Covalent binding of hyper-activated *Rhizomucor miehei* lipase (RML) on hetero-functionalized siliceous supports

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Abstract

Physical adsorption onto hydrophobic supports has proven to be an effective way to improve the activity of lipases. Covalent binding, on the other hand, enhances the active lifetime of the immobilized biocatalysts. To combine the benefits of adsorption and covalent binding, immobilization of RML on new hetero-functional supports are reported. For this, chemical modification of silica and silica mesoporous nanoparticles was performed by the simultaneous use of two coupling linkers; Octyltriethoxysilane (OTES) for hydrophobic interaction and glycidoxypyltrimethoxysilane (GPTMS) for covalent linkage of RML. Altering the GPTMS/OTES ratio makes possible to have different amount of octyl and epoxy groups on the supports. The results showed that immobilization of RML on octyl-functionalized supports produces specific activity almost 1.5-2 folds greater than the specific activity of the free enzyme. The observed hyper-activation decreased with increasing epoxy groups on the supports confirming the enhancement of covalent nature of the attachment. Leaching experiment was also confirmed positive effect of the presence of epoxy groups on the supports. Regarding the specific activity of the immobilized preparations and desorption percentages of RML from each support, the most suitable carrier obtains from the functionalization of the supports in presence of GPTMS and OTES in the ratio of 1:1.

Keywords: Covalent immobilization, Physical adsorption, *Rhizomucor miehei* lipase, SBA-15, Silica gel.

1. Introduction

Lipases (glycerol ester hydrolase, E.C. 3.1.1.3) have gained special attention in industry due to their availability, stability in organic and aqueous media and wide range of natural and non-natural substrates with high chemo-, regio- and stereoselectivity [1, 2]. They can catalyze esterification, hydrolysis and transesterification to produce many useful chemical products including alcohols, esters, acids and amine under mild reaction conditions [3]. Lipases are very likely the most used enzymes in organic chemistry and are known as one of the leading biocatalysts with proven potential for contributing to the multibillion dollar underexploited lipid bio industry [4]. In the past years, a better understanding of catalytic behaviors of lipases together with the progress of molecular engineering have led to significant improvement in functional properties of these enzymes.

Lipases have two different structural forms [5]. In the "close" form, the active site of lipases is isolated from the medium by a helical oligopeptide chain called "lid". The other structure is called "open" form in which the lid displaced and the active site exposed to the reaction medium (Fig. 1). The later is considered as the lipase most active form. In aqueous media, the lipase is in equilibrium between these two structural forms. However upon interaction with a hydrophobic surface such as drops of oils, hydrophobic proteins and hydrophobic supports, the open form of the lipase becomes adsorbed to it and this equilibrium shifts towards the open form [6]. These unique properties open a versatile method for either purification or immobilization of lipases on hydrophobic supports via interfacial activation at low ionic strength [7]. These conditions expose the internal face of the lid to the reaction medium thus fixing their open conformation on the hydrophobic support after immobilization.

73 The main purposes of enzyme immobilization are usually stabilization and re-utilization of the
74 enzyme under process conditions [8]. **Lipases** can be immobilized by several methods such as
75 adsorption, cross-linking, covalent attachment and physical entrapment [9, 10]. Hydrophobic
76 adsorption of lipases on solid supports is a simple method for both purification and
77 immobilization purposes [7]. This method usually leads no conformational change of the enzyme
78 and almost no reagents are required for immobilization. Immobilization by adsorption on
79 hydrophobic supports may also influence the catalytic properties as reported, for example, in the
80 hydrolysis of racemic ibuprofen esters the immobilized lipases showed an improvement of the
81 enantioselectivity [5, 11]. Furthermore there are several reports on hyper-activation (mostly
82 against small and hydrophobic substrates) of lipases immobilized on hydrophobic supports [12].
83 **However, it has the disadvantage that the adsorbed enzyme may leak from the carrier due to**
84 **weak binding forces. It means that small change in temperature, pH and ionic strength or even**
85 **the presence of substrates and organic solvents** may cause to release of the immobilized enzyme
86 and subsequent contamination of the reaction medium and losing one of the advantages of
87 employing immobilized enzymes. The first attempt of using not-releasable hydrophobic
88 adsorption of lipases has been reported by Guisan and co-workers [13]. They have proposed the
89 crosslinking of the immobilized enzymes on octyl-agarose beads with aldehyde-dextran as a way
90 to prevent the release of immobilized enzyme from the support. The rationale behind their idea
91 was that intermolecular-cross-linked lipases would only desorb if all of the cross-linked lipase
92 molecules were simultaneously released from the support. There is also another report on using
93 hetero-functional supports for covalent immobilization of chemically-modified lipases on
94 glyoxyl-octyl agarose after initial adsorption via hydrophobic interaction [14]. In this way

covalent attachment of lipases is performed at relatively harsh condition (pH 9-10) which would be undesirable for the enzymes with lower pH stability.

