Process Biochemistry 46 (2011) 1083–1089

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Immobilization of *Rhizopus oryzae* lipase on silica aerogels by adsorption: Comparison with the free enzyme

Nadia Kharrat[a], Yassine Ben Ali[a], Sana Marzouk[b], Youssef-Talel Gargouri[a,*], Maha Karra-Châabouni[a]

[a] *Laboratoire de Biochimie et de Génie Enzymatique des Lipases, ENIS route de Soukra, BPW, 1173 Sfax-Tunisia, Tunisia*
[b] *Laboratoire de chimie industrielle II, ENIS route de Soukra, BPW, 1173 Sfax-Tunisia, Tunisia*

ARTICLE INFO

Article history:
Received 23 September 2010
Received in revised form
29 December 2010
Accepted 25 January 2011

Keywords:
Rhizopus oryzae lipase
Immobilization
Adsorption
Silica aerogels
Stability
Esterification

ABSTRACT

Rhizopus oryzae lipase (ROL) was immobilized by physical adsorption onto silica aerogels. The functional properties of immobilized lipase were determined and compared to the soluble lipase ones. The optimum temperature for both free and immobilized lipase activities was 37 °C. We found that the immobilization of *R. oryzae* lipase onto silica aerogels increased remarkably its stability at high temperatures and within a wade pH range. Besides the immobilized enzyme exhibited a high tolerance to apolar solvent and retained its fully activity in suspension after 4 months of storage at 4 °C. This immobilized biocatalyst is applied in n-butyl oleate synthesis by esterification of oleic acid with n-butanol, using hexane as an organic solvent. The best conversion yield of the ester butyl oleate was obtained with the immobilized lipase (80% versus 35% with the free lipase). This catalytic esterification has been carried on the presence of hexane at 37 °C with oleic acid to butanol molar ratio of 1:1 and 450 IU of immobilized lipase. Furthermore, the reuse of the lipase immobilized by adsorption allowed us to observe that its can achieved 12 successive cycles, without a significant loss of its catalytic activity. Such results revealed good potential for recycling under non-aqueous system.

1. Introduction

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are among the most popular enzymes in biocatalysis with a broad variety of applications in fine chemistry, pharmaceutical industry and in the food industry owing to their usefulness in both hydrolytic and synthetic reactions [1,2]. Most of lipases used in industrial processes were in the immobilized state. Indeed, the immobilization of enzymes to solid support materials is of great interest due to some important advantages such as: improvement of the activity and stability of enzymes, possibility recovery of the immobilized enzyme at the end of the reaction and thus its potential reuse and accomplishment of the enzyme-catalyzed reaction under a continuous process [3]. Numerous papers reported different lipases immobilization techniques such as physical adsorption of the enzyme on a carrier material [4], its entrapment or microencapsulation in a solid support [5,6] and covalence binding to a solid matrix [7,8]. Typical materials used for these purposes include chitin, cellulose, polyalanine and silica [9]. Silica aerogels are extremely porous materials with high specific surface areas. It exhibits interesting physico-chemical properties, including very high specific surface areas (500–1000 $m^2 g^{-1}$) [10]. Due to economical considerations, Silica aerogels can be considered as the suitable carrier for biocatalysts immobilization.

The selection of an immobilization strategy is based on effectiveness of enzyme utilization, cost of the immobilization procedure, toxicity of immobilization reagents and the desired final properties of the immobilized biocatalyst [11]. The immobilization could resolve some problems when using enzymes as industrial biocatalysts: enzyme recovery, enzyme stability, enzyme selectivity, inhibition by the medium or products. In all these cases, the use of a battery of immobilization procedure permits the control the support-enzyme interaction and increases the possibilities of success. Among immobilization techniques, adsorption may have the highest commercial potential compared to other techniques due to its relatively low cost, simpliness and it allows retaining of high catalytic activity. This method offers the reusability of expensive supports after inactivation of immobilized enzyme [12–14]. However, adsorption is generally not a very tight interaction and the protein will desorbs from support during washing and other steps. Thus, immobilization via adsorption requires an electrostatic interaction between support and enzyme. The lipase immobilization by adsorption on various supports was reported in numerous studies. Macroporous resin was used as support for the immobilization of *Candida* sp. 99–125 lipase by physical adsorption [15]. Foresti and Ferreira [16] reported the immobilization of *Candida antarctica* B lipase, by adsorption, onto polypropylene coated glass balls, and used to synthesize ethyl oleate. Torres et al. [17]

* Corresponding author. Tel.: +216 74 675055; fax: +216 74 675055.
E-mail address: ytgargouri@yahoo.fr (Y.-T. Gargouri).

1359-5113/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2011.01.029

reported the immobilization of the same lipase by adsorption on polyethylene–agarose.

In this study, we describe the physical adsorption of *Rhizopus oryzae* lipase (ROL) isolated and produced in our laboratory [19], onto silica aerogels. The stability and the activity of the immobilized lipase were investigated. Finally, we have tested the ability of this enzyme to synthesize 1-butyl oleate used in industrial applications.

2. Materials and methods

2.1. Materials

The n-hexane, the 1-butanol, and the dioxane (99.5% of purity) were purchased from Prolabo (Paris, France). The oleic acid was purchased from Fluka (Buchs, Switzerland). The pH-Stat was from Metrohm (Herisau, Switzerland). The shaker (Certomat H/HK) was from B. Braun Biotech (Goettingen, Germany). The spectrophotometer UVmini 1240 was from Shimadzu (Japan). The centrifigeuse was from Hermle (Germany).

The strain was isolated from olive in decomposition and it was identified as *R. Oryzae* [19].

