2.28 ± 0.01 and 2.20 ± 0.23 U/mg$_{protein}$. Hence, no difference was observed between the powder and the monolith pieces. The activity was slightly lower with the monolith which indicated limitation by diffusion. In all further studies, the aerogels were then used as broken to pieces of a few mm, which was more convenient for recycling and sample taking.

3.5. Diffusion of the substrates in the aerogels

It is also possible to estimate the adsorption rate of the substrates on these aerogels. For this purpose, aerogel monoliths (mass ≈200 mg, volume ≈0.51 cm^3) were pre-equilibrated at $a_w = 0.81$, then placed in 10 ml isooctane (10 ml) in which either 0.5 mmol lauric acid, or 1 mmol 1-octanol was added and the solutions also pre-equilibrated at $a_w = 0.81$. These mole numbers were selected to reproduce the initial conditions prevailing in a standard catalytic test. The diffusion tests were carried out in the same experimental conditions as a standard catalytic test and the evolution of each substrate concentration in the isooctane was followed by GC. From these data, it was possible to calculate the rate at which each substrate adsorbed on the aerogel. The results are reported in Fig. 2. First, it clearly appears that the rate of adsorption of these substrates was much faster than the esterification rates. Secondly, it also appears that aerogels absorbed a maximum of about 25% of the initial amount of both substrates, after 1 h. Adsorption of the product octyl laurate was also studied, but the results showed that within the precision of the gas chromatograph, this product was not adsorbed.

An important question is to know whether substrate molecules can participate in an esterification reaction with the lipase, when they are adsorbed inside a gel. Actually, the data in Fig. 2 shows that a significantly higher percent of lauric acid was taken up by an aerogel containing the enzyme (≈40% of the substrate) than by an aerogel without enzyme (≈25%). Besides, when lauric acid was the only substrate present in the adsorption experiment with an aerogel containing some enzyme, the formation of methyl laurate was clearly identified by gas chromatograph couple to mass spectrum (GC–MS) and already reported in a previous study [4]. This product could indeed only result from the esterification between lauric acid and the methanol produced during gel synthesis which remained adsorbed inside a dry aerogel as shown previously by solid state NMR study [21]. Adsorption of alcohols, 1-octanol as well as methanol, occurs on the gel silanol Si–OH functionalities. Once this methanol has been consumed, methyl laurate no longer forms. This was checked after washing the aerogel in

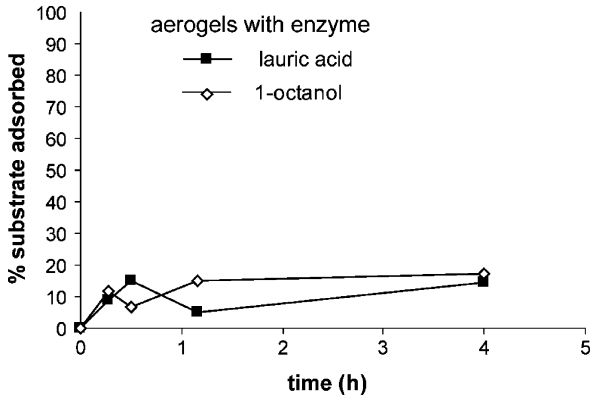

Fig. 3. Adsorption of lauric acid or 1-octanol in solution in isooctane, on recycled aerogels.

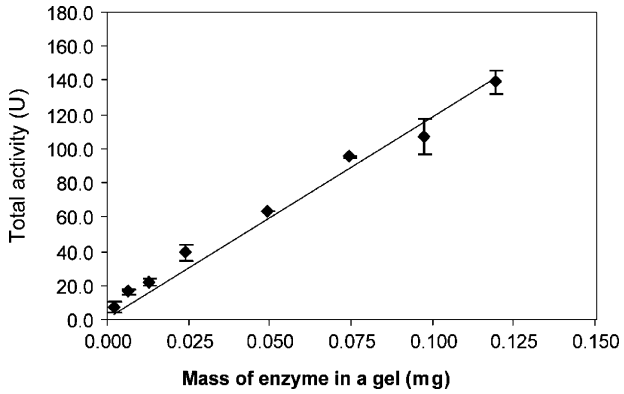

Fig. 4. Influence of the mass of enzyme encapsulated in an aerogel on the total activity.

isooctane and performing a second adsorption test. In this second test, the final adsorption of lauric acid was drastically lowered to ≈15% of initial amount of substrate introduced (Fig. 3). Actually 10% "un-adsorbed substrates" would be sufficient to provide an un-limiting supply of substrates to the enzyme, which is consistent with the present adsorption studies.

