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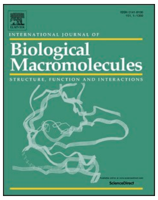

The combination of covalent and ionic exchange immobilizations enables the coimmobilization on vinyl sulfone activated supports and the reuse of the most stable immobilized enzyme

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ABSTRACT

The coimmobilization of lipases from *Rhizomucor miehei* (RML) and *Candida antarctica* (CALB) has been intended using agarose beads activated with divinyl sulfone. CALB could be immobilized on this support, while RML was not. However, RML was ionically exchanged on this support blocked with ethylendiamine. Therefore, both enzymes could be coimmobilized on the same particle, CALB covalently using the vinyl sulfone groups, and RML via anionic exchange on the aminated blocked support. However, immobilized RML was far less stable than immobilized CALB. To avoid the discarding of CALB (that maintained 90% of the initial activity after RML inactivation), a strategy was developed. Inactivated RML was desorbed from the support using ammonium sulfate and 1% Triton X-100 at pH 7.0. That way, 5 cycles of RML thermal inactivation, discharge of the inactivated enzyme and re-immobilization of a fresh sample of RML could be performed. In the last cycle, immobilized CALB activity was still over 90% of the initial one. Thus, the strategy permits that enzymes can be coimmobilized on vinyl sulfone supports even if one of them cannot be immobilized on it, and also permits the reuse of the most stable enzyme (if it is irreversibly attached to the support).

1. Introduction

Lipases are among the most employed enzymes both, in industry and academia [1–6], because of their high activity and stability in a wide diversity of reaction media and conditions [7–12] and the lack of cofactors. Moreover, lipases accept a wide variety of substrates (they have low specificity), although, in certain cases, they exhibit a high enantiospecificity or regio- or enantio-selectivity [13]. In fact, lipases are recurrent examples of enzymes with promiscuous activities [14].

They have a specific catalytic mechanism, called interfacial activation [15]. It is based on the existence of two conformational structures, one where the active center is isolated from the medium by a lid (closed form), and an open form, where the lid moves and exposes a large hydrophobic pocket [15]. This open form can strongly adsorb on drops of hydrophobic substrates, and also on any other hydrophobic surface (such as other lipases [16,17], hydrophobic proteins [18,19] or supports [20–22]). Simultaneously, their active center flexibility makes lipase properties easily alterable by genetic alterations [23–25], chemical modification [26,27] or immobilization following different protocols [28–34].

One important application of lipases is related to the modification of their physiological substrates, oils and fats, with interest in the production of fatty acids, structured lipids [35], biodiesel [36–38] biosurfactants [39–41] or biolubricants [42–46]. Oils and fats are in fact multifunctional and heterogeneous (mono, di or triglycerides, different fatty acids) substrates [47]. The lipase specificity becomes now a problem that can prevent the total transformation of an oil in the target product [47]. It has been reported that the joint utilization of different lipases may be advantageous for both increasing the final yield and the reacting velocity [48,49,58–67,50,68–70,51–57]. Moreover, when the experimental conditions change during the reaction course (pH, substrate concentration, substrate/product concentrations ratio), it may

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also be advantageous to use some different lipases [47].

A further step is the coimmobilization of all utilized lipases, which some authors claim as convenient for these reactions [71–73]. However, standard enzyme coimmobilization has several drawbacks (as it has been recently reviewed) [74]. For example, the same chemistry and protocol should be involved in the immobilization of all enzymes. That may be a problem if the best protocol for one enzyme is unsuitable for the other enzyme, as immobilization is no longer just a strategy to recover and reuse enzymes, but a powerful instrument to enhance enzyme features [75]. That way, an adequate enzyme immobilization may increase enzyme activity (mainly under tough conditions) and stability (increasing the operational window of the enzyme) [76–85]. Moreover, the enzyme selectivity and specificity may be altered [77], the inhibitions lowered [86] or the resistance to chemicals augmented [87]. Furthermore, purification and immobilization may be coupled [88]. That way, it is possible that all advantages of the immobilization to enhance the enzyme features are not utilized if the immobilizations protocols are reduced to those useful for all enzymes [74,75].