2.2. Support

Silica aerogels were provided by the Laboratory of Industrial Chemistry II (ENIS), they were produced as described by Marzouk et al. [18]. Aerogels was obtained by attack of sol with a solution of sodium hydroxide. In the obtained sodium metasilicate sol SiO_2–Na_2O, we substitute the sodium ions with ammonium ones using ions exchange resin. The ammonium metasilicate sol transforms into hydrogel after destabilization with chloric acid 2 M or acetic acid 2 M. The obtained hydrogel is changed in alcoogel by continuous washing with pure ethanol in a soxhlet during 10 days. By hypercritical drying of alcoogels in an autoclave at 350 °C, they transform into silica aerogel. The approximate values of the pore size are between 5 and 100 nm, the specific surface area is Sp 110 $m^2\ g^{-1}$ and the specific pore volume is Vp 4 $cm^3\ g^{-1}$. The silica aerogels are used as solid supports for lipase immobilization.

2.3. Immobilization of lipase

R. oryzae lipase was produced and partially purified as described by Ben Salah et al. [19]. At the end of the cultivation period, the mycelium was removed by filtration. The supernatant was brought to 65% saturation with solid ammonium sulphate $(NH_4)_2SO_4$ followed by centrifugation for 20 min at 8000 rpm and 4 °C. The pellet was resuspended in 50 mM sodium acetate buffer pH 6 containing 100 mM NaCl and 2 mM benzamidine. Then, the enzyme solution was centrifuged at 8000 rpm for 10 min and the supernatant containing the lipase (crude lipase) was used for immobilization.

Adsorption of crude lipase was carried out in batch by adding to a suspension containing 1% (w/w) of silica aerogels, lipase dissolved in acetate buffers (pH 6) containing 13,000 IU/ml. The adsorption was conducted for 1 h at 4 °C in a shaking water bath. The lipase-adsorbed onto silica aerogels was washed with 20 ml of acetone priorly chilled at −20 °C, filtered through a Buchner funnel and dried at room temperature. The immobilized lipase was stored at 4 °C until use. The yield of the immobilized lipase activity was defined as the ratio of the adsorbed activity recovered at the end of the immobilization period divided by the total soluble lipase activity initially added to 1 g of the support.

2.4. Determination of protein concentration

Protein concentrations were determined as described by Bradford [20] using bovine serum albumin ($E^{1\%}_{1\,cm} = 6.7$) as reference.

2.5. Lipase activity

The activities of the free and immobilized lipase were measured titrimetrically using a pH-Stat (Metrohm, Herisau, Switzerland), under the standard assay conditions described previously using olive oil emulsion as substrate [21]. Briefly, the reaction mixture contains 10 ml of emulsified olive oil (1 ml of olive oil and 9 ml of arabic gum at 10%), 20 ml of distilled water and 50 μl of bovine albumin serum 12.5%. The reaction was carried out at 37 °C and at pH 8.5. The amount of free fatty acid released during hydrolysis was estimated by titration with 0.01 N NaOH solution. Activity was expressed as units per ml of broth (enzymatic solution). One international unit (IU) of lipase activity was defined as the amount of enzyme that catalyses the liberation of 1 μmol of fatty acid from olive oil as substrate per min at pH 8.5 and at 37 °C.

2.6. Characterization of immobilized lipase

2.6.1. Effect of temperature on the free and the immobilized lipase activities and stabilities

The effect of temperature on the relative activity of the free and immobilized ROL was determined at pH 8.5 in temperature range varying from 25 to 50 °C, using olive oil emulsion as substrate. Relative activities were calculated as the ratio of the enzyme activity measured at different temperatures to the maximal activity of the enzyme measured as described above.

The thermal stability assays were performed by the incubation for 1 h of the same amount of immobilized or free ROL at various temperatures (25–60 °C). After cooling to room temperature, the activity of enzyme was measured under standard conditions (pH 8.5, 37 °C) as described above. Residual activities were calculated as the ratio of the activity of enzyme measured after incubation to the maximal activity of the enzyme.

2.6.2. Effect of pH on the free and the immobilized lipases activities and stabilities

The effect of pH on the free and immobilized lipase activities was studied in the pH range varying from 7.5 to 11 at 37 °C using olive oil emulsion as substrate. Relative activities were calculated as the ratio of the activity of enzyme measured at different pH to the maximal activity of enzyme, at pH 8.5 and 37 °C.

The effect of pH on the enzyme stability was determined by incubating immobilized and free lipase to different pH values ranging from 3 to 12 for 1 h at 4 °C, by using different buffers: glycine–HCl 50 mM (pH 3–4), sodium acetate 50 mM (pH 5–6), phosphate 50 mM (pH 7), Tris–HCl 50 mM (pH 8–9) and boric acid 50 mM (pH 10–12). Then the lipase activity was measured under the standard conditions (pH 8.5, 37 °C). Residual activities were calculated as the ratio of the activity of enzyme measured after incubation to the maximal activity of the enzyme.

2.6.3. Effect of organic solvents on the stability of immobilized lipase

The effect of organic solvents on the stability of immobilized *R. oryzae* lipase (ROLi) was determined using solvents of polarity (log *P*) ranging from −0.23 to 3.5. The immobilized lipase was incubated with the experimental solvent for 1 h at 37 °C. Then, the solvent was removed by centrifugation. The immobilized lipase was dried under vacuum and then assayed for lipase activity using oil olive emulsion as substrate under standard conditions. The residual activity of the solvent-treated lipase was expressed as the ratio of the activity of treated immobilized lipase to the activity of the untreated immobilized lipase.

2.7. Esterification assay

The esterification reactions were performed in screw-capped flasks with a molar ratio of oleic acid to butanol 1:1 (0.4 mmole of oleic acid and 0.8 mmole of butanol), 450 IU of immobilized lipase dissolved in 4 ml of anhydrous n-hexane. The reaction mixture was shaken for 8 h at 220 rpm at 37 °C in a shaking incubator.

Aliquots of 200 μl were withdrawn periodically from the reaction mixture. The immobilized enzyme was removed by centrifugation at 2000 rpm for 5 min, then the supernatant residual acids contents were assayed by titration with 0.5 N sodium hydroxide, using phenolphthalein as an indicator and 2 ml of ethanol as a quenching agent. The conversion (%) of ester synthesis was calculated based on the conversion of the acid to ester after a given time.