In summary, the present adsorption data completed by previous experimental reports seem to indicate that the lipase can actually perform esterification with an adsorbed substrate such as an alcohol, methanol or 1-octanol.

3.6. Stirring rate

Determination of the optimum stirring speed is important to avoid limitation of the reaction by transport of the substrates and products from the bulk to the gel. With the shaking bath used, the data showed that the activity levelled out at a shaking rate of 150 rpm. Actually, in all further studies, a shaking rate of 180 rpm was selected to work in optimum conditions.

3.7. Influence of the mass of enzyme encapsulated in an aerogel

In this part of the study, the enzyme was introduced during the gel synthesis, as a concentrated enzyme solution, completed by an appropriate volume of water to maintain a constant molar ratio of water to Si. The mass of enzyme ranged from 0 to 0.125 mg per aerogel sample (0 to 0.6 mg/g aerogel). As Fig. 4 shows, the total catalytic activity was a reasonably linear function of the total mass of enzyme. That is to say, in this range, no marked limitation by diffusion due to a too high mass of enzyme was observed. Consequently, further work was carried out with a mass ranging from 0.025 to 0.05 mg enzyme/g aerogel. Similar studies were made by Pencreac'h and Baratti [9,10,22] for the same enzyme immobilized by adsorption on Accurel, who also observed limitation by diffusion beyond 0.12 mg/g of support.

3.8. Mass of free enzyme

Catalytic tests were made with an increasing mass of free (commercial) enzyme, from 5 to 60 mg. The results are presented in Fig. 5 and show a reasonable linear fit, indicating no marked limitation by diffusion due to a too high mass of enzyme. The kinetics study with free enzyme was further on made with 10 mg commercial powder.

3.9. Enzymatic kinetics mechanism

Each data point used to determine the kinetics was the average of results obtained with two new samples, specially made for this data point. In order to determine the kinetic model, it is common to plot v_0 as a function of $[S]_0$, where $[S]_0$ represents the initial substrate concentration and v_0 the initial ester formation rate (i.e., the catalytic activity), for each of the two substrates (lauric acid and 1-octanol). With the encapsulated and free enzyme, these graphs (Figs. 6 and 7) showed a maximum for lower values of v_0. According to the different kinetics models presented by Segel [5], this type of graph suggested a model such as "Bi-Bi Ping-Pong with inhibition by both substrates", which would therefore prevail for the free as well as the encapsulated enzyme.

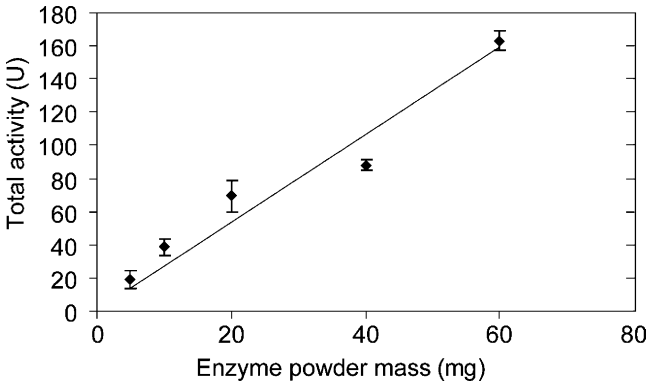

Fig. 5. Influence of the mass of free enzyme on the total activity.

