International Journal of Biological Macromolecules 206 (2022) 580–590

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Coimmobilization of lipases exhibiting three very different stability ranges. Reuse of the active enzymes and selective discarding of the inactivated ones

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ARTICLE INFO

Keywords:
Enzyme coimmobilization
Coimmobilized enzyme stability
Selective enzyme desorption
Reuse of coimmobilized stable enzymes

ABSTRACT

Lipase B from *Candida antarctica* (CALB) and lipases from *Candida rugosa* (CRL) and *Rhizomucor miehei* (RML) have been coimmobilized on octyl and octyl-Asp agarose beads. CALB was much more stable than CRL, that was significantly more stable than RML. This forces the user to discard immobilized CALB and CRL when only RML has been inactivated, or immobilized CALB when CRL have been inactivated. To solve this problem, a new strategy has been proposed using three different immobilization protocols. CALB was covalently immobilized on octyl-vinyl sulfone agarose and blocked with Asp. Then, CRL was immobilized via interfacial activation. After coating both immobilized enzymes with polyethylenimine, RML could be immobilized via ion exchange. That way, by incubating in ammonium sulfate solutions, inactivated RML could be released enabling the reuse of coimmobilized CRL and CALB to build a new combi-lipase. Incubating in triton and ammonium sulfate solutions, it was possible to release inactivated CRL and RML, enabling the reuse of immobilized CALB when CRL was inactivated. These cycles could be repeated for 3 full cycles, maintaining the activity of the active and immobilized enzymes.

1. Introduction

The use of coimmobilized enzymes is becoming increasingly popular because the multi-enzymatic cascade reactions are taking a higher relevance day by day [1–4], and the use of coimmobilized enzymes produces some kinetic advantages in these instances [5–7]. Moreover, the use of immobilized enzymes in chemoenzymatic cascade reactions may also be found. [8–10]. This has evolved to the design of plurizymes [11], bearing one biological active center and one organometallic catalyst. [12]

Enzyme coimmobilization generates some technological problems in the preparation of an appropriate biocatalyst, that has been recently reviewed [13]. Because of these problems, it may not be taken for granted that the kinetics advantages [2,14,15] are enough to compensate the coimmobilization problems, it may require a careful economic analysis. That is far from the scope of this paper. It could be that the effect is just in the initial reaction rates, with a scarce saving of time in the overall reaction cycle, although in some instances the kinetic incidence may be higher, depending on the kinetic properties of the enzymes, particles size, etc. [13]. The main effect of the enzyme coimmobilization is to decrease or even eliminate the lag time of the reactions, and this may be critical when the product of the first enzyme is unstable, or if the product can inactivate the enzymes and must be rapidly transformed [3,16–23].

If the use of several enzymes requires some in situ reaction to reach the synergic effect, only using coimmobilized enzymes this may be achieved [24–26]. Moreover, using lytic enzymes to protect a target immobilized enzyme from microbial contamination, both enzymes must be coimmobilized [27,28]. Additionally, if the researcher wants to use immobilized cofactors, and that way, to reuse these expensive molecules or use them in a continuous reactor, all enzymes (cofactor users and cofactor regenerating ones) must be coimmobilized if a porous support is utilized [29–34]. That way, enzyme coimmobilization in some instances is not only advantageous, but fully necessary. However, the research in this area pays scarce attention to the problems generated when utilizing enzymes with very dissimilar stabilities. Using conventional coimmobilization strategies, when the least stable enzyme(s) becomes/become inactivated, the whole immobilized combi-biocatalyst, perhaps with

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https://doi.org/10.1016/j.ijbiomac.2022.02.084
Received 12 January 2022; Received in revised form 15 February 2022; Accepted 15 February 2022
Available online 24 February 2022

some immobilized enzymes still fully active, must be discarded [13,35]. There are some recent proposals to solve this problem when the enzymes involved have two stability ranges [36–43]. In these coimmobilization strategies, the most stable enzymes are first immobilized on the support via an immobilization strategy different to the one utilized to immobilize the least stable enzymes. For example, the coimmobilization of lipases on octyl agarose via interfacial activation has been reported, its coating with polyethylenimine (PEI) and the immobilization of a second enzyme, with much lower stability, via ion exchange on the PEI bed [36–38]. After inactivation of the least stable enzymes, they can be released by incubation at high ion strength and the lipase immobilized via interfacial activation on the support maybe recovered and reused to build a new combi-biocatalyst. Other proposed alternative is the use of glyoxyl-octyl agarose beads to solve the specific case of coimmobilization of lipases presenting different stabilities [40,41]. Here, a covalent immobilization protocol is employed to immobilize the most stable lipases, following their previous immobilization via interfacial activation [44], and after reducing the glyoxyl groups on the support, the least stable lipase could be immobilized just via interfacial activation in this chemically inert support [40,41]. Employing detergents, the inactivated enzymes may be desorbed from the support, while the most stable enzymes, covalently immobilized, remained attached to the support [40,41]. These stable and fully active immobilized lipases could be utilized to produce a new combi-biocatalyst identical to the initial one. As most cases use just two coimmobilized enzymes, these previous strategies may be enough to solve the problem.

Due to the complication of coimmobilization, the coimmobilization of more than 2 enzymes is not so popular in literature, but it is possible to find many examples where more than two enzymes are coimmobilized [45–65]. In these very interesting papers, the problem of dissimilar enzyme stabilities is not analysed. However, it is possible that if many different enzymes are coimmobilized, the involved enzymes may present more than two stability ranges. The reuse of the intermedium and most stable enzymes may be performed to build new combi-lipases following the previous strategies after inactivation of the least stable enzymes, just releasing the inactivated enzyme and using two different immobilization strategies [36–42]. However, if the difference in the stabilities of the intermedium and the most stable enzymes is high enough, one should finally discard the most stable immobilized enzyme, perhaps almost fully active, when the intermedium enzyme resulted inactivated.

To solve this situation, it is necessary to develop strategies that permit to co-immobilize enzymes using three different protocols in the same particle, protocols that should retain the active enzymes in an immobilized form while enabling the release of the inactivated enzymes under adequate conditions. A first approach will be to couple both strategies described above, using heterofunctional octyl supports and their coating with PEI for the lipase by lipase immobilization [10–14].

In this paper, we have utilized lipases as model enzymes for a first approach to solve this enzymes stabilities differences problem. There are many examples in the design or use of coimmobilized lipases [66].

The use of several lipases to get the full modification of heterogeneous substrates, such as oils and fats, has been shown to be advantageous, as the enzyme specificity and regioselectivity becomes a problem to get the full modification of this kind of substrates [67–76]. If is expected that an increase in the number of the lipases used, involving different selectivities and specificities, can permit higher final modification yields [77–79].