On the other hand, covalent immobilization of enzymes is one of the most economical immobilization methods [15]. This type of irreversible immobilization is preferred when leaching of enzyme from support is a main concern. In other words, covalent binding of an enzyme onto a support is especially useful when there is a strict requirement for the absence of the enzyme in the product. There are however, some drawbacks in covalent immobilization of enzymes. For example, the conditions for covalent attachment are usually much more complicated compared to the physical adsorption. Therefore covalent immobilization in most cases alters the structure of the protein, resulting in remarkable loss of activity [16].

We here describe the simultaneous use of physical adsorption and covalent attachment for immobilization of *Rhizomucor miehei* lipase in order to gain the advantages of both methods. In this way, two new hetero-functional silica-based supports (SBA-15, silica), bearing epoxy and octyl groups on their surfaces were used for immobilization in extremely mild condition. This makes possible immobilization of the enzyme first via a hydrophobic adsorption followed by covalent attachment of the hyper-activated enzyme to avoid undesired enzyme release. We also report the controllable hetero-functionalization of silica and SBA-15 with determination of the percentage of each functional group on the support and the effect of different percentages of epoxy and octyl groups on the properties of the immobilized RML. RML is a stable and very interesting enzyme with a molecular size of 31,600 Da and a isoelectric point (pI) of 3.8. It has 35 residues of aspartic and glutamic groups and 10 residues of lysine moieties [17].

2. Materials and methods

2.1. Materials

The lipase from *Rhizomucor miehei* was supplied by Sigma (20000 U/g lipase, 12 mg of protein/ml). *p*-Nitrophenyl butyrate (*p*-NPB), tetra ethyl ortho silicate (TEOS), polyuronic acid (P123), glycidoxypopyltrimethoxysilane, sucrose laurate and Octyltriethoxysilane were from Sigma. Dioxane, 1-propanol, 2-propanol and silica gel (70-230 mesh) were purchased from Merck. Fourier transform infrared spectra (FT-IR) were recorded on a Bomem FT-IR-MB-series instrument with a KBr pellet technique. Thermogravimetry (TGA) and differential thermal analysis (DTA) were carried out from 10 °C to 800 °C at a heating rate of 20 °C/min in air atmosphere using a STA 503M system from Bahr GmbH, Germany.

2.2. Methods

2.2.1. Preparation of the silica nanoparticles

Pure siliceous SBA-15 was prepared using a reported procedure [18]. Pluronic P123 triblock copolymer (EO20–PO70–EO20, BASF) was used as template. Briefly 4 grams of Pluronic P123 were added to 144 mL of an aqueous solution of HCl (2 M) at 40 °C. Successively, TEOS was added dropwise (mass ratio TEOS/P123= 2:1) and then stirred for 2 h. Afterwards, the mixture was transferred to Teflon-lined sealed container and kept at 100 °C for 48 h. The white solid was filtered, washed with distilled water and calcined at 550 °C to remove the template.

2.2.2. Homo and hetero-functionalization of SBA-15 and silica gel

The dry silica gel particles (1 g) or mesoporous silica materials (0.5 g) were dispersed in 50 mL of dry toluene; then 1 mL of GPTMS/OTES in different proportions and 200 µL Et₃N were added. The resulting mixture was refluxed under nitrogen atmosphere and vigorously stirred for 4 h. The modified supports were collected by filtration and washed thoroughly with THF. Finally

the modified particles were dried at 120 °C for 8 h. The functionalization of the supports was confirmed by IR spectroscopy and TGA-DTA analysis.