2.8. Reusability of the immobilized R. oryzae lipase

The esterification of oleic acid with butanol was conducted under these conditions (butanol/oleic acid molar ratio equal to 1; enzyme amount equal to 450 IU; hexane volume of 3 ml and reaction time of 8 h) using *R. oryzae* lipase immobilized onto silica aerogel. This immobilized ROL was reused many times for consecutive cycles. At the end of each batch, the immobilized lipase was removed from the reaction medium, washed with n-hexane in order to remove any substrate or product retained in the support and dried at room temperature. Then, the immobilized lipase was used again for another reaction cycle using fresh substrates.

2.9. Statistical analysis

Experimental results were given as mean value ± SD of three parallel measurements. All statistical analysis were conducted using Microsoft Excel.

3. Results and discussion

3.1. Adsorption isotherm of ROL onto silica aerogels

To evaluate the loading capacity of the support, 1 g of silica aerogels particles were loaded with different initial protein amounts (4300–11,876 μg). As shown in Fig. 1, the adsorbed protein amount increased as more initial protein amount was loaded onto the support to reach a maximum value of 10,000 $\mu g\ g^{-1}$ which is equivalent to 13,000 IU attesting that the adsorbing support surface is

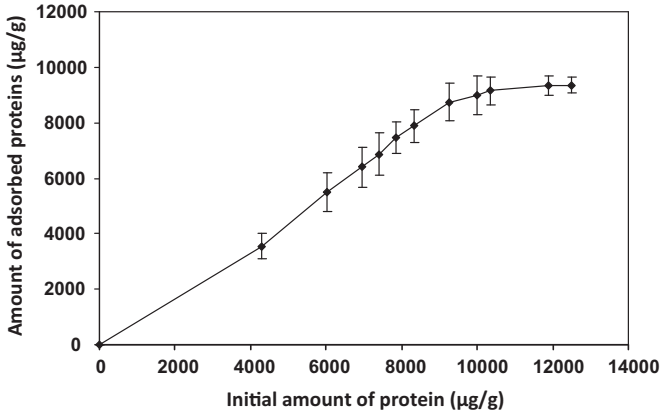

Fig. 1. Adsorption isotherm of ROL onto silica aerogels using different initial amount of protein. Lipase activity was measured by pH-stat using olive oil emulsion as substrate at pH 8.5 and 37 °C.

saturated with protein molecules. The immobilized yield of ROL to silica aerogels reached 95%. This result shows that silica aerogels have excellent adsorption properties due to its large specific surface area (Sp 110 $m^2\ g^{-1}$) and high porosity (Vp 4 $cm^3\ g^{-1}$) which allow the adsorption of a high amount of protein. In addition, silica aerogels are hydrophobic mesoporous materials with methoxy groups (–OCH3)*x*, so the adsorption enzyme-support involves multiple interactions through hydrogen, ionic and hydrophobic interaction.

Ghamgui et al. [22] reported that the immobilization of the same lipase onto $CaCO_3$ displayed a similar immobilization yield (93.75%). However, Karra-Chaâbouni et al. [23] reported that the same *R. oryzae* lipase immobilized on cellulose fibers, displayed an immobilization yield of about 70%.

3.2. Adsorption kinetic of ROL onto silica aerogels

The immobilization yield was measured at different incubation time of an enzymatic solution (13,000 UI) with 1 g of support at 4 °C. As shown in Fig. 2, a 60 min of incubation time of the enzymatic solution with the support was considered ideal to reach the maximum adsorption of the enzyme onto silica aerogels (Fig. 2). A decrease of the adsorbed activity was observed after 120 min of incubation. This desorption is probably due to the disruption of the weak physical forces linking the enzyme to its support. Basri et al. [24] showed that the best immobilization yield of *Candida rugosa* lipase onto Amberlite XAD7 was obtained after 30 min of incubation.

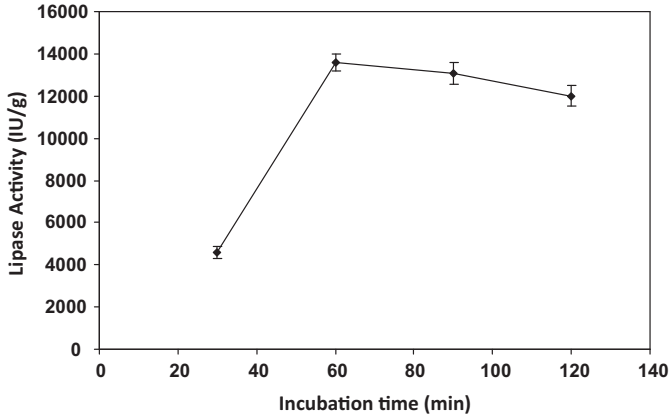

Fig. 2. Kinetic of ROL adsorption on the silica aerogels. The activity was measured using olive oil emulsion as substrate at pH 8.5 and 37 °C.