Lipases are interfacial enzymes, which suffer large conformational changes between an open and a closed structures [80–85]. The open form of the lipase is usually the active one, and exposes to the medium a large hydrophobic pocket, that becomes adsorbed to the drop of oils enabling the interfacial activity of the lipases [80–85]. Following a similar mechanism, lipases may become strongly adsorbed to any hydrophobic surface even at low ionic strength, and the immobilization of lipases on hydrophobic supports has become a much utilized strategy to produce biocatalysts of these enzymes [86–90]. The immobilization of lipases on octyl-agarose has produced biocatalysts in many instances more stable than the commercial ones, and more than biocatalysts stabilized by multipoint covalent immobilization [91–93].

The use of heterofunctional octyl-vinyl sulfone supports enables the covalent immobilization of the enzymes previously adsorbed by interfacial activation, being the interfacially activated enzyme irreversibly attached to the support producing a further stabilization of the immobilized enzyme [94] and enabling to alter the enzyme features and even the inactivation pathway [95]. This immobilization strategy will be used for the most stable lipase. This method is more efficient to prevent the enzyme release than glyoxyl-octyl agarose, and the covalent bonds involved next to 100% of the immobilized enzyme molecules [94]. This advantage derived from the features of vinyl sulfone: it may react with a wider range of groups of the enzyme (e.g., primary amino groups, thiol, phenol and imidazole) [96] than glyoxyl, and the spacer arm is longer, facilitating the reaction with the enzyme in the presence of the octyl groups layer. Moreover, the blocking with aspartic acid of the support will make the adsorption of the PEI bed on the biocatalyst stronger, and this can reduce the risks of PEI release (see below). Next, the enzyme having an intermedium stability will be immobilized on the biocatalyst just via interfacial activation on the chemically inert support [36,39]. In this paper, we have selected as blocking agent Asp acid, because the anionic character of the group should not become a serious problem to release (after its inactivation) the intermedium-stable enzyme at pH 7 (higher than the isoelectric point of most enzymes). Next, the surfaces of both enzymes and any free area of the octyl-Asp support will be coated with PEI. This PEI coating has been reported to improve the stability of many enzymes [97–101] and specifically of lipases immobilized via interfacial activation [102,103]. Finally, the immobilization of the least stable enzyme will be performed on the PEI bed via ion exchange. That way, we will have the 3 lipases immobilized via 3 different immobilization protocols, and the two enzymes bearing the lower stability levels, in reversible but different forms.

As model lipases, those from *Candida rugosa* [104,105] and *Rhizomucor miehei* [106] and the isoform B of the lipase from *Candida antarctica* [107–109] have been selected.

2. Materials and methods

2.1. Materials

Branched polyethylenimine (PEI) (MW 25,000), Triton X-100, L-Aspartic acid (Asp), *p*-nitrophenyl butyrate (*p*-NPB), divinyl sulfone (DVS) were obtained from Sigma Aldrich. Liquid CALB (the isoform B from *C. antarctica*) (12 mg of enzyme per mL), and RML (lipase from *R. miehei*) (Palatase 20,000 L) (3.7 mg of protein per mL) solutions were kindly donated by Novozymes (Spain). CRL (lipase from *C. rugosa*) in powder formulation (3.2% of protein content) was acquired from Sigma, Spain. To quantify the proteins concentrations, the BCA dye reagent staining was used, being the Absorbance determined at 595 nm, utilizing bovine serum albumin as reference [110]. Ammonium sulfate (AS) was purchased from Fisher (Alcobendas, Madrid). Low molecular weight (LMW) calibration kit for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (14.4–97 kDa) and octyl-Sepharose Cl-4B beads were acquired from GE healthcare. The other compounds were of analytical grade.

2.2. Methods

2.2.1. Immobilization of enzymes on octyl agarose beads

The immobilization of lipases on octyl agarose beads, using loadings over the capacity of the support to ensure the full coating of its surface with enzymes molecules (CALB (12 mg/g), CRL (30 mg/g) or RML (20 mg/g)), was performed by interfacial activation [86–90]. The immobilization was performed at 25 °C in 100 mL of a suspension formed by

lipase in 5 mM sodium phosphate solution at pH 7.0, where 10 g of support were added. When there were no changes in the supernatant *p*-NPB activity for 1 h, the immobilized lipase was vacuum filtered, washed with abundant distilled water and were put in the refrigerator at 6 °C until their use.

2.2.2. Preparation of octyl-VS-agarose beads

20 mL of a solution composed of 0.350 M DVS in 0.333 M sodium carbonate was prepared and the pH was adjusted to 11.5. Then, 1 g of octyl-agarose beads was added. During 2 h this suspension was maintained under orbital stirring, being later thoroughly washed with distilled water and put in a fridge at 6 °C.

2.2.3. Immobilization of the most stable lipase on octyl-VS-agarose support

For individual biocatalysts, CALB was immobilized using a load higher than the support capacity (12 mg/g), using the protocol previously described [94]. The liquid commercial solution of the lipase was diluted in 5 mM sodium acetate at 25 °C and pH 5.0 (to reduce the vinyl sulfone groups reactivity) in a ratio 1/10. After this first immobilization (via interfacial activation), CALB biocatalyst was recovered by filtration and washed with distilled water to eliminate the excess of enzyme and suspended in 0.1 M sodium carbonate at pH 10.0, to favour the enzyme-support covalent reaction, for 4 h. Finally, the biocatalyst was blocked using 2 M aspartic acid at 25 °C and pH 8.0 utilizing a relation 1 g of biocatalysts per 10 mL of blocking agent solution. After 24 h, the biocatalyst was filtered and washed abundantly with distilled water and put in storage at 6–8 °C. Immobilization yield is defined as the percentage of lipase activity (determined by the *p*-NPB assay) immobilized on the support.

2.2.4. Immobilization of the intermediate stable lipase on octyl-VS-Asp-agarose support

Octyl-VS-Asp-agarose was prepared by blocking Octyl-VS- Agarose with Asp as described above to have a support without chemically reactive groups. 300 mg of lipase from *Candida rugose* solution dissolved in 100 mL of 5 mM sodium phosphate at pH 7.0 and 25 °C and subsequently added to 10 g of support under gently stirring. The process was followed by measuring enzyme activity in the suspension and supernatant employing *p*-NPB. CRL was offered using a load that excess that of the support, to entirely cover the support surface with the enzyme, when the activity in the supernatant was constant after 1 h, the immobilized enzyme was filtered, washed and kept in a fridge.

2.2.5. Incubation of immobilized enzymes with PEI

1 g of the different biocatalysts was added to 10 mL of a solution 10% (w/v) of PEI at 4 °C and pH 7.0 for 24 h [111,112]. Next, the composites were filtered and washed with abundant water to eliminate the non-immobilized PEI molecules and stored at 6–8 °C. These PEI coating of the immobilized enzymes should permit the immobilization of most enzymes via ion exchange at pH 7 [111].