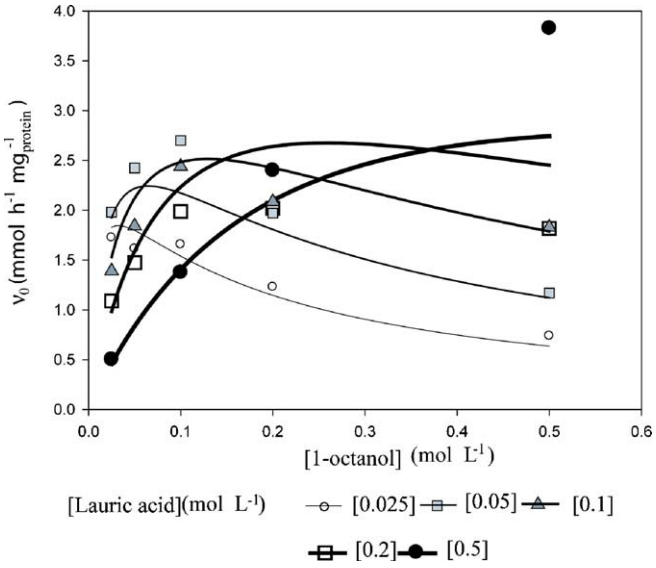

Fig. 6. Activity v_0 vs. the initial 1-octanol concentration for increasing initial lauric acid concentrations. Experimental data points and curves from the theoretical equation with the best fitted parameters, for encapsulated enzyme.

The data were then fitted to the corresponding direct Eq. (1) given below, of a Bi–Bi Ping Pong type mechanism with inhibition by alcohol and acid [5]. Presently, the fitting was done with the software "Sigma Plot 8"® of the society SSPS, using all data points.

$$\frac{v_0}{V_{max}} = \frac{[acid][alcohol]}{K_{m_{acid}}[alcohol](1+[alcohol]/K_{i_{alcohol}}) + K_{m_{alcohol}}[acid](1+[acid]/K_{i_{acid}}) + [acid][alcohol]} \quad (1)$$

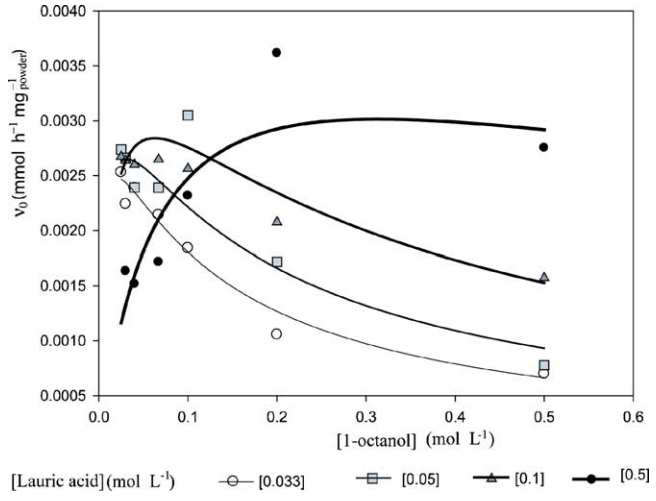

Fig. 7. Activity v_0 vs. the initial 1-octanol concentration for increasing initial lauric acid concentrations. Experimental data points and curves from the theoretical equation with the best fitted parameters, for free enzyme.

v_0 is the initial ester formation rate (activity) (1000 U/mg); V_{max} the maximal rate, Michaelis Menten parameter (1000 U/mg); []: Initial concentration of 1-octanol or lauric acid (mol/l); K_m the Michaelis constant, for the alcohol or the acid (mol/l); K_i the inhibition constant, for the alcohol or the acid (mol/l).