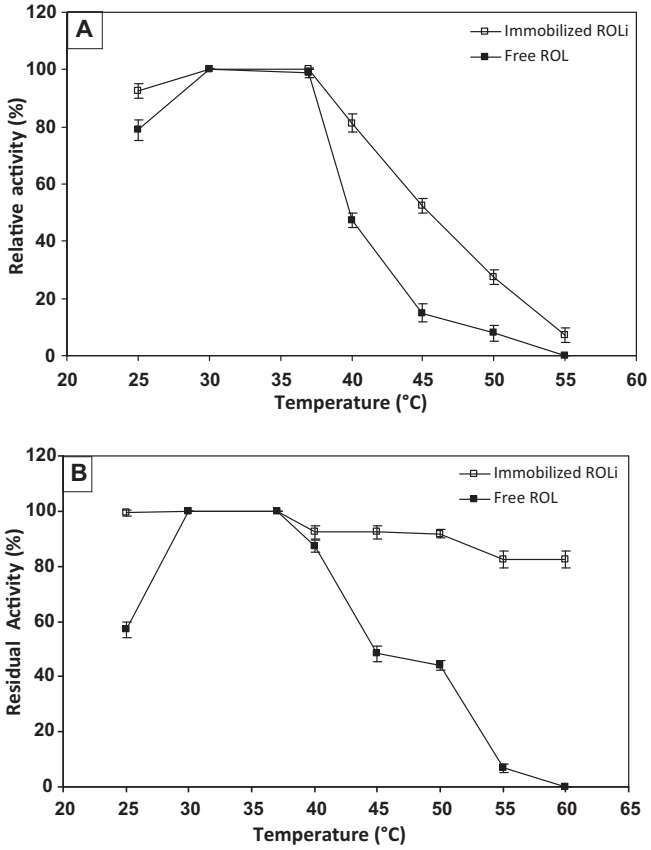

Fig. 3. Effect of temperature on the (A) activity and (B) stability of the free and immobilized ROL. Enzymes activities were assayed at pH 8.5 using 10 ml of olive oil emulsion as substrate, 20 ml of distilled water and 50 µl of bovine albumin serum 12.5%. Enzymes were incubated 1 h at different temperatures for stability study.

The immobilization onto silica aerogels was carried out with 13,000 IU g^{-1} of support during 60 min and gave rise to the highest immobilization yield (95%). This result could be explained by the presence of micropores of the silica aerogels that protect the lipase against alterations of the microenvironment. Gao et al. [9] have reported that the immobilization yield of *C. rugosa* lipase on methyl-modified silica aerogels by physical adsorption is only 56.4%.

3.3. Biochemical properties of free and immobilized lipase

3.3.1. Effect of the temperature on lipase activity and stability

The immobilization of lipases onto solid support contributes to increase their thermostability and to extend their biotechnology potential, since running bioprocesses at elevated temperature is advantageous because of higher diffusion rate, lower substrate viscosities, increased reactant solubilities and reduced risk of microbial contamination [25].

The temperature dependence of the lipolytic activity of free or immobilized ROL was studied at pH 8.5 in the range of 25–50 °C. The activity profiles of free and immobilized lipase at different temperature are represented in Fig. 3A. The optimum temperature for both free and immobilized lipases was found to be 37 °C. Beyond 40 °C, the relative activity of the free lipase declined rapidly, while the immobilized lipase seemed to be more stable to heat. Indeed, the immobilized lipase retained 50% of its activity at 45 °C and the soluble one retained only 20% of its activity and was completely inactivated at 55 °C. Silica aerogels may be protecting the enzyme against thermal denaturation. Similar result was reported in our previous study, we did not notice changes of the optimum temper-

ature for the ROL immobilized onto oxidized cellulose fibers, and in the same time a significant improvement of its thermal stability was observed [23]. However, Kılınç et al. [26] have noted a change in the optimum temperature of porcine pancreatic lipase activity after its immobilization by adsorption on chitin and chitosan and subsequent cross-linking with glutaraldehyde.

Thermal stability of soluble and silica aerogels-immobilized ROL was determined by measuring the residual activity as function of temperature in the range of 25–60 °C (Fig. 3B). Immobilized enzyme was inactivated at a much slower rate than the free enzyme after incubation at 60 °C for 1 h, in this condition, the immobilized lipase retained 83% of its activity while the free lipase was completely inactivated. These data indicate that the thermal stability of lipase was enhanced by the described immobilization method. This could be due to the lipase location inside the support micropores which offer a good protection against alterations. Abdul Rahman et al. [27] found that the thermal stability of *C. rugosa* lipase was enhanced after its immobilization onto natural kaolin by physical adsorption method. De oliveira et al. [28] have showed that the *C. rugosa* lipase immobilized on styrene-divinylbenzen have lost 50% of its activity after only 1 h of heat treatment at 60° C. Under the same conditions, the free enzyme lost 94% of its activity [28]. It is often found that immobilized enzyme has a higher thermal stability than free enzyme due to restriction of its conformational flexibility attributed to its multiple attachment point of enzyme on the support which limit the conformational alterations and movements under various temperatures [29].

3.3.2. Effect of pH on lipase hydrolytic activity and stability

The effect of pH on the immobilized enzyme activity depends on the enzyme, the immobilization method and the carrier used. Effect of pH (7.5–11) on the activity of free and immobilized lipase was studied. As shown in Fig. 4A, the immobilization did not cause a shift on the optimal pH for the activity, in fact the highest enzyme activity was obtained for both forms of the enzyme at pH 8–8.5. Beyond pH 9, we noted a decrease in the activity for free and immobilized enzyme. Usually the immobilization of enzyme on a cationic carrier cause a shift on the optimum pH to the acidic range, while, the immobilization on an anionic carrier cause a shift to the basic pH values [30]. In general, the immobilized enzymes have either a broader or the same pH range of high activity than free enzyme [31–33].

The ROL immobilized onto silica aerogels remained stable and kept about 95% of its activity within a large range of pH varying from 5 to 9 (Fig. 4B). The free enzyme looses 60% of its activity at pH 5 and 30% of its activity at pH 9. This result indicates that the immobilization procedure improve the lipase stability over a broader pH range. This result is consistent with other works [34–36]. Krajewska et al. [37] showed that the immobilization of urease significantly improved its stability to pH.