2.2.6. Immobilization of RML in PEI layer

RML was immobilized on octyl-VS-Asp-PEI-agarose beads using 20 mg enzyme/g of support in 5 mM sodium phosphate at pH 7.0 and room temperature. A mild mechanical stirring was used during the immobilization, which was maintained for 2 h. After immobilizing the enzyme, the biocatalysts were recovered by filtration and washed with distilled water.

2.2.7. Building of the three-combi-biocatalyst

The immobilization protocols described above were used, just changing the offered enzyme loadings in certain cases. The immobilization protocol described above using octyl-VS-Sepharose support was utilized to immobilize CALB, but offering only 4 mg/g, to ensure that the support was not fully covered with this enzyme to permit the later immobilization of CRL. Then, 17 mg of CRL was offered to the biocatalyst, to fully coat the surface of the support with both enzymes, using 50 mM Tris buffer to fully prevent the adsorption of CRL molecules on the immobilized CALB molecules. Next, this combi-biocatalyst was coated with PEI and RML was immobilized as described above for octyl-VS-Asp-PEI-agarose.

In some instances, the already immobilized enzyme was inactivated before adding the next enzyme using diethyl *p*-nitrophenylphosphate (D-*p*NPP). After immobilizing the next enzyme, this permitted to analyse its activity and stability without any interferences of the previously immobilized enzyme(s). To this purpose, 2 g of the biocatalysts containing the enzymes that we want to inactivate (CALB or CALB and CRL) was suspended at 25 °C in 40 mL of 100 mM sodium phosphate pH 7.0. Then, solid D-*p*NPP was added up to a concentration of 20 mM. These D-*p*NPP additions were performed each 30 min for 3 times, until the activity of the immobilized enzymes was under 5% of initial one. Then, the inactivated biocatalyst was washed several times with 10 volumes distilled water.

2.2.8. Desorption of immobilized CRL coated with PEI from octyl-VS-Asp-agarose

Different solutions were prepared in 50 mM Tris buffer at pH 7.0: 0.5 M, 3.0 M or 4.0 M ammonium sulfate containing some Triton X-100 (0, 0.1%, 0.25%, 0.5% or 1% (v/v)) or only Triton X-100 (0.1%, 0.25%, 0.5% and 1% (v/v)) were tested. Then, 1 g of the different biocatalysts were added to 10 mL of the desorption solution at 25 °C for 2 h. Finally, the biocatalysts were washed 10 times using 10 volumes of the respective desorption solutions to eliminate the released enzyme, following by 100 times washes with 10 volumes of distilled water to eliminate all the detergent molecules from the biocatalyst [40], finally it was stored at 6–8 °C until its use.

2.2.9. RML desorption from the PEI bed

1 g of combi-biocatalysts was incubated in 10 mL of a solution of 0.5 M ammonium sulfate in 50 mM Tris buffer at pH 7.0. The incubation was carried out at room temperature for 2 h. Afterwards, the biocatalysts were washed 10 times utilizing 10 mL of desorption solution per gram of biocatalysts to ensure the complete elimination of released enzyme and then 10 washes with distilled water. Finally it was stored at 6–8 °C until their use.

2.2.10. Thermal inactivation, desorption and reuse of immobilized lipase to build new combi-biocatalysts

After analysing several temperatures of inactivation, we selected 60 °C in order to inactivate only the least stable enzyme, and 67 °C, to inactivate the intermedium and the least stable enzymes. Experiments were performed in 10 mM sodium phosphate buffer at pH 7.0 in a proportion of support and inactivation medium 1/10 (w/v). At defined times, samples of the suspensions were taken and their remaining *p*-NPB activities were determined. Residual activities were calculated as the percentage of the biocatalysts initial activity. After the inactivation of the least stable lipases, the biocatalysts were incubated in a desorption solution composed by 0.5 M ammonium sulfate to release only RML from the support, or 0.5 M ammonium sulfate with 0.25% (v/v) of Triton X-100 to release for RML and CRL. Finally, the biocatalysts were recovered by filtration, when CRL and RML were inactivated and released, CRL was coimmobilized with the immobilized CALB, coimmobilized CALB-CRL were incubated with PEI and then, RML was immobilized. These cycles were repeated several times.

2.2.11. SDS-PAGE analysis

SDS-PAGE experiments were carried out according to Laemmli [113]. The biocatalyst samples were boiled for 8 min in a solution composed of 4% SDS (w/v) and 10% mercaptoethanol (v/v), to get a final protein content of 0.3–0.5 mg/mL. The suspensions were centrifuged at 10,000 rpm to discard the support. 15 μL aliquots of supernatants or 5 μL of LMW-SDS Marker (14.4–97 kDa) were utilized in the

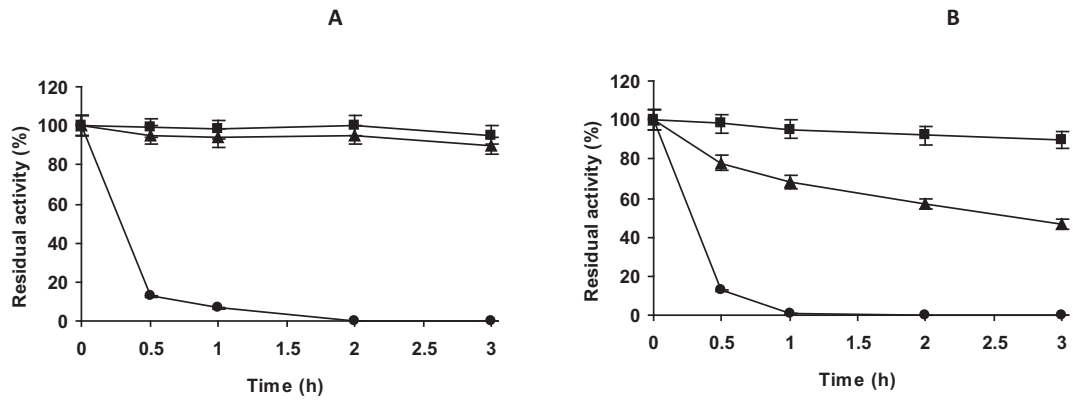

Fig. 1. Inactivation courses of different lipases immobilized on octyl-VS-ASP agarose at pH 7 and 60 °C **(A)** or **(B)** 67 °C. Squares: CALB; Triangles: CRL; Circles: RML. Other specifications are described in Methods.

experiments. The samples were injected using 12% polyacrylamide gels, they were run at 100 V. Proteins were stained by Coomassie blue dye.