Another usually overlooked difficulty is the possibility of using coimmobilized enzymes with very different stabilities. In these cases, it is possible that the most stable enzyme remains fully active while the activity of least stable one is under the threshold value to discard it. That means that a immobilized enzyme may be discarded even maintaining the initial activity [74,75]. There are several recent methodologies aiming to solve this problem [89–93]. For example, the use of octyl-glyoxyl agarose beads enabled the covalent immobilization of the most stable enzyme, while the least stable ones were immobilized only via interfacial activation [89,90]. This way, after the inactivation of the least stable enzyme, this could be released by incubation in detergents, and the most stable enzyme could be reused to build a new combilipase [89,90]. Other strategy involved the coating of the most stable enzymes after their immobilization with polyethylenimine, and the immobilization on this polymeric bed of the least stable enzyme [91–93]. After the least stable enzyme inactivation, it could be released via incubation at high ionic strength and the most stable immobilized enzyme could be reused to prepare a new combienzyme [91–93]. Other possibility is the use of vinyl sulfone supports, that also enables the coimmobilization of enzymes using different immobilization phenomena [94–102]. This support is very adequate to stabilize enzymes via multiple covalent immobilization [78,103,104]. Thus is due to the high reactivity of vinyl sulfone with different moieties of the amino acids: imidazole of His, thiol groups of Cys, ϵ-amino of Lys, phenol of Tyr [103,104]. The advantages and possibilities of this support to stabilize enzymes via multipoint covalent attachment have been recently reviews [78]. After enzyme immobilization, a blocking step is recommended to avoid undesirable enzyme-support reactions [103,104]. This step permitted to introduce the least stable enzyme in the support groups able to immobilize via a reversible immobilization mechanism (e.g., ion exchange) [105]. That way, the blocking step becomes a critical advantage to solve the coimmobilization drawback based in dissimilar enzyme stabilities. This strategy may be used with any enzyme (not being reduced to lipases like the use of octyl agarose [89,90]) and do not require the further modification of the immobilized enzymes (like the use of enzymes coated with PEI [91–93]).

As model enzymes, we have selected the lipase B from *Candida antarctica* (CALB) [106–110] and the lipase from *Rhizomucor miehei* (RML) [111,112] that are among the most employed lipases in the literature and have quite dissimilar stabilities when immobilized on octyl agarose [91].

2. Materials and methods

2.1. Materials

The liquid protein formulations Palatase® 20,000 L (lipase from *Rhizomucor miehei*, 3.7 mg of protein per mL of enzyme solution) (RML) and Lipozyme® CALB L (lipase B from *Candida antarctica*, 7.7 mg of protein per mL of enzyme solution) (CALB) were a kind gift of Novozymes (Alcobendas, Spain). Agarose beads standard matrix (4% BCL) was acquired from ABT (Madrid, Spain). Low molecular weight (LMW) calibration kit for SDS electrophoresis (14.4–97 kDa) was supplied by GE Healthcare (Alcobendas, Spain). *p*-Nitrophenyl butyrate (*p*NPB), Triton X-100, ethylenediamine (EDA) and diethyl *p*-nitrophenylphosphate (D-*p*NPP) were from Sigma Aldrich Spain (Madrid, Spain). Ammonium sulfate and divinyl sulfone were from Thermo Fisher scientific Spain (Madrid, Spain). Other solvents and chemicals were of analytical grade. Bradford method was employed to determine protein concentration [113], utilizing bovine serum albumin as reference.

2.2. Methods

The data are the results of at least 3 independent assays, they are utilized as mean values and standard deviation.

2.2.1. Determination of enzyme activity

The enzymatic activity was determined by determining the augment in the absorbance at 348 nm provoked by the *p*-nitrophenol (*p*NP) produced by the hydrolysis of *p*NPB at 348 nm (isobestic point). A Jasco spectrophotometer (V-730) (Jasco, Madrid, Spain) was utilized to perform the measurements. Under these conditions the *p*NP extinction coefficient is 5150 M^{-1} cm^{-1} [114]. In a cuvette containing 2.5 mL of 25 mM sodium phosphate at pH 7.0, 50 μL of 50 mM *p*NPB (dissolved in acetonitrile) was added. 50 μL of free or immobilized enzyme was then added. The temperature was controlled at 25 °C under magnetic stirring. One enzyme activity unit (U) was defined as μmol of substrate hydrolysed per minute under the assay conditions.