2.2.3. Determination of epoxy groups on the support

Determination of the epoxy groups on each support was carried out as follows: 100 mg of the support was added to 1.5 mL of 1.3 M sodium thiosulphate solution and then the solution was titrated by addition of 0.1 M hydrochloric acid until neutralization. The amount of epoxy groups was calculated from the amount of hydrochloric acid needed in order to maintain neutrality of the mixture [19]. The same reaction was performed using unmodified particles as blank.

2.2.4. Enzyme immobilization on homo/hetero functionalized silica

Homo and hetero functionalized silica supports (100 mg) were added in 10 mL of 25 mM sodium phosphate buffer at pH 7 containing (0.5 mg/mL) of RML under gently stirring at 25 °C for 24 h. Periodically, samples of the supernatant were withdrawn and analyzed for determination of the protein concentration by the activity assay and Bradford's method [20]. Finally the immobilized RML derivatives were filtered and washed by distilled water and stored at 4 °C.

2.2.5. Enzyme immobilization on homo/hetero functionalized SBA-15

Different homo and hetero functionalized SBA-15 supports (100 mg) were suspended in 3 mL of solution of the enzyme (1.7 mg/mL) in sodium phosphate buffer (pH 7) under gently stirring at 25 °C for 24 h. The protein concentration in supernatant was periodically determined by the activity assay Bradford's method. The final derivative was washed with distilled water and stored at 4 °C.

2.2.6. Enzymatic activity assay

The activities of the soluble lipase and its immobilized preparations were analyzed spectrophotometrically by measuring the increment in absorbance at 348 nm ($\epsilon = 5150 \text{ M}^{-1}\text{cm}^{-1}$) [21]. The increase in absorbance produced by the release of *p*-nitrophenol in the hydrolysis of *p*-NPB in 25 mM sodium phosphate buffer at pH 7 and 25°C. Briefly, 0.05-0.2 mL of the lipase suspension or solution (blank or supernatant without further dilution) was added to 2.5 mL of substrate solution (0.8 mM) under magnetic stirring. Spontaneous hydrolysis of *p*-NPB was measured using 2.5 mL of substrate solution (0.8 mM) in the absence of RML as control. Enzymatic activity is given as 1 μmol of *p*-nitrophenol released per minute per mg of the enzyme (IU) under the conditions described above.

2.2.7. Determination of the amount of protein bound to the carriers

The amount of the protein in supernatant and blank was determined by the Bradford's method. The reported yields of immobilization were calculated as the ratio of the amount of protein bound on to the support to the initial amount. Yields were expressed as percentage.

2.2.8. Thermal inactivation of different RML immobilized preparations

Free enzyme and the immobilized preparations of RML were incubated in 25 mM sodium phosphate at pH 7 and different temperatures. The suspension of each sample was withdrawn periodically and their activities were measured using the *p*-NPB assay.

2.2.9. Stability of different RML immobilized preparations in presence of organic solvents

Free enzyme and the immobilized preparations were incubated in a total volume of 1 mL solution containing 25 mM sodium phosphate buffer pH7 and 10% and 20% of dioxane, 1-propanol and 2-propanol at 25 °C. The activity of each sample was measured using the *p*-NPB assay.

2.2.10. Desorption of RML from the supports

The immobilized preparations (100 mg) were suspended in 5 mL of 0.1-2% (w/v) of sucrose laurate in 100 mM sodium phosphate buffer solution at pH 7.0 for 2 h. The desorption course was monitored by periodically checking the enzymatic activity of supernatant and suspension.

2.2.11. Determination of kinetic parameters

Kinetic parameters of the free and immobilized preparations of RML were determined using different p-NPB concentrations in the range of 0.02-1 mM in 25 mM sodium phosphate buffer (pH 8.0) respectively. K_M and V_{max} values of free and immobilized lipases were calculated via non-linear regression fitting of the Michaelis-Menten equation using Prism 6 (Graphpad Software).

3. Results and Discussion

3.1. Preparation, characterization and functionalization of the supports

The textural properties of the modified and unmodified siliceous materials were assessed by N_2 adsorption-desorption experiments (Fig. 2). As Table 1 shows the pore size distribution and the pore volume for SBA-15 material are 8.6 nm and 1.05 cm³/g, respectively and the surface area of SBA-15 is 544 m²/g calculated by the BET method. In the case of octyl-functionalized SBA-15, the surface area, pore volume and the pore size are 522 m²/g, 0.85 cm³/g and 7.8 nm respectively. Obviously, the decrease in surface area, pore volume and pore diameter of the functionalized SBA-15 is due to the presence of organic groups on the surface of this material. As can be concluded from Table 1, almost the same properties are observed after

functionalization of this support with epoxy and octyl residues in the ratio of 1:1. Further decrease in surface area, pore volume and pore diameter of the octyl-functionalized support after immobilization of RML also clearly confirms the presence of RML not only on the external surface of the support but also inside the pores.