2.2.12. Enzyme activity versus p-NPB

The augmentation of absorbance produced by the released *p*-nitrophenol resulted in the hydrolysis of *p*-NPB was quantified at 348 nm (isosbestic point of *p*-NP, ε under these conditions is 5150 M − 1 cm − 1) during 90 s [114]. The cuvette contained 2.5 mL of 25 mM sodium phosphate at 25 °C and pH 7.0. Then, 50 μl of *p*-NPB solution (at a concentration of 50 mM, dissolved in acetonitrile) was introduced in the cuvette and to start the reaction, 50 μL of the enzyme solution or suspension was added under magnetic stirring. The enzymatic activity unit (U) is given as μmol of hydrolysed *p*-NPB per minute.

3. Results

3.1. Stability of the different lipases used in this study when immobilized on octyl-VS-Asp agarose

First, CALB, RML and CRL were immobilized on octyl-VS-Asp agarose (Fig. 1-S) and their stabilities were analysed at different temperatures (Fig. 1). As an excess of enzyme was used in all cases, immobilization yields were around 40–60%, while the activity of this suspension slightly increased, even when the volumetric activity was quite high and some substrate diffusional problems could be expected (Fig. 1-S).

Regarding the stabilities, Fig. 1 shows that CALB biocatalyst remained almost fully active when incubated at either 60 or 67 °C for 3 h, while RML became readily inactivated at both temperatures. CRL activity was almost not affected when incubated at 60 °C, while lost more than 50% of the initial activity after 3 h of incubation at 67 °C. That way, although the 3 enzymes could be coimmobilized using the same support, CALB and CRL would be almost fully active when RML becomes fully inactivated. Moreover, CRL lost 50% of the activity while CALB remained almost fully active if the temperature was higher. That way, the preparation of a combilipase formed by the 3 selected enzymes was a representative example of the possibility of using enzymes having 3 very different stability ranges. In this case, CALB was the most stable enzyme, CRL was the enzyme exhibiting a in between stability, while RML exemplified the least stable enzyme.

Following our strategy, CALB should be covalently immobilized on octyl-VS support, while CRL should be reversibly immobilized on the support after blocking with Asp to eliminate the risks of covalent bonds formation, and after their coating with PEI; RML could be immobilized over the previously immobilized enzymes.

3.2. Effect of the PEI coating on the activity and stability of immobilized CALB and CRL

To reach our goal, first, it was necessary to check the effect of the PEI coating on the features of CALB and CRL immobilized on octyl-VS-Asp agarose. The activity of CRL and CALB immobilized on octyl-VS-Asp agarose beads slightly increased after the PEI coating (by around a 15%). Moreover, the stability of CRL slightly increased after this treatment (see Fig. 2), although there was still a significant inactivation of the enzyme after 3 h at 67 °C (higher than 40%). CALB maintained its

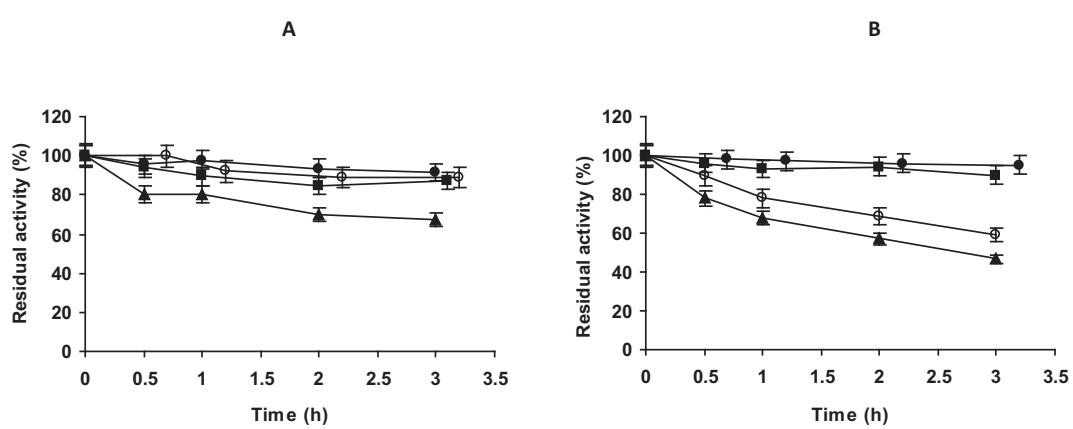

Fig. 2. Inactivation courses of different lipases immobilized on octyl-VS-ASP agarose at pH 7 and 60 °C **(A)** or **(B)** 67 °C. Solid squares: Octyl-VS-ASP-CALB; Solid triangles: Octyl-VS-ASP-CRL; Solid circles: Octyl-VS-ASP-CALB-PEI. Empty circles: Octyl-VS-ASP-CRL-PEI. Other specifications are described in Methods.

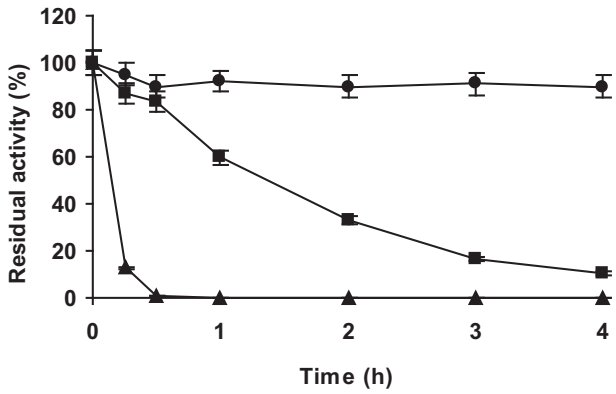

Fig. 3. Inactivation courses of different immobilized lipase biocatalysts at pH 7.0 and 60 °C. Solid squares: Octyl-Vs-Asp-RML; Solid triangles: Octyl-VS-ASP-PEI-RML; Solid circles: Octyl-VS-ASP-CRL. Other specifications are described in Methods.

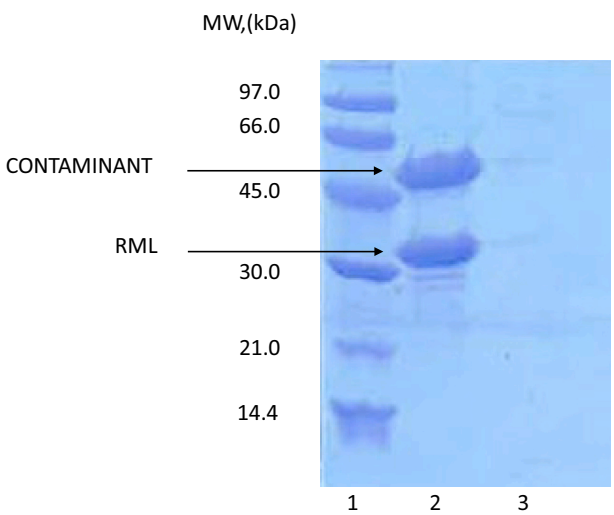

Fig. 4. SDS-PAGE analysis of octyl-VS-ASP-PEI-RML of the effect of the incubation and washing with ammonium sulfate. Lane 1: Low weight molecular markers; Lane 2: initial preparation; Lane 3: after incubation in 0.5 M ammonium sulfate. Other specifications are described in Methods.

activity at both temperatures for 3 h after this treatment. That way, this treatment could be considered adequate for the development of our coimmobilization protocol.