3.3.3. Effect of organic solvents on lipase stability

Several organic solvents have various physicochemical effects on enzymes. It is well known that the enzyme activity is strongly affected by the nature and the polarity of the organic solvent which may cause the denaturation of the enzyme and thus leads to the loss of its catalytic activity. The organic solvents change the native conformation of the enzyme by disrupting of the hydrogen bond and hydrophobic interactions, leading to alterations of activity and stability [38]. In this study, the tolerance of immobilized lipase to organic solvent was studied. Residual activity was measured versus time in different organic solvents (polar and non-polar) with polarity index (log *P*) ranging from −0.23 to 3.5 (Fig. 5). The log *P* value of a solvent is the parameter used to describe its polarity and its possible effects on enzyme activity. *P* is the partition coefficient of

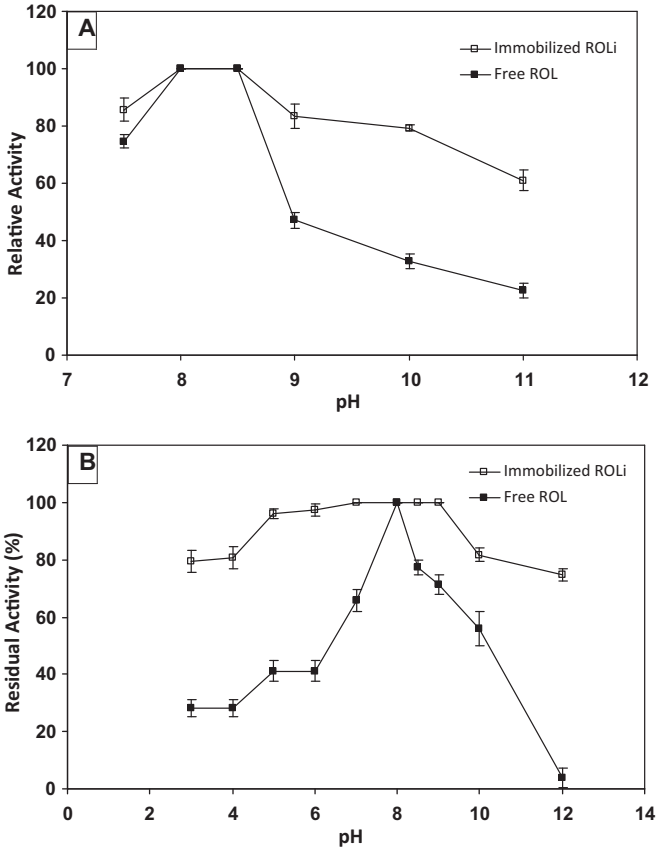

Fig. 4. Effect of pH on the (A) activity and (B) stability of the free and immobilized ROL. Enzymes activities were assayed at 37 °C using 10 ml olive oil emulsion as substrate, 20 ml of distilled water and 50 μl of bovine albumin serum 12.5%. Enzymes were incubated 1 h at different pH for stability study.

a given solvent between water and octanol in a two-phase system [39].

Fig. 5 shows that the hexane and the tert-butanol are tolerated by the silica aerogels-immobilized ROL. Indeed, ROLi maintained 100% of its maximal activity after a long incubation period. As against, the ROLi looses 50% of its activity in the presence of dioxane after 150 min of incubation. Also, we found that the inhibition is less pronounced when the solvent is more hydrophilic. This could be explained by the fact that hydrophilic solvents such as acetoni-

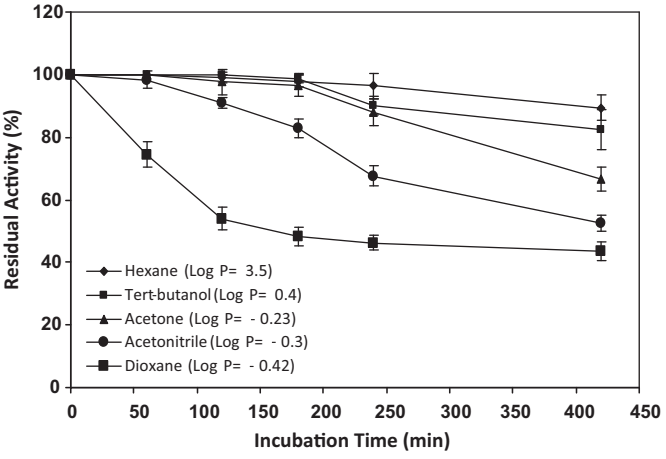

Fig. 5. Effect of organic solvents on the stability of immobilized ROL. Enzymes activities were assayed with olive oil emulsion as substrate at pH 8.5 and at 37 °C. The *P* value of each used organic solvent is shown.

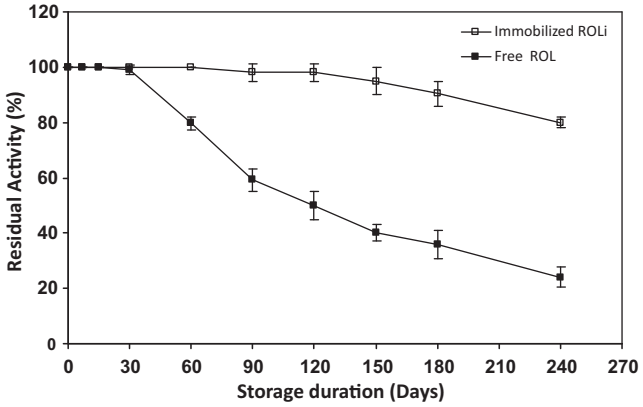

Fig. 6. Residual activity of free and immobilized ROL as affected by incubation at 4 °C after a storage period of 240 days.

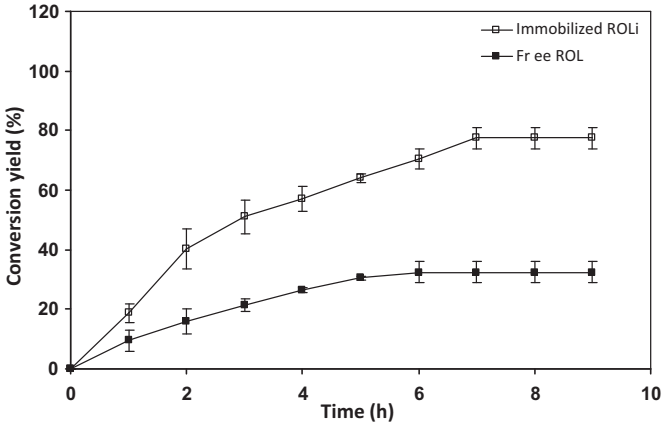

Fig. 7. Synthesis of n-butyl oleate by the free and the immobilised ROL. Reaction conditions are the following: 450 IU of lipase, 4 ml hexane, an butanol/oleic acid molar ratio of 1 at 37 °C and stirred at 220 rpm.

trile and dioxane can remove the water layers that hydrate the enzyme preparation and thus distort the catalytic conformation of the enzyme [40]. These results are on line with several studies showing the ability of many lipases to remain active exclusively in the presence of hydrophobic solvents [41].