Fig. 3 shows SEM images of SBA-15 and the derivative obtained from immobilization of RML on octyl-functionalized SBA-15. Both images exhibit a rather wavy or short rod-shaped morphology. By considering TGA, FT-IR and N₂ adsorption–desorption isotherms, it can be concluded that the modification still preserving the textural properties of the parent SBA-15.

FT-IR spectroscopy was used to confirm the functionalization of the supports. For unmodified SBA-15 and silica the broad band between 3200 cm⁻¹ and 3600 cm⁻¹ could be ascribed to the O–H stretching frequency of silanol groups. As Fig. 4 shows, remarkable decrease in intensity of this band after functionalization together with appearance of aliphatic C–H stretch band at 2855–2940 cm⁻¹ clearly demonstrate successful functionalization of silica.

The surface chemistry of silica-based materials gives opportunity to easy hetero-functionalization of their surfaces with different functional groups to obtain new generation of multi functional supports for enzyme immobilization. In order to introduce different amounts of epoxy and octyl groups on the surface of the supports, different proportions of GPTMS and OTES were used. The use of various ratios of GPTMS and OTES allows the density of the functional groups present on the supports surfaces to be controlled. Quantification of oxirane groups on the supports was performed by using the reaction between epoxy groups on the support and sodium thiosulphate and subsequent measurement of the released hydroxide ion by titration with 0.1 M hydrochloric acid. The results revealed that the amount of epoxy groups on

the surface of silica and SBA-15 is about 348 and 1248 μmol per gram of each support, respectively while using GPTMS as the only grafting agent. High degree of functionalization of SBA-15 compared to silica can be attributed to more surface area of this support. As can be seen from Table 2 and 3 altering the GPTMS /OTES ratio makes possible to have different amounts of octyl and epoxy groups on the supports which can be used for immobilization of lipases in more controlled way. The results also show that the use of equal ratios of two linkers does not cause to the equal grafting of the linkers neither for silica nor for SBA-15.

3.2. Immobilization of RML on functionalized-silica

The lipase attachment on the supports was followed by changes in the activity of supernatant during immobilization. Immobilization of 5mg of RML per 1 g of octyl-functionalized silica was performed with 100% yield and produced 4.6 specific activity which is almost 2 fold greater than the specific activity of the free enzyme. Around 20-25 fold hyperactivation for RML has previously been reported in the presence of different detergents (TritonX-100, sucrose laurate) [22]. The observed hyper-activation can be attributed to the interfacial activation mechanism of lipases in the presence of hydrophobic surfaces. Furthermore, since this method of immobilization is based on the mild physical adsorption of RML, the method causes little or no conformational change of the enzyme or destruction of its active center. The other silica supports with different ratios of epoxy/octyl moieties were also subjected for immobilization of RML. The results showed almost fully immobilization of the enzyme on all the supports with 95-100% yield determined by both activity assay and the Bradford s method. As can be seen from Table 2, increasing the number of epoxy groups decreases the specific activity of the immobilized enzyme. The epoxy groups on the supports promote the covalent linkage of the enzyme on the support. They can react with many nucleophilic groups present on the protein surface; in

particular with terminal amino group and carboxylic groups [23, 24]. The reaction between epoxy groups and carboxylic groups produces an ester bond, although this reaction proceeds in a much slower fashion [25]. With the large number of epoxy groups, the total hydrophobicity of the surface also decreases, leading to diminished specific activity of the immobilized enzyme.