3.3. Immobilization of RML on octyl-VS- Asp-PEI

The immobilization proceeded very rapidly (Fig. 2- S) and the enzyme activity increased by 60%. However, the stability of RML immobilized on this support decreased when compared to the stability of the enzyme immobilized on octyl-VS-Asp agarose beads (Fig. 3). The economic analysis of the advantages of the possibility of reusing CRL and CALB versus the disadvantages of the decrease in the RML stability may depend on many factors, out of the scope of this paper. Thus, we have continued using RML as a model enzyme for our strategy. Next, the experiment was repeated using octyl-VS-CALB-Asp-CRL-PEI after inactivation of the immobilized enzymes using D-*p*NPP (see Methods section), with almost identical results (not shown results).

That way, it was possible to get the enzyme per enzyme immobilization strategy proposed in our strategy. However, before the strategy may be considered possible, it was necessary to ensure that it was possible to achieve the release of RML from the PEI bed without

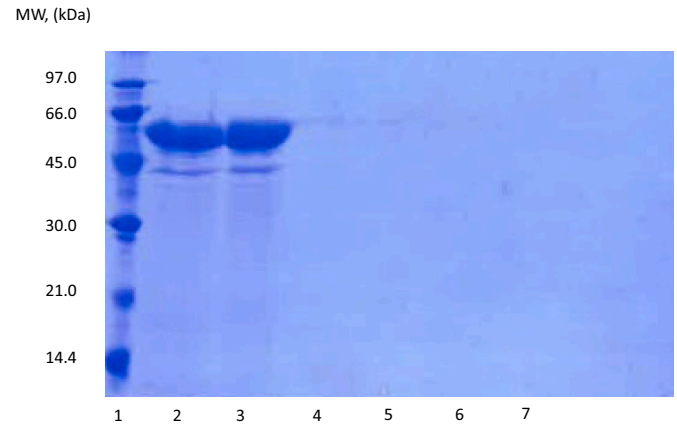

Fig. 5. SDS-PAGE analysis of octyl-VS-ASP-CRL after incubation and washing with different concentrations of Triton X-100 (v/v) solutions. Lane 1: Low weight molecular markers; Lane 2: initial preparation; Lane 3: after incubation in 0.05 M Tris buffer; Lane 4: after incubation in 0.1% (v/v) of Triton X-100; Lane 5: after incubation in 0.25% (v/v) of Triton X-100; Lane 6: after incubation in 0.5% (v/v) of Triton X-100; Lane 7: after incubation in 1% (v/v) of Triton X-100. Other specifications are described in Methods.

affecting the activity of immobilized CALB and CRL coated with PEI and also of CRL coated with PEI from the octyl-VS-Asp agarose combi-biocatalysts without affecting the activity of immobilized CALB.

3.4. Desorption of RML from octyl-VS-Asp-PEI support

The immobilization of RML on octyl-VS-Asp-PEI-RML should be mainly via ion exchange. We did not detect any enzyme release from the support during the washings, confirming that this polymeric bed was able to produce a very intense multipoint ionic exchange able to prevent enzyme release under mild conditions [111]. The simplest way to release the enzyme from this support is via the washing of the biocatalyst using high ionic strength. As this situation is similar to the described in reference [38], we tried the same conditions in this desorption. The incubation in 500 mM ammonium sulfate solutions resulted enough to eliminate more than 98% of RML activity from the support. The SDS-PAGE analysis confirmed that RML could not be detected in the support after the washing (Fig. 4). This figure shows two bands, as the commercial RML preparation presented RML and a contaminant, and the immobilization via ion exchange involved both proteins.

The incubation of octyl-VS-CALB-Asp-CRL under these conditions had not effect on its activity even after 24 h. As expected, no release of CRL or CALB could be detected under these conditions.

3.5. Desorption of CRL from octyl-VS-Asp-CRL-PEI

In this instance, CRL was immobilized via interfacial activation versus the octyl layer in the support. Later, some ion interactions between the enzyme and the support may be established, making the enzyme release harder [115]. However, we have observed that more than 95% of the CRL activity could be released from the support by using just 0.1% (v/v) Triton X-100 in 50 mM Tris buffer. This was confirmed by SDS-PAGE (Fig. 5).

The coating of the immobilized CRL with PEI could make some way more complex the release of the enzyme, as PEI can generate some intermolecular ionic crosslinking making harder the enzyme release [45,46]. This reinforcement of the lipase immobilization is positive in terms of immobilized enzyme stability, but now it may be a problem [38]. In fact, the incubation only in a detergent solution was not enough to release CRL after the PEI coating. However, combining Triton X-100 and 0.5 M sodium sulfate, 100% of CRL could be released from the support as confirmed by SDS-PAGE (Fig. 6A). If 3 or 4 M ammonium