2.2.2. Preparation of vinyl sulfone agarose support

A volume of 400 mL of 333 mM sodium carbonate at pH 11.5 was mixed with 15 mL of divinyl sulfone (final concentration was 0.35 M). This mixture was vigorously stirred until a homogeneous solution was achieved. Next, 20 g of agarose beads was added. The suspension was submitted to mild stirring for 2 h. After, the activated support was vacuum filtered employing a sintered glass funnel, exhaustively washed with distilled water and stowed at 6–8 °C.

2.2.3. Enzyme immobilization

Immobilizations were characterized by immobilization yield (proportion of immobilized enzyme in %) and expressed activity (observed activity/theoretic activity) [115].

2.2.3.1. Immobilization of lipases on vinyl sulfone agarose beads. Briefly, 1 g of vinyl sulfone agarose beads per 10 mL or 3 mL of enzyme solutions (2 mg of enzyme per g of support for RML and 1 mg of enzyme per g of support for CALB) was utilized [116]. The immobilization of CALB on this support and the features of the immobilized enzyme is described in [116]. The immobilization was carried out in 10 mM sodium carbonate at pH 9.0 (RML) or pH 10.0 in the presence of 0.3% (v/v) Triton X-100 (CALB) at 25 °C. The detergent was employed to eliminate lipase interfacial activation adsorption on the support [116,117]. After enzyme immobilization, the CALB biocatalyst was washed to eliminate the detergent. The immobilized CALB biocatalyst was incubated in 100 mM sodium carbonate at pH 10.0 and 25 °C for 72 h. As a reaction end point, the heterogeneous CALB biocatalyst was incubated in 2 M of ethylenediamine at pH 10.0 and 25 °C for 24 h to modify the residual vinyl sulfone moieties. Finally, using a sintered glass funnel, the immobilized preparation was vacuum filtered, washed with abundant distilled water and placed in a fridge at 6–8 °C [116].

2.2.3.2. Enzyme immobilization of RML via ion exchange on vinyl sulfone agarose beads blocked with ethylendiamine. 1 g vinyl sulfone agarose beads blocked with ethylendiamine was added to 10 mL of enzyme solution (2 mg of enzyme per g of support of RML) in 10 mM sodium phosphate at pH 7.0 and 25 °C. Next, the biocatalyst was vacuum filtered employing a sintered glass funnel, washed with distilled water and placed in a fridge at 6–8 °C

2.2.4. *Desorption of RML from aminated agarose beads*

The enzyme is ionically exchanged on the support, and the use of high ionic strength should be enough to release the enzyme from the support [81–93]. The release of the enzyme in the presence of growing concentrations of ammonium sulfate was studied. To this goal, ammonium sulfate solutions at different concentrations (0 mM, 50 mM, 100 mM, 250 mM, 500 mM or 1000 mM) in 10 mM Tris at pH 7.0 and 25 °C were prepared, in some instances adding 1% (v/v) Triton X-100. Then, 1 g of ionically exchanged RML was incubated in 10 mL of these solutions. After 2 h, the incubated biocatalysts were vacuum filtered employing a sintered glass funnel, washed 4 times utilizing 20 mL of the same incubation solution per gram of biocatalysts. The activities of the supernatant before and after the enzyme desorption were measured utilizing the *p*-NPB assay described above. The washed biocatalysts were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as detailed below.

2.2.5. *SDS-PAGE of the immobilized enzymes*

SDS-PAGE analyses were carried out following the method described by Laemmli [118]. The concentration gel was prepared utilizing 5% polyacrylamide, while the resolution gel was obtained using 12% polyacrylamide. After preparing the rupture buffer (8% (w/v) SDS and 10% mercaptoethanol (v/v)), the immobilized enzymes were added (calculating a maximum final protein concentration of 0.25–0.3 mg of protein per mL of solution). This suspension was boiled for 8 min, and later, they were centrifuged. This protocol releases the no-covalently enzyme molecules presented in the support [77]. Finally, 8 μL of low molecular weight (LMW) marker and 15 μL samples of the supernatants were loaded in the gel. A current of 100 V was employed in the experiments. The gels were tainted with Coomassie brilliant blue.