3.3.4. Effect of storage temperature on lipase activity

The ability to be stored for a period of time at a certain temperature is an important parameter that must to be considered when using immobilized lipases. The immobilization of enzyme to a support often limits its freedom to undergo drastic conformational changes, and hence results in increased stability towards denaturation [29]. Silica aerogels-immobilized lipase retained 100% of its catalytic activity when stored at 4 °C whereas the activity of the free lipase decreased to 50% after a storage period of 150 days (Fig. 6). Moreover, the decrease of the activity over time is more pronounced in the case of the free ROL which retained only 40% of its activity after 5 months, while immobilized *R. oryzae* lipase remained stable and active during the same period and kept up to 80% of its activity after 8 months of storage. This confirms that the silica aerogels provide better stability to the enzyme, due to its porosity, which protects the three-dimensional conformation of the lipase and the active site of any distortion or structural changes that can affect its catalytic activity. In addition, multiple attachments of the enzyme to the support, preventing any intermolecular process such as proteolysis and aggregation and therefore, creating a more rigid enzyme molecule [42].

3.4. Esterification activity of the immobilized ROL

After immobilization of ROL on silica aerogels, we tested its ability to catalyze esters synthesis in organic microaqueux media. We took the synthesis of butyl oleate by direct esterification of butanol and oleic acid in hexane, as a reaction model. This ester is used in the field of oleochemistry biodiesel as an additive and as a poly vinyl chloride plasticizer.

3.4.1. Kinetic synthesis of butyl oleate with immobilized ROL

As seen in Fig. 7, a high conversion yield of 80% was obtained with the immobilized lipase. Meanwhile, the free lipase allowed only 35% of conversion yield. So we have shown not only that the immobilization of ROL onto silica aerogels improved its activity and its thermal stability but also increased the performance of this enzyme to catalyze reactions in a synthesis organic unconventional media. Several studies have described the production of esters using various immobilized lipases but the conversion yield varied considerably with strain [43–45].

3.4.2. Effect of the repeated use of immobilized ROL

The reuse of the enzyme constitutes the main advantage of the process of biocatalysts immobilization. The immobilized enzyme is an important parameter for repeated applications in batch reactors or continuously. The reusing of the lipase for several reactions allowed the reduction of the reaction cost and making an economically feasible process. Moreover, the use of an immobilized enzyme permits to greatly simplify the design of the reactor and the control of the reaction [46]: the filtration of the enzyme stops the reaction; it is possible to use any kind of reactor, etc. However, the idea of enzyme reuse implicitly means that the stability of the final enzyme preparation should be high enough to permit this reuse [3].

To check this parameter, silica aerogels-adsorbed lipase was used in subsequent cycles in the esterification reaction of oleic acid with n-butanol under the same experimental conditions as described above and using 450 IU of lipase. At the end of each batch, the immobilized enzyme was removed from the reaction medium and washed with n-hexane in order to remove any substrate or product retained in the support. Then, the immobilized lipase was consecutively reused after each reaction cycle. As shown in Fig. 8, we noted that immobilized ROL on silica aerogels was reused 11 times with the same efficiency (conversion yield of 80%), starting from the cycle number 12, the conversion yield begin to decrease. This result show that the immobilization of ROL by adsorption on silica aerogels allowed not only a good summary of activity in the

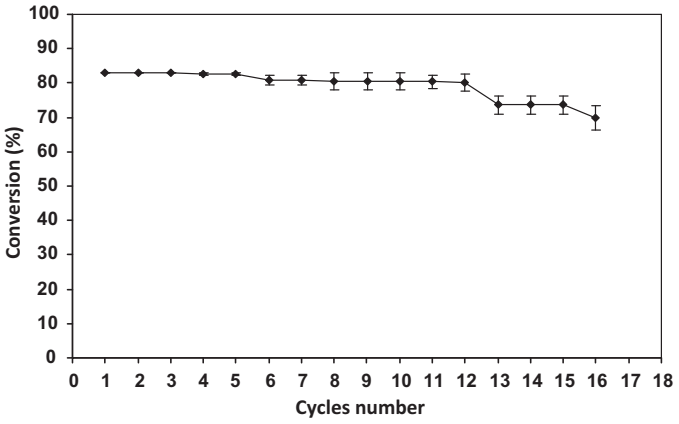

Fig. 8. Effect of repeated use of immobilized ROL on the conversion rate of butyl oleate. Reaction conditions are following: 450 IU of lipase, 4 ml hexane, an butanol/oleic acid molar ratio of 1 at 37 °C and stirred at 220 rpm. The incubation time was 8 h for each cycle.

presence of organic solvent but also the reuse of the enzyme in several catalytic cycles. The reuse of the same lipase immobilized on calcium carbonate and oxidized cellulose was carried out during the synthesis of butyl oleate and allowed to perform 6 cycles and 3 cycles, respectively [22,23]. Blanco et al. [47] showed that the lipase from *C. antarctica* B immobilized on the mesoporous silica allowed a quantitative conversion even after 15 reaction cycles. Two commercial lipases (*Burkholderia cepacia* and *C. antarctica*) were encapsulated in silica aerogels. These immobilized lipases were applied in biodiesel synthesis by transesterification of sunflower seed oil with methyl acetate, the reuse of these lipases allowed 11 cycles and kept only 60% of their initial activity [48]. The activity of α-chymotrypsin immobilized to magnetic particles did not significantly change after 12 repeated uses during the proteolytic cleavage of porcin pepsin [49].