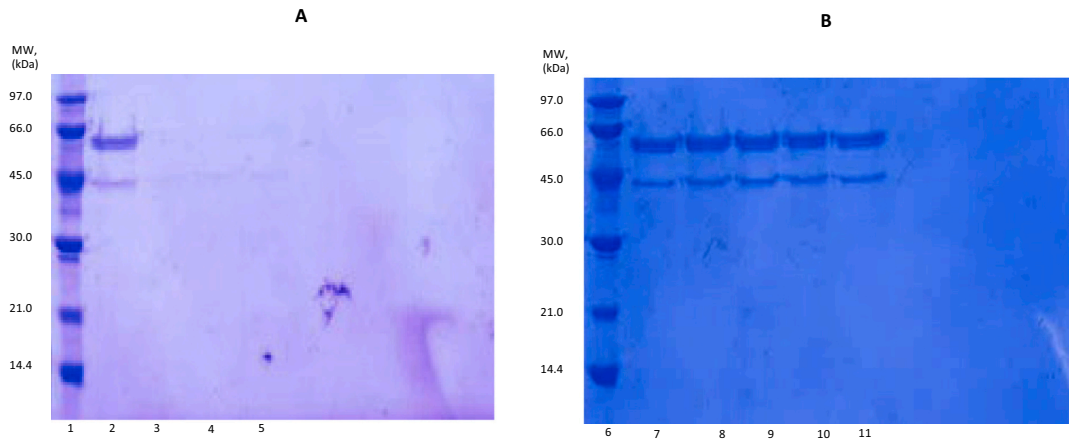

Fig. 6. SDS-PAGE analysis of octyl-VS-ASP-CRL-PEI after incubation and washing in ammonium sulfate (AS)/Triton X-100 solutions. Lane 1: Low weight molecular markers; Lane 2: initial preparation; Lane 3: after incubation in 0.5 M AS with 0.25% (v/v) of Triton X-100; Lane 4: after incubation in 0.5 M AS with 0.5% (v/v) of Triton X-100; Lane 5: after incubation in 0.5 M AS with 1% (v/v) of Triton X-100; Lane 6: Low weight molecular markers; Lane 7: initial preparation. Lane 8: after incubation in 3 M AS with 0.1% (v/v) of Triton X-100; Lane 9: after incubation in 3 M AS with 0.25% (v/v) of Triton X-100; Lane 10: after incubation in 4 M AS with 0.1% (v/v) of Triton X-100; Lane 11: after incubation in 4 M AS with 0.25% (v/v) of Triton X-100; Other specifications are described in Methods.

Table 1
Activities of the different biocatalyst in the hydrolysis of *p*-NPB at pH 7 and 25 °C. Experiments were conducted as described in Methods. The values are given as average and standard deviation.

Layers	Activity with *p*NPB (U/g)
OCTYL-VS-CALB-ASP	65.22 ± 2.61
OCTYL-VS-CALB-ASP-CRL	145.55 ± 7.28
OCTYL-VS-CALB-ASP-CRL-PEI	68.21 ± 2.73
OCTYL-VS-CALB-ASP-CRL-PEI-RML	127.50 ± 5.74
OCTYL-VS-CALB-ASP-CRL-PEI-RML (1° Cycle, 60 °C)	97.15 ± 4.18
OCTYL-VS-CALB-ASP-CRL-PEI-RML (1° Cycle, 67 °C)	66.89 ± 2.61

sulfate were used, the enzyme remained almost fully immobilized (Fig. 6B), very likely because the high ionic strength reinforced the lipase immobilization via interfacial activation.

The incubation of octyl-VS-CALB-Asp-PEI under these conditions had no effect on its activity even after 24 h. The covalent immobilization of all CALB molecules on octyl-VS-CALB-Asp was confirmed as not protein band could be detected from the biocatalyst when using it to perform a SDS-PAGE experiment (not shown result).

That way, the different pieces of the puzzle have been successfully achieved, and next, the octyl-VS-CALB-Asp-CRL-PEI-RML biocatalyst was build.

3.6. *Performance of octyl-VS-CALB-Asp-CRL-PEI-RML: cycles of inactivation, inactivated enzyme(s) release and fresh enzyme(s) re-immobilization*

The immobilization of the different enzymes was confirmed using D-*p*NPP inactivated formulations of the previous biocatalyst (that is, D-*p*NPP-CALB to immobilize CRL, and D-*p*NPP-CALB-CRL-PEI before immobilizing RML). The activity of these preparations was similar to those obtained using the corresponding supports (octyl-VS-Asp for CRL and octyl-VS-Asp-PEI for RML).

The activity evolution versus *p*-NPB of the fully active combi-biocatalyst is show in Table 1.

In the immobilization of CRL, the concentration of Tris was increased to 50 mM, as, using only 5 mM, some CRL activity was adsorbed on a support fully coated with CALB treated with D-pNPP, (around 10% of the amount of CRL immobilized in a naked octyl agarose). Although this immobilization was very weak and most of the CRL was washed way, we prefer to fully prevent the CRL adsorption. CRL contribution to the

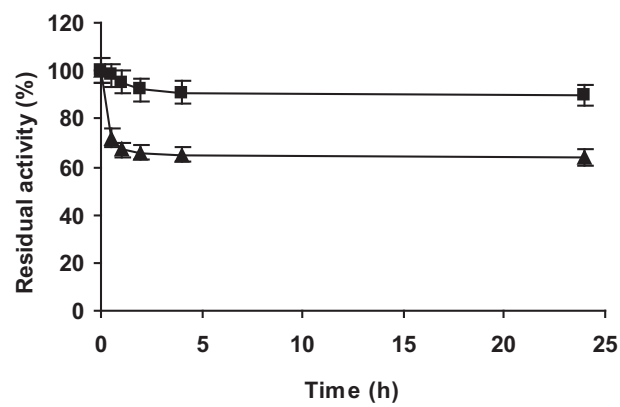

Fig. 7. Inactivation courses of different combi-biocatalysts at pH 7.0 and 60 °C. Solid line, solid squares: Octyl-VS-CALB-ASP-CRL-PEI; solid line, solid triangles: Octyl-VS-CALB-ASP-CRL-PEI-RML. Other specifications are described in Methods.

activity of the combi-biocatalyst is double than that of CALB, being the contribution of RML slightly lower than that of CALB. As we have 3 different stability ranges, the combi-biocatalyst has been inactivated under 2 different conditions.

First, we have inactivated it for 3 cycles at 60 °C, where mainly RML

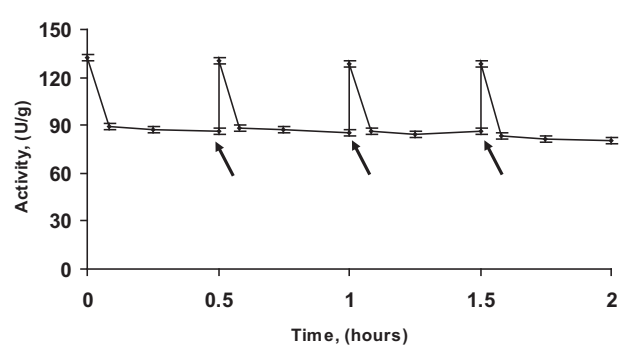

Fig. 8. Cycles of thermal inactivation of octyl-VS-CALB-ASP-CRL-PEI-RML at 60 °C and pH 7.0, RML desorption by incubation and washing with 0.5 M AS (see the text) and immobilization of a new batch of RML (indicated with an arrow). Other specifications can be found in the Materials and methods section.