In addition to activity studies, the immobilized preparations were evaluated in enzyme desorption experiments (Table 2 and 3). This experiment shows the amount of enzyme physically adsorbed on the surface of the supports. The immobilized preparations were treated for 2h at room temperature with a 2% (v/v) solution of sucrose laurate in phosphate buffer (100 mM; pH = 7.0). At this condition, the support fully functionalized with epoxy groups shows no leaching of the enzyme from the carrier; showing that the immobilization of RML on this support performs only via covalent bonds. On the other hand the same condition causes to desorption of almost 50% of the immobilized enzyme from the surface of silica-octyl, confirming that the weak and reversible physical adsorption is the main mechanism of immobilization of the lipase on this support. However, 50% of the enzyme molecules **remain adsorbed** at this condition most likely because of porous structure of siliceous materials. Fully desorption of RML from the surface of non siliceous supports reported by Palomo et al. clearly confirms the effect of porous structure of carriers on the leaching of immobilized enzymes [12]. Very few studies have been published about immobilization of RML on hydrophobic supports with the reported leakage amounts lower or similar than the amounts obtained in this work with silica-octyl-RML. Typically, the use of a porous support is advantageous since the enzyme is adsorbed within the pores and at the outer surface of these materials as well. However the release of the main part of immobilized enzyme from the support would restrict the operation of this derivative under industrially relevant conditions.

Leaching experiment under the same conditions was performed for the derivatives of RML immobilized on the silica with different ratios of epoxy/octyl groups. The results clearly show the positive effect of the presence of epoxy groups on preventing the release of RML from the support. In fact with introducing 92 μmol of epoxy groups on 1 g of this support, the leaching amount significantly decreases (8 %) compared to the derivative obtained from immobilization of RML on octyl-silica (47 %). Although further increasing in epoxy groups leads to decreasing in specific activity of the immobilized enzyme, negligible leakage of the enzyme compensate the negative effect of covalent immobilization on activity of RML. Regarding the two important parameters of the immobilized enzyme (specific activity and desorption percentage), the most suitable biocatalyst is the derivative of immobilization of RML on the support with 92 μmol of epoxy groups. As Table 2 shows this support is obtained from the incubation of silica in presence of GPTMS and OTES in the ratio of 1:1.

3.3. Immobilization of RML on functionalized-SBA-15

Immobilization of RML on homo and hetero-functionalized SBA-15 was also investigated in terms of immobilization yield, specific activity and desorption percentage in order to combine the benefits of physical adsorption and covalent binding. High immobilization yield obtained for all the supports ranging from 91 to 100%. The homo-functionalized SBA-octyl shows the highest specific activity with almost 1.5 and 2 fold increased activity compared to the soluble enzyme and SBA-epoxy-RML respectively. With SBA-octyl the interfacial activation mechanism of the lipase leads to immobilization of the enzyme with open configuration of its active site favoring the highest activity. As expected increasing the number of epoxy groups enhances the covalent linkage of the enzyme molecules, thus lowering the specific activity of the immobilized enzyme. For example, while the immobilization of RML on SBA-15 (fully functionalized with epoxy

groups) shows 25% decreased in specific activity compared to the free enzyme, the enzyme immobilization with combined hydrophobic and covalent attachment on the support with 453 μmol of epoxy groups presents almost 1.5 fold improved specific activity. Leaching experiments on the immobilized preparations also showed that the enzyme immobilized on the supports with the lower number of epoxy groups are simply leaked out from their surfaces. For example more than 60% of RML releases from the surface of SBA-octyl after only 2h incubation in presence of 2% sucrose laurate.

3.4. The stability of the immobilized derivatives

The stability of the soluble and some of immobilized preparations of RML was investigated through incubation in 10 and 20% of three organic solvents (1-propanol, 2-propanol and dioxane); simulating the conditions of an enzymatic reaction. Normally the use of organic solvents in enzymatic reactions can increase solubility of the substrate in water. However, the solvent may cause conformational changes in the enzyme structure and changing its catalytic properties. This is due to the fact that a few amounts of water molecules are required for enzymatic function, and some organic solvents with log P values below 2 tend to strip this essential water and decrease the catalytic activity of enzyme [26]. In order to evaluate the effect of enzyme immobilization on stability of the lipase, the above-mentioned solvents with log P < 2 were used. The results show that the soluble enzyme loses 30-40% of its activity after 24h incubation in presence of 10% of these solvents (Fig. 5). On the other hand the immobilized derivatives remain completely active at the same condition. Increasing the percentage of the solvents to 20% clearly unravels the difference in the stability of the biocatalysts. For example while the free enzyme is completely inactivated in presence of 20% dioxane, silica-octyl-RML and SBA-octyl-epoxy-RML retain 100% of their activities. A little loss in activity is also