Direct fit with this full equation, which comprises five parameters, ended with a very large standard deviation for most kinetics parameters except V_{max}. The numerical values were meaningless and they are not reported. Nevertheless, this first fitting showed the values of $K_{m_{alcohol}}$ as well as $K_{i_{acid}}$ were very small, for the encapsulated as well as the free enzyme. Hence, inhibition by the Lauric acid was strong. Consequently the ratio $[acid]/K_{i_{acid}}$ was $\gg 1$, so that Eq. (1) could be rewritten with four parameters as (2)

$$\frac{v_0}{V_{max}} \approx \frac{[acid][alcohol]}{K_{m_{acid}}[alcohol](1+[alcohol]/K_{i_{alcohol}}) + K[acid][acid] + [acid][alcohol]} \quad (2)$$

where $K = K_m/K_i$

With this simplification, a fit with the direct rate equation for the same data points ended with significant lower standard deviations for the four parameters, including K (Table 3). It must be mentioned that fitting with the inverse rate as a function of the inverse concentrations, corresponding to the so-called Lineweaver–Burk transformation method, showed as good a fit as by non-linear regression. Indeed, no observable difference could be observed on the graphs, when plotting the fitted equations determined from the inverse or direct rate.

According to the graph in Fig. 6, the fit between the experimental data points and the theoretical model can be considered as overall acceptable in the case of the free enzyme. With the encapsulated enzyme, the fit in the graph appears qualitatively acceptable, except when both concentrations of lauric acid and 1-octanol were high (e.g. when both concentrations = 0.5 mol/l). In the latter conditions, the esterification rate increased again, an effect which was only observed with

Table 3
Numerical values of the kinetics constants obtained by best fitting of the experimental data points with the theoretical activity Eq. (2) of a "Bi—Bi Ping Pong model with inhibition by 1-octanol and lauric acid", for encapsulated enzyme and free enzyme

Statistical R^2	Encapsulated enzyme		Free enzyme	
	0.744		0.845	
Constant	Average	Stand dev.	Average	Stand. dev.
V_{max} (mmol/mg$_{protein}$ h)	5.07	1.37	0.44[a]	0.06[a]
$K_{m_{acid}}$ (mol/l)	0.0243	0.0135	0.0111	0.006
$K_{i_{alcohol}}$ (mol/l)	0.0815	0.0454	0.0319	0.0162
$K' = K_{m_{acid}}/K_{i_{alcohol}}$	0.499	0.243	0.1370	0.0424

[a] Value estimated from V_{max} (mmol/mg$_{powder}$ h) $\approx$ 0.0044, assuming the powder contains 1% active protein by mass.

the encapsulated enzyme, not with the free one. This suggests that some other kinetics model involving the gel might be more appropriate.

Other studies on the mechanism and kinetics of esterification with a lipase were published in the early 1990's [23–25]. They showed an excellent agreement with a Bi–Bi Ping Pong mechanism and inhibition by alcohol. One study also showed that the thermodynamic activity of water a_w was not sufficient to predict the activity of an enzyme [26]. Other studies, with the lipase from *B. cepacia*, were made by adsorption on polypropylene beads [9–11]. In the latter case, it was reported that the lipase activity increased by up to 18.9-fold after immobilization, in a hydrolysis reaction.

However, all these studies were made with lipase immobilized by adsorption on commercial beads, probably synthesized in large batches. Hence, the dispersion in the sample preparation was minor. With the present aerogel, two new small samples (≈200 mg each) were made for each data point. Besides, immobilization was not on the pore surface of macroporous beads, but inside a mesoporous gel texture with a much higher specific surface area. This induced sample to sample structure and texture variations during gel synthesis. Such variations were indeed the major source of dispersion of the experimental data points with the encapsulated enzyme, which was ≈10% as previously mentioned, for apparently identical samples made and tested according to the same protocols.

On the other hand, by comparison with these previous kinetics studies made with organic polymer beads, the main focus was presently on the support material, i.e. the aerogel, and how this aerogel modifies the enzyme activity. The net result is that, starting from a given total powder activity (in U) for a given mass of enzyme powder, this total activity was significantly increased in an aerogel, a result consistent with the well known results by Reetz et al. on so-called "ambigels" (present generic name for gels dried by evaporation without shrinkage, because of a high density of hydrophobic groups). Simply, in aerogels, supercritical drying makes it possible to avoid shrinkage with a lower proportion of hydrophobic groups.