4. Conclusion

The key step in the enzymatic process consists on the successful immobilization of the enzyme allowing its recovery and reuse. The effectiveness of an immobilization process depends on the support used. This work focuses on the immobilization of ROL onto silica aerogels by physical adsorption. The immobilization carried out at 4 °C during 60 min gave rise to the highest immobilization yield (95%). The silica aerogels-immobilized lipase displayed a better stability than the free form. Indeed, immobilization of the enzyme enhanced its stability towards to the temperature and pH. Moreover the immobilized enzyme was shown to be well tolerant to apolar solvent like hexane and improved storage stability.

In order to evaluate the efficiency of immobilized enzyme for the esterfication reaction, we used oleic acid and butanol as reagents in hexane solvent. Our results showed that the highest yield was obtained at 37 °C with a molar ratio of oleic acid to butanol 1:1 and 450 IU of immobilized lipase. This system could be reused 11 times without a significant loss of the activity. Therefore, the silica aerogels may have a promising future as support for biocatalysts in various syntheses.

Acknowledgements

This work is part of a doctoral thesis by Nadia KHARRAT. This work received financial support from “Ministère de l’enseignement supérieur et de la recherche” granted to the “Laboratoire de Biochimie et de Génie Enzymatique des Lipases”.

References

[1] Sharmaa R, Chistib Y, Banerjeea UC. Production, purification, characterization, and applications of lipases. Biotechnol Adv 2001;19:627–62.
[2] Bornscheuer UT, Bessler C, Srinivas R, Krishna SH. Optimizing lipases and related enzymes for efficient application. Trends Biotechnol 2002;20:433–7.
[3] Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microbiol Technol 2007;40:1451–63.
[4] Zhen-Gang W, Jian-Qin W, Zhi-Kang X. Immobilization of lipase from *Candida rugosa* on electrospun polysulfone nanofibrous membranes by adsorption. J Mol Catal B: Enzym 2006;42:45–51.
[5] Antczak T, Mrowiec Bialon J, Bielecki S, Jarzebski AB, Malinowski JJ, Lachowski AI, Galas E. Thermostability and esterification activity of *Mucor javanicus lipase* entrapped in silica aerogel matrix and in organic solvents. Biotechnol Technol 1997;11:9–11.
[6] El Rassy H, Perrard A, Pierre AC. Application of lipase encapsulated in silica aerogels to a transesterification reaction in hydrophobic and hydrophilic solvents: bi–bi ping–pong kinetics. J Mol Catal B: Enzym 2004;30:137–50.
[7] Yong-Xiao B, Yan-Feng L, Yong Y, Liu-Xiang Y. Covalent immobilization of triacylglycerol lipase onto functionalized novel mesoporous silica supports. J Biotechnol 2006;125:547–82.
[8] Hong J, Xu D, Gong P, Yu J, Ma H, Yao S. Covalent-bonded immobilization of enzyme on hydrophilic polymer covering magnetic nanogels. Micropor Mesopor Mater 2008;109:470–7.
[9] Gao S, Wang Y, Wang T, Luo G, Dai Y. Immobilization of lipase on methyl-modified silica aerogels by physical adsorption. Bioresour Technol 2009;100:996–9.
[10] Novak Z, Habulin M, Krmelj V, Knez Ž. Silica aerogels as supports for lipase catalysed esterifications at sub- and supercritical conditions. J Supercrit Fluids 2003;27:169–78.
[11] Panzavolta F, Soro S, D’Amato R, Paocci C, Cernia E, Russo MV. Acetylenic polymers as new immobilization matrices for lipolytic enzymes. J Mol Catal B: Enzym 2005;32:67–76.
[12] Arica MY, Bayramoglu G. Polyethyleneimine-grafted poly(hydroxyethyl methacrylate-co-glycidyl methacrylate) membranes for reversible glucose oxidase immobilization. Biochem Eng J 2004;20:73–7.
[13] Bellezza F, Cipiciani A, Costantino U. Esterase activity of biocomposites constituted by lipases adsorbed on layered zirconium phosphate and phosphonates: selective adsorption of different enzyme isoforms. J Mol Catal B: Enzym 2003;26:47–56.
[14] Amounas M, Magne V, Innocent C, Dejean E, Seta P. Elaboration and chemical reactivity of enzyme modified ion exchanging textiles. Enzyme Microbiol Technol 2002;31:171–8.
[15] Gao Y, Tan TW, Nie KL, Wang F. Immobilization of lipase on macroporous resin and its application in synthesis of biodiesel in low aqueous media. Chin J Biotechnol 2006;22:114–8.
[16] Foresti ML, Ferreira ML. Analysis of the interaction of lipases with polypropylene of different structure and polypropylene-modified glass surface. Colloids Surf, A 2007;294:147–55.
[17] Torres R, Ortiz C, Pessela BCC, Palomo JM, Mateo C, Guisàn JM, Fernàndez-Lafuente R. Improvement of the enantioselectivity of lipase (fraction B) from *Candida antartica* via adsorption on polyethylenimine-agarose under different experimental conditions. Enzyme Microbiol Technol 2006;39:167–71.
[18] Marzouk S, Rachdi F, Fourati M, Bouaziz J. Synthesis and grafting of silica aerogels. Colloids Surf, A 2004;234:109–16.
[19] Ben Salah R, Fendri K, Gargouri Y. La lipase de *Rhizopus oryzae*: production, purification et caractéristiques biochimiques. Rev Fr Corps Gras 1994;41:133–7.
[20] Bradford A. Rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[21] Gargouri Y, Piéroni G, Rivière C, Saunière JF, Lowe PA, Sarda L, Verger R. Kinetic assay of human gastric lipase with short and long chain triacylglycérol emulsion. Gastroenterology 1986;91:919–25.
[22] Ghamgui H, Karra châabouni M, Gargouri Y. 1-Butyl oleate synthesis by immobilized lipase from *Rhizopus oryzae*: a comparative study between n-hexane and solvent-free system. Enzyme Microbiol Technol 2004;35:355–63.
[23] Karra-Châabouni M, Bouaziz I, Boufi S, Botelho do Rego AM, Gargouri Y. Physical immobilization of *Rhizopus oryzae* lipase onto cellulose substrate: activity and stability studies. Colloids Surf, B 2008;66:168–77.
[24] Basri M, Zin Wan Yunus WM, Yoong WS, Ampon K, Razak CNA, Salleh AB. Immobilization of lipase from *Candida rugosa* on synthetic polymer beads for use in the synthesis of fatty esters. J Chem Technol Biotechnol 1996;66:169.
[25] Hasan F, Ali Shah A, Hameed A. Industrial applications of microbial lipases. Enzyme Microbiol Technol 2006;39:235–51.
[26] Kılınç A, Mustafa T, Seçil Ö, Azmi T. Immobilization of pancreatic lipase on chitin and chitosan. Prep Biochem Biotechnol 2006;36:153–63.
[27] Abdul Rahman MB, Tajudin SM, Hussein MZ, Abdul Rahman RN, Salleh AB, Basri M. Application of natural kaolin as support for the immobilization of lipase from *Candida rugosa* as biocatalsyt for effective esterification. Appl Clay Sci 2005;29:111–6.
[28] De Oliveira PC, Alves GM, De Castro HF. Immobilisation studies and catalytic properties of microbial lipase onto styrene-divinylbenzene copolymer. Biochem Eng J 2000;5:63–71.
[29] Bayramoglu G, Yalçin E, Arica MY. Immobilization of urease via adsorption onto L-histidine-Ni (II) complexed poly (HEMA-MAH) microspheres: preparation and characterization. Process Biochem 2005;40:3505–13.
[30] Tien-Chieh HR, Giridhar Shao-Hua C, Wen-Teng WB. Immobilization of *Candida rugosa* lipase on chitosan. J Mol Catal B: Enzym 2003;26:69–78.
[31] Leaka J, wlodarezyk J, Zaborska W. Polyaniline as a support for urease immobilization. J Mol Catal B: Enzym 1999;6:549–56.
[32] Guo Z, Bai S, Sun Y. Preparation and characterization of immobilized lipase on magnetic hydrophobic microspheres. Enzyme Microb Technol 2003;32:776–82.
[33] Karout A, Chopard C, Pierre AC. Immobilization of a lipoxygenase in silica gels for application in aqueous media. J Mol Catal B: Enzym 2007;44:117–27.
[34] Huang XJ, Ge D, Xu ZK. Preparation and characterization of stable chitosan nanofibrous membrane for lipase immobilization. Eur Polym J 2007;43:3710–8.
[35] Ye P, Xu ZK, Wu J, Innocent C, Seta P. Poly (acrylonitrile-co-maleic acid) membranes functionalized with gelatin and chitosan for lipase immobilization. Biomaterials 2006;27:4169–76.
[36] Petkar M, Lali A, Caimi P, Daminati M. Immobilization of lipases for non-aqueous synthesis. J Mol Catal B: Enzym 2006;39:83–90.
[37] Krajewska B, Leszko M, Zaborska W. Urease immobilized on aminated butyl acrylate-ethylenedimethacrylate copolymer. Macromol Mater Eng 1990;179:21–33.
[38] Cremonesi P, Carrea PJ, Ferrarra L, Antonini E. Enzymatic dehydrogention of testosterone coupled to pyruvate in a two-phase system. Eur J Biochem 1974;44:401–7.