observed for SBA-octyl-RML and silica-octyl-epoxy-RML at the same condition retaining almost 80% of their activities. In 1-propanol the immobilized preparations are strongly affected by solvent inactivation, showing after 24 h of incubation less than 30% of residual activity. The results also show that immobilization of RML greatly enhances the enzyme stability, resulted in no activity loss for SBA-octyl-RML, silica-octyl-epoxy-RML and silica-octyl-RML in the presence of 2-propanol. The results of investigation on thermal stability of the immobilized derivatives also showed significant improvement in stability of RML after immobilization. Fig. 6 shows thermal inactivation of the soluble enzyme and four selected immobilized derivatives after 2h incubation at 45 °C, 50 °C and 55 °C. RML remains completely active at 45 °C both in free and immobilized form. Furthermore, while the free enzyme loses 30% of its initial activity at 50 °C, the immobilized preparations loses only 10-12% of their activities at the same condition. Losing of almost 70% of activity of the free enzyme is observed after 2h incubation at 55 °C. As can be seen from Fig. 6, the immobilized preparation obtained from immobilization of RML on SBA-octyl-epoxy remains completely active at 55 °C. This support was obtained from the incubation of SBA-15 in presence of GPTMS and OTES in the ratio of 1:1. The results also revealed that the derivatives obtained from physical adsorption/covalent immobilization have higher stabilities compared to the derivatives obtained from physical adsorption. However, there is an example of increasing the thermal and co-solvent stability of RML after immobilization on hydrophobic supports in comparison with covalent attachment [12].

3.5. Kinetic study

Kinetic parameters for the activity of the free and two immobilized preparations of RML ($V_{\max}$, and K_m) were determined using the Michaelis–Menten plot by *p*-NPB as the substrate (Fig. 7). The protein amount for the free enzyme and the immobilized derivatives were 5 and 3.5 µg,

respectively. The K_m values for SBA-octyl-epoxy-RML and silica-octyl-epoxy-RML were 0.1135 and 0.1141 mM, respectively which were lower than K_m value of the free enzyme (0.1954 mM). The lower value of K_m represents higher affinity between enzymes and substrates. The V_{max} value for SBA-octyl-epoxy-RML and silica-octyl-epoxy-RML were 0.064, 0.061 mM min^{-1} and for the free lipase was 0.083 mM min^{-1} . The observed results are in accordance with the values reported on the immobilization of polyphenol oxidase [27].

4. Conclusion

The new hetero-functional octyl/epoxy supports, showed a great potential to be used in the immobilization of lipases. These supports have ability to combine the benefits of adsorption and covalent binding of lipases. Their preparation involves the dual functionalization of silica and SBA-15 with a mixture of GPTMS and OTES. The density of the grafted epoxy groups over the supports surface can be controlled by using certain amount of GPTMS and OTES during functionalization. Immobilization of RML on octyl homo-functionalized supports shows hyper activation of the enzyme due to fixation of its open form on the hydrophobic supports. However in this way the immobilization is based on weak physical adsorption and is quite reversible as showed by the release of most enzyme molecules in presence of sucrose laurate. On the other hand immobilization on the supports with different amounts of epoxy groups and subsequent leaching experiment on immobilized preparations showed that increasing the epoxy groups promotes higher degree of covalent attachment of the enzyme. Regarding the results of activity and leaching experiments it was also found that the immobilization of RML on SBA-15 and silica hetero-functionalized by incubation in presence of GPTMS and OTES in the ratio of 1:1 produces the best immobilized preparations. That way, the immobilized RML presented lower contamination of the reaction media and better thermal stability of the enzyme than the

derivatives obtained from simple physical adsorption. The activity of these derivatives is also higher than the activity of the free enzyme. Kinetic study of free RML and SBA-octyl-epoxy-RML and silica-octyl-epoxy-RML showed lower K_m values for the immobilized preparations; indicating that immobilization of RML via hydrophobic/covalent attachment resulted in change of affinity for the substrate.

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Figure captions:

Fig. 1. Conformational changes of lipases.