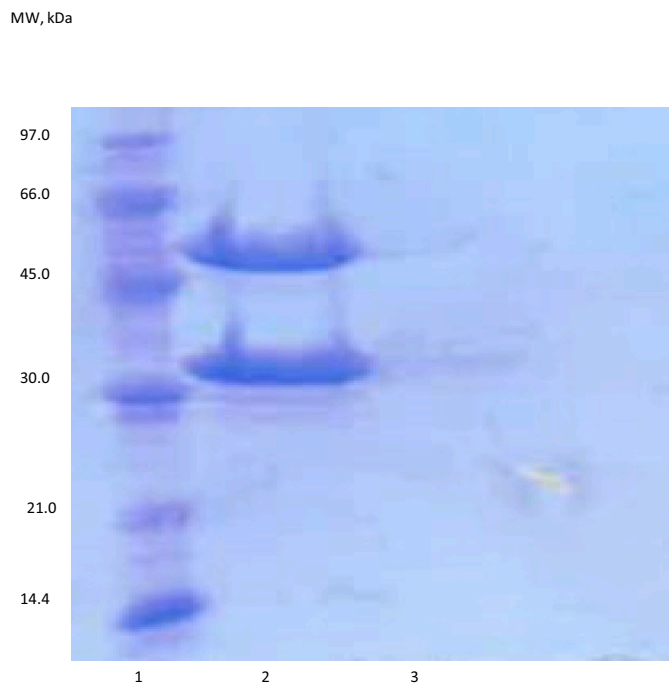

Fig. 9. SDS-PAGE analysis of octyl-VS-CALB-ASP-PEI-RML after 3 cycles of thermal inactivation at 60 °C and pH 7.0 and incubation and washing with ammonium sulfate (AS) and immobilization of fresh RML. The figure shows the biocatalysts after the last washing. Lane 1: Low weight molecular markers; Lane 2: initial preparation; Lane 3: after incubation in 0.5 M ammonium sulfate. Other specifications are described in Methods.

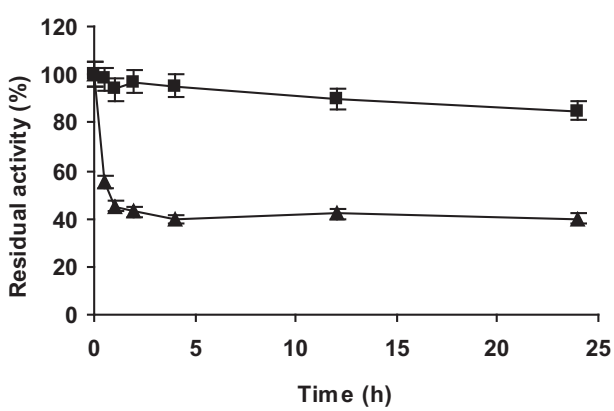

Fig. 10. Inactivation courses of different combi-biocatalysts at pH 7.0 and 67 °C. Solid squares: Octyl-VS-CALB-ASP-PEI; Solid triangles: Octyl-VS-CALB-ASP-CRL-PEI-RML. Other specifications are described in Methods.

is inactivated while CRL and CALB maintained almost full activity (Fig. 1). This was confirmed by inactivating RML immobilized on D-*p*NPP inactivated CALB/CRL biocatalysts. Inactivated RML was released from the support using 0.5 M ammonium sulfate, and after re-incubating the biocatalyst in PEI (as some PEI molecules were released from the biocatalysts, as previously shown [38]) fresh RML was offered.

Fig. 7 shows the inactivation course of the triple combi-biocatalyst at 60 °C. The activity deceased very rapidly to reach around 65% (approximately the percentage of activity that is contributed by RML) in only 1 h. Then, there was a slow decrease of the enzyme activity, which corresponded to the slow inactivation of CRL. Using the octyl-VS-CALB-Asp-CRL-PEI double combi-biocatalyst, this second slow inactivation may be easily visualized. That way, if the triple biocatalyst was incubated for 0.5 h at 60 °C, washed with 0.5 M ammonium sulfate, re-

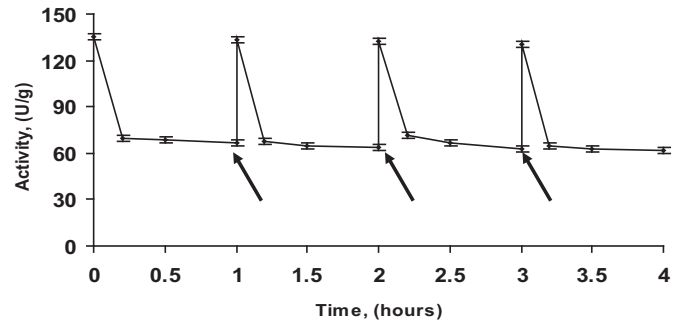

Fig. 11. Cycles of thermal inactivation at 67 °C and pH 7.0 of octyl-VS-CALB-ASP-CRL-PEI-RML, CRL and RML desorption by incubation in 0.25% (V/V) Triton/0.5 M AS and enzyme by enzyme immobilization of a fresh new batch of both enzymes (indicated with an arrow). Other specifications can be found in the Materials and methods section.

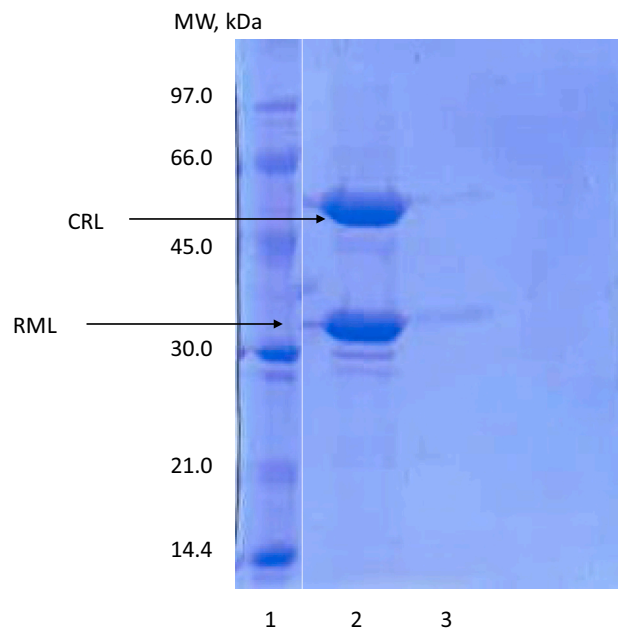

Fig. 12. SDS-PAGE analysis of octyl-VS-CALB-ASP-PEI-RML after 3 cycles of thermal inactivation at 67 °C and pH 7.0 and incubation and washing with ammonium sulfate (AS) and immobilization of fresh CRL, incubation in PEI solution and fresh RML immobilization. The figure shows the biocatalysts after the last washing. Lane 1: Low weight molecular markers; Lane 2: initial preparation; Lane 3: after incubation in 0.5 M AS/0.25% of Triton X-100. Other specifications are described in Methods.

incubated in PEI and a new and fresh batch of RML was immobilized, the activity of the triple combi-biocatalyst could be maintained almost unaltered after 3 full cycles (Fig. 8), reusing coimmobilized CRL and CALB to build a new combilipase. As CRL has a molecular weight similar to that of the contaminant of RML, we performed a similar experiment using octyl-VS-CALB-Asp-PEI-RML (using an excess loading of CALB to fully coat the support) just to check the intensity of the RML bands after the washings in the third cycle (Fig. 9). It was visible that only a very slight protein band corresponding to RML may be detected, showing that we can rebuild an almost identical biocatalyst for these 3 cycles.