[39] Laane C, Boeren S, Vos K, Veeger C. Rules for optimization of biocatalysis in organic solvents. Biotechnol Bioeng 1987;30:81–7.
[40] Lima VMG, Krieger N, Mitchell DA, Fontana JD. Activity and stability of a crude lipase from *Penicillium aurantiogriseum* in aqueous media and organic solvents. Biochem Eng J 2004;18:65–71.
[41] Dalla-Vecchia R, Sebrão D, Da Garça Nascimento M, Soldi V. Carboxymethylcellulose and poly (vinyl alcohol) used as a film support for lipases immobilization. Process Biochem 2005;40:2677–82.
[42] Basri M, Ampon K, Wan Yunus WMZ, Razak CNA, Salleh AB. Immobilization of hydrophobic lipase derivation on to organic polymer beads. J Chem Technol Biotechnol 1994;59:37.
[43] Deng L, Tan TW, Wang F, Xu XB. Enzymatic production of fatty acid alkyl esters with a lipase preparation from *Candida sp.* 99–125. Eur J Lipid Sci Technol 2003;105:727–34.
[44] Kaieda K, Samukawa T, Matsumoto T, Ban K, Kondo A, Shimada Y, Noda H, Nomto F, Ohtsuka K, Izumoto E, Fukuda H. Biodiesel fuel production from plant oil catalyzed by *Rhizopus oryzae* lipase in a water containing system without an organic solvent. J Biosci Bioeng 1999;88:627–31.
[45] Kaieda M, Samukawa T, Kondo A, Fukuda H. Effect of methanol and water contents on production of biodiesel fuel from plant oil catalyzed by various lipases in a solvent-free system. J Biosci Bioeng 2001;91:12–5.
[46] Bickerstaff GF. Immobilization of enzymes and cells: methods in Biotechnology, vol. l. Totowa: Humana Press; 1997.
[47] Blanco RM, Terreros P, Fernandez-Perez M, Otero C, Diaz-Gonzalez G. Fonctionalization of mesoporous silica for lipase immobilization: characterization of the support and the catalysts. J Mol Catal B: Enzym 2004;30:83–93.
[48] Orçaire O, Buisson P, Pierre AC. Application of silica aerogel encapsulated lipases in the synthesis of biodiesel by transesterification reactions. J Mol Catal B: Enzym 2006;42:106–13.
[49] Sustrova B, Novotna L, Kucerova Z, Ticha M. Immobilization of α-chymotrypsin to magnetic particles and their use for proteolytic cleavage of porcine pepsin A. J Mol Catal B: Enzym 2009;60:22–8.