Overall, regarding the present aerogel, the type of kinetics model fitting best the data was not changed by encapsulation (i.e. Bi–Bi Ping Pong with inhibition by both substrates, in particular by the alcohol) except when both substrate concentrations were high. Mainly, V_{max} was of the order of 10^3 times lower, per mg of powder for the free enzyme, than per mg of protein for the encapsulated one. Even if one considers that 1 mg of so-called free enzyme only contained ≈1% protein, this still made V_{max} 10 times lower for the free lipase than for the encapsulated one, per mg of protein. This is probably an effect of using an organic solvent in which free enzyme agglomerate, while this agglomeration is avoided when using aqueous an enzyme solution to synthesize the gel, as discussed further on in the text. This result is also globally consistent with a previous report regarding the transesterification of vinyl laurate [6].

Most studies on sol–gel encapsulation of enzyme have shown that encapsulation does not change the kinetics mechanism type, but only the magnitude of the kinetics constants. Moreover the effect depends largely on the encapsulation medium and the enzyme. Usually, in xerogels, the Michaelis constant K_m increases and the present results are consistent with this. In a classical quasi-equilibrium Michaelis type interpretation of the kinetics, and for give substrate S,

$$K_m = \frac{[E][S]}{[ES]} \quad (3)$$

where [S], [E] and [ES] stand for the concentrations of, respectively, the substrate, enzyme free of substrate and enzyme–substrate complexes, in the solution. A higher K_m is then considered as an indication of a weaker affinity of the enzyme for the substrate [27–30].

For a given enzyme, i.e. the present lipase, it may seem difficult to admit that K_m should be modified, unless the substrate itself were modified before being fixed by the enzyme. This in turns suggests that a possible transformation of the substrate by the gel may be worth considering. Such a possibility would indeed be very similar to that which prevails in traditional chemical catalysis, for instance in the isomerization of paraffins by strong acidic catalysts such as chlorided alumina or a zeolite of the type mordenite. Metals such as Pt deposited on the acidic oxide do not by themselves catalyze such reactions. However they can adsorb and dissociate dihydrogen molecules, so that the resulting monoatomic hydrogen can migrate in surface towards the acidic catalytic oxide sites and regenerate them by destroying the deactivating carbonaceous residues. Hence they accelerate the catalytic process, a phenomenon known as "spillover" [31]. Actually, in an esterification reaction by a lipase, the enzyme performs the same final transformation (formation of an ester) as an acid catalyst such as H_2SO_4 in traditional chemical synthesis. Moreover, in a silica aerogel, both substrates (alcohol and carboxylic acid) can easily adsorb on silanols Si–OH. Considering that at neutral pH the surface of silica carries an excess of negative charges (zero point charge at pH ≈ 2.5), an alcohol molecule ROH could easily adsorb by hydrogen bonding on a negatively charged surface siloxane ≡Si–O^- or even on a neutral silanol to ≡Si–OH, and lose a proton to produce respectively a neutral siloxane ≡Si–OH or a protonated silanol ≡Si–OH_2^+, plus an alkoxy anion RO^-. This anion would in turn diffuse very fast in surface towards the enzyme, due to repulsion by negative silica surface charge. This suggestion is consistent with the fact that the lipase was also able to perform an esterification reaction with adsorbed methanol molecules, in spite of the fact this methanol remained adsorbed on silica even after acetone dialysis and subsequent CO_2 supercritical drying.

As for V_{max}, it has usually been found to decrease for most xerogels [32] except in recent studies with a few enzymes where it could drastically increase. This is the case with some lipases [2,33,34] and the present study fits within this second category. V_{max} is related to the total concentra-

tion of enzyme [E_0] available to complex the substrate, by the relationship.

$$V_{max} = k_{cat}[E_0] \quad (4)$$

where the catalytic constant rate k_{cat} represents the rapidity (or frequency) with which an enzyme accomplishes a catalytic cycle. A reason for V_{max} increase could come from the real maximum concentration of enzymes available for a cycle [E_0]. By using an encapsulation process where the enzyme is introduced as an aqueous solution, the lipase is well dispersed in the gel and does not agglomerate. Encapsulation also enables to well disperse the enzyme in an organic medium, which is not the case when using free enzyme, which is not soluble and tends to agglomerate.