In a second experiment, the triple combi-biocatalyst was inactivated at 67 °C, conditions where both RML and CRL were inactivated. Fig. 10 shows the inactivation course of octyl-VS-CALB-Asp-CRL-PEI-RML and octyl-VS-CALB-Asp-CRL-PEI, showing that the activity corresponding to CRL decreased significantly under these conditions, while that of octyl-VS-CALB-Asp-PEI remained almost unaltered. That way, incubating the

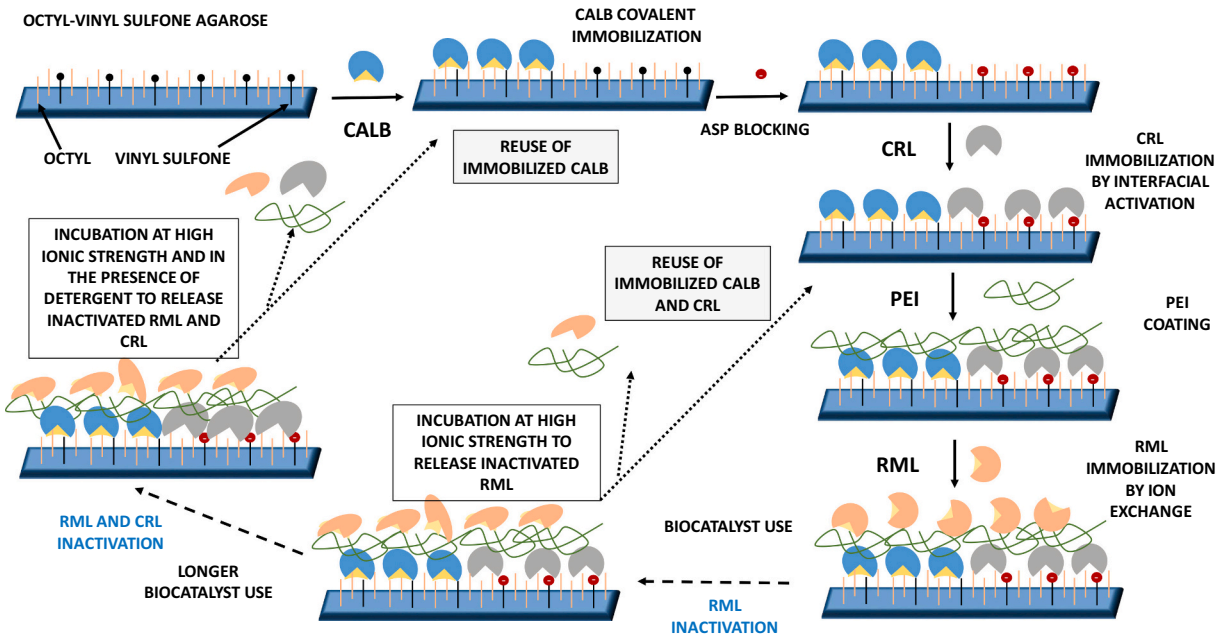

Fig. 13. Schematic representation of the proposed strategy.

triple combi-biocatalyst at 67 °C for 60 min, releasing RML and CRL by washing in 0.5 M ammonium sulfate/0.1% (v/v) Triton X-100 solutions, followed by an exhaustive washing with water to eliminate the detergent [40], immobilization of a fresh batch of CRL; incubation in PEI to coat all immobilized enzymes with this cationic polymer, and immobilization of a fresh batch of RML, immobilized CALB could be reused in 3 successive cycles to rebuild a new triple combilipase (Fig. 11). Fig. 12 shows that even after 3 cycles, the bands of CRL and RML were almost undetectable in the SDS-PAGE showing the success of the protocol to rebuild a new combi-biocatalyst similar to the initial one.

The strategy is summarized in Fig. 13.

4. Conclusions

Coimmobilization of enzymes must be applied only when the gains compensate the technological problems. Obviously, the higher the number of coimmobilized enzymes, the higher the complexity. However, using lipases, coimmobilization of many different enzymes can be performed using a hydrophobic support. However, in this paper, we have shown that the problem of coimmobilizing enzymes with different stabilities is a real one that can make discarding fully active and immobilized enzymes after inactivation of the least stable enzymes compulsory. The discarding of immobilized and active enzymes is economically negative, as we should add to the cost of the enzyme, that of the immobilization process, and of the support if the immobilization is not using reversible strategies. This paper shows an example involving 3 lipases that have stabilities very different each other. The standard coimmobilization of the 3 enzymes makes necessary to discard two fully active enzymes when the least stable enzyme has been inactivated, while the previous strategies to solve this problem make necessary to discard the most stable immobilized enzyme in a fully active way after inactivation of the intermedium-stable enzyme. The proposed strategy, involving the enzyme by enzyme immobilization and the use of three different immobilization protocols, two of them reversible is fully new and permits to release the inactivated enzymes in a selective form while enabling the reuse of the active and immobilize enzymes to build a new combi-biocatalyst similar to the initial one. The produced combi-lipases could have interest in the full modification of oils and fats, as has been discussed in introduction.

The results in this paper clearly exemplify the importance of the problem, even though it is usually ignored in the literature, making clear that enzymes coimmobilization should be utilized when the advantages clearly surpass the problems derived from enzyme coimmobilization, or when it is strictly necessary to get the desired results. Considering the increasing complexity of the cascade reaction that has been developed, every day involving more enzymes, it is possible to envisage situations where the number of involved enzymes may be high enough to have situations where the enzymes may be classified even in more than 3 ranges of stabilities, that way enzyme immobilization strategies that can be compatible with each other and that can permit the reuse of the release of the inactivated enzymes and the reuse of the fully active immobilized enzymes should be a research topic that gain importance in the future. Only with a better understanding of the enzymes immobilization and the problems derived of the enzyme coimmobilization, these opportunities could be fully explored.

CRediT authorship contribution statement

Diego Carballares performed the experiments. Roberto Fernandez-Lafuente and Javier Rocha-Martin designed and supervised the experiments. All authors contributed to the writing and final editing of the paper.

Acknowledgments

We gratefully recognize the financial support from Ministerio de Ciencia e Innovación-Spanish Government (project number CTQ2017-86170-R). Mr. Diego Carballares thanks to Ministerio de Ciencia e Innovación-Spanish Government by a FPI. Mr. Martinez (Novozymes Spain) is gratefully recognized for the supply of enzymes. Prof. Guisan support is recognized. The help and suggestions from Dr. Ángel Berenguer (Departamento de Química Inorgánica, Universidad de Alicante) are gratefully recognized.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.02.084.

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