Fig. 2. Nitrogen adsorption isotherms for the a) unfunctionalised SBA-15 and (b) SBA-octyl-RML. All these materials show a typical type IV adsorption–desorption isotherm which corresponds to the meso-structure of these materials

Fig. 3. Scanning electron micrographs a) unfunctionalised SBA-15 and b) SBA-octyl-RML

Fig. 4. Characterization of the support: a) FT-IR spectrum of SBA-15, b) FT-IR spectrum of SBA-octyl-epoxy

Fig. 5. Co-solvent stability of free RML and immobilized preparations in presence of 20 % of organic solvents. Experimental condition: Incubation of each biocatalyst in 1 mL solution containing 25 mM sodium phosphate buffer (pH 7.0) and 20% of three organic solvents at 25°C. Initial activity of RML and each immobilized derivatives was determined in 1 mL of sodium phosphate buffer 25 mM (pH 7.0) at 25 °C and set as 100%.

Fig. 6. Thermal stability of free RML and immobilized preparations at 45°C, 50 °C, 55 °C. Experimental condition: Increasing certain amount of each biocatalyst in 1mL of sodium phosphate buffer 25 mM (pH 7.0) and incubation at different temperatures for 2h. Initial activity of RML and each immobilized derivatives was determined in 1 mL of sodium phosphate buffer 25 mM (pH 7.0) at 25 °C and set as 100%.

Fig. 7. Michaelis-Menten plot for the free and immobilized preparations of RML

Figure 1

Fig. 1.

Figure 2

Fig. 2.

(a)

(b)

Fig. 3.

(a)

(b)

Figure 4

Fig. 4.

Figure 5

Fig. 5.

Figure 6

Fig. 6.

Figure 7

Fig. 7.

Table 1. Textural properties of different supports.

Compound	Mean pore (Å) diameter	Total pore volume (mLg ⁻¹)	Surface area(m ² g ⁻¹)
SBA-15	8.6	1.05	544
SBA-octyl	7.8	0.98	522
SBA-octyl-epoxy (1:1)	7.7	0.85	475
SBA-octyl-epoxy-RML	7.0	0.82	431

Table 2. Immobilization of RML on functionalized silica

Biocatalyst ^a	Octyl:Epoxy ^b	Specific activity ^c	Immobilization yield ^d (%)	Desorbition (%)	epoxy groups ^e (μm)
Silica-epoxy	1:0	1.7	95	0	348
Silica -octyl	0:1	4.6	100	47	0
Silica-octyl-epoxy	1:1	3.0	100	8	92
Silica-octyl-epoxy	1:2	2.4	100	5	148
Silica-octyl-epoxy	1:4	2.2	97	0	165
Silica-octyl-epoxy	1:5	1.81	98	0	240
Silica-octyl-epoxy	2:1	2.1	99	5	140
Silica-octyl-epoxy	4:1	2.2	100	4	145

^aImmobilization was performed as described in the experimental section.

^bThe ratio of GPTMS and OTES in the reaction mixture

^cSpecific activity (U/mg lipase) is expressed as micromole of substrate hydrolyzed per minute per mg of RML. The specific activity of the soluble RML was 2.1

^dYield is defined as the percentage of the soluble enzyme that becomes attached to the support.

^e The quantification of oxirane groups was performed using a 1.3 M solution of sodium thiosulphate as described in the experimental section

Table 3. Immobilization of RML on functionalized SBA-15

Biocatalyst ^a	Octyl:Epoxy ^b	Specific activity ^c	Immobilization yield ^d (%)	Desorbition (%)	epoxy groups ^e (μm)
SBA-epoxy	1:0	1.6	91	2	1248
SBA-octyl	0:1	3.4	100	61	0
SBA-octyl-epoxy	1:1	3.2	97	9	453
SBA-octyl-epoxy	1:2	2.7	96	9	620
SBA-octyl-epoxy	1:4	2.3	96	5	670
SBA-octyl-epoxy	1:5	1.8	94	0	730
SBA-octyl-epoxy	2:1	2.5	95	0	586
SBA-octyl-epoxy	4:1	2.9	96	21	263

^aImmobilization was performed as described in the experimental section.

^bThe ratio of GPTMS and OTES in the reaction mixture

^cSpecific activity (U/mg lipase) is expressed as micromole of substrate hydrolyzed per minute per mg of RML.
The specific activity of the soluble RML was 2.1

^dYield is defined as the percentage of the soluble enzyme that becomes attached to the support.

^e The quantification of oxirane groups was performed using a 1.3 M solution of sodium thiosulphate as described in the experimental section