3.10. Recycling study

As explained previously, it was not convenient for this part to pre-equilibrate the reaction medium at constant water activity a_w between each catalytic tests, with metal salts. Hence, all tests were simply carried out in water saturated isooctane. A water saturated hydrocarbon should have a water thermodynamic activity a_w close to 1, while the hygrometry of the vapor in equilibrium with water saturated isooctane was measured to be close to $a_w = 0.39$, which was roughly the hygrometry prevailing in the laboratory most of the year. Nevertheless, this surprising finding may be significant. Indeed, considering that water is virtually un-soluble in isooctane (no data are available to our knowledge), the diffusion of a very small population of water molecules is sufficient to reach equilibrium in changing humidity conditions. Hence, a previously water saturated isooctane solution can very fast return to a state with an a_w close to that prevailing in the laboratory, when in contact with air. In such case, a first catalytic test was virtually made by pre-equilibrium with the laboratory humidity. On the other hand, after this first catalytic test, the water produced by the esterification reaction may have remained trapped inside the gel, bringing the gel a_w to a value closer to the optimal enzyme catalytic activity.

Besides a significant enhancement of the catalytic activity after one recycling, the data in Fig. 8 show a gradual decline during further tests. The initial activity was recovered at the eighth test. To determine if the gradual loss of activity after the second test was due to a loss of enzyme in the reaction medium (phenomenon of lixiviation), an aerogel was withdrawn from a reaction medium after 2 h catalysis in a test. It was then observed that the esterification reaction stopped. On the other hand, if the washed aerogel was introduced in a new fresh reaction medium, the esterification started again with the same initial rate as when used in the previous reaction medium. This proves that catalysis is not due to free enzyme. Nevertheless, the loss of activity could also be due to some enzyme inactivated enzyme, possibly desorbed during the process. In anyway, an aerogel is also partially crushed by the agitation process during a test and some of the finest

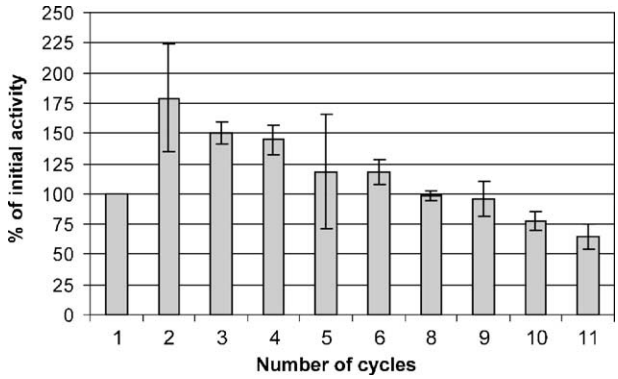

Fig. 8. Evolution of the catalytic activity of an aerogel, by comparison with the activity during the first test, after several recycling.

particles were lost during washing by filtration and recycling. The exact mass of gel lost was un-practical to assess exactly, because the initial sample was dry, while the gel was wet with isooctane after a test. Nevertheless a mass not exceeding 10% of the initial gel mass was estimated to be lost after several tests.

3.11. Influence of the temperature

All data reported so far were obtained at 30 °C. However, the effect of temperature was studied. Catalytic tests were carried out at temperatures ranging from 20 to 70 °C, both with encapsulated enzyme and free enzyme. The data reported in Fig. 9 were obtained from the initial conversion curves were linearity as a function of time appeared experimentally acceptable, as shown in Fig. 10, given a probable deactivation of the enzyme as the temperature increases. They show that free and encapsulated enzyme had a similar behavior and an optimum catalytic activity around 40 °C. These results are consistent with previous results indicating an optimal temperature of 37 °C for the same enzyme, although in a different reaction [8]. The determination of an activation energy E_a, according to a variation of the Arrhenius type has been attempted in the temperature range 20–40 °C. The results were compared with those determined for a traditional homogenous chemical catalysis by 0.85 mmol H_2SO_4, in the

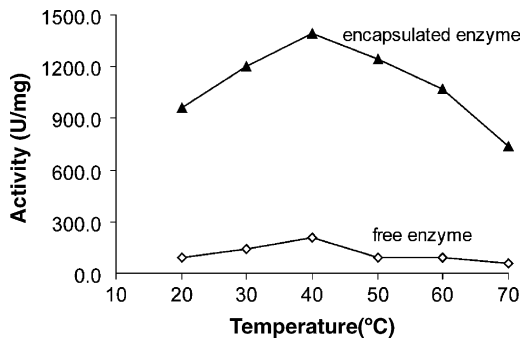

Fig. 9. Variation of the catalytic activity of free enzyme and encapsulated enzyme as a function of the reaction temperature.

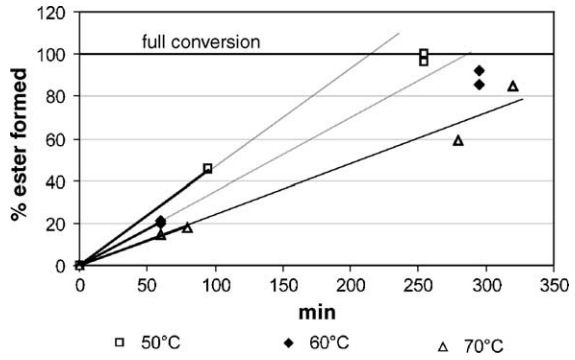

Fig. 10. Conversion kinetics to the ester at temperatures above the optimum temperature.

same solvent and with the same substrate concentrations. The corresponding apparent activation energies were respectively 10.4 kJ/mol for the free enzyme, 14.2 kJ/mol for the encapsulated one and 18.2 kJ/mol for H_2SO_4. These values are somewhat low by comparison with that reported by Pencreac'h for hydrolysis in *n*-heptane with the same lipase (24.7 kJ/mol). The loss of activity beyond 40 °C after encapsulation was also surprising. Hence we checked if this was a reversible or an irreversible deactivation. For this purpose, catalytic tests were carried out at 30, 60 and 70 °C during 5 h, the gels were then recycled and used at 30 °C. It seems that the lipase was not permanently deactivated, and more amazingly that all activities were multiplied by a factor of the order of 1.7 during the second test. This is consistent with the data on recycling (increase of activity after the first use).

It has been well documented that in silica gels, the conformational motion of enzyme molecules is slowed down by the gel network [35–38]. This fact is sufficient to explain that the enzyme becomes more stable at higher temperature, when encapsulated, because conformational changes leading to denaturation are slowed down as well. In the present study on aerogels, the optimal temperature was not changed by encapsulation. That is to say the aerogel did not improve the thermal stability of the lipase and hence possibly had a lower effect on the freedom of motion of the enzyme. This can come from the fact that an aerogel network is more loose than a xerogel network, hence interactions between the enzyme and the aerogel network are not so strong, so that the effect of protection against denaturation is lost to some extent and the freedom of motion of the enzyme can be considered to be less altered by an aerogel.

4. Conclusion

The esterification kinetics of lauric acid with 1-octanol was studied with the free lipase from *Burkohlderia cepacia*, as well as the lipase encapsulated in an aerogel comprising a moderate proportion of hydrophobic CH_3 groups. The experimental data fits at least qualitatively well with a kinetics model known as "Bi-Bi Ping-Pong with inhibition by both substrates", except at high substrate concentration for the encapsulated enzyme. Aerogel encapsulation has the effect of drastically increasing the Michaelis kinetics constants V_{max}, as well as all K_m and K_i. Small aerogel monoliths with a size of a few mm do not show limitation diffusion and they improve the storage stability of the enzyme.

Acknowledgements

The authors are grateful to the company Amano, for gracefully providing the lipase used in the present study. They are also very grateful to Professor J. Baratti from the University of Marseille Luminy (France) for many advices on lipases, and to the referees for many remarks to improve the paper.

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