2.2.6. *Thermal inactivations of the lipase biocatalysts*

Biocatalysts were incubated in 10 mM Tris at pH 7.0. The pH was settled at 25 °C, thus some variations in the actual pH value could occur. However, this is not relevant for our objectives. Tris was employed to prevent the destabilization of immobilized lipase promoted by the presence of phosphate anions [119,120]. Periodically, aliquots of the inactivation suspensions were taken and their remaining activities were determined utilizing the *p*NPB method detailed above. This value was expressed taking the biocatalysts initial activity as 100%.

2.2.7. *Reuses of the most stable enzyme to build new combi-lipase biocatalyst: Cycles of inactivation, release of the inactivated least stable enzyme, coimmobilization of a new batch of the least stable enzyme and reusing the most stable and immobilized enzyme*

After the inactivation of the least stable lipase at 53 °C in 10 mM Tris at pH 7.0, the biocatalysts were incubated in 10 mM Tris containing 500 mM ammonium sulfate and 1% Triton X-100 (1 g of biocatalyst in 10 mL of desorption mixture), washed 4 folds with 10 volumes of this solution and 50 times with 10 volumes of distilled water to eliminate the detergent [90]. Then, a fresh batch of lipase was coimmobilized. The cycle of RML immobilization, incubation at 53 °C, and release of the partially inactivated RML was repeated five times, reutilizing the immobilized CALB.

2.2.8. *Irreversible inhibition by D-pNPP of immobilized CALB*

The biocatalyst was suspended in 100 mM sodium phosphate at pH 7.0 and 25 °C in a relation 1:20. To have an inactive CALB biocatalyst, solid D-*p*NPP was added to have a concentration of 30 mM [121]. Each 15 min, new batches of D-*p*NPP were added to reduce the residual activity of the biocatalysts under 5%. Next, the biocatalyst containing an irreversibly inhibited enzyme was washed with abundant distilled water to eliminate all of D-*p*NPP.

3. Results and discussion

3.1. *Immobilization of CALB and RML on vinyl sulfone activates agarose beads*

CALB was immobilized on vinyl sulfone agarose beads (Fig. 1A), with detergent to prevent its immobilization via interfacial activation on the support, as previously described [116]. Using a ratio of 1 g of support per 10 mL of enzyme solution, the immobilization was very slow, that way 1 g of support per 3 mL of enzyme was used (Fig. 1B). All enzyme activity was immobilized after 24 h and it was maintained fairly close to the initial activity. Fig. 2 shows that all CALB molecules were covalently immobilized on the support.

However, RML is not immobilized on this support, even in absence of detergent (Fig. 3A). This result agrees with previous trials of immobilizing this enzyme on this support [117]. That way, this support seems to be not valid to coimmobilize both enzymes. However, it was possible to rapidly adsorb RML on the support blocked with ethylendiamine via anion exchange. Moreover, this promoted an increment of RML activity of 2.5 folds (Fig. 3B).

3.2. *Characterization of the thermal stability of immobilized CALB and RML*

The inactivation courses of both immobilized biocatalysts at pH 7.0 and different temperatures are shown in Fig. 4. RML ionically exchanged on ethylendiamine blocked support was much less stable than CALB covalently immobilized on vinyl sulfone. Both preparations hardly became inactivated at 50 °C (Fig. 4A), while at 53 °C CALB maintained 90% of the initial activity after 2 h, and RML was almost fully inactivated after only 30 min (Fig. 4B). At 60 °C (Fig. 4C), RML was inactivated in only 15 min, but CALB also lost a significant percentage of the initial activity (CALB immobilized on octyl agarose remained fully active under these conditions). That way, 53 °C and pH 7.0 were conditions where CALB was almost fully active while RML became almost fully inactivated were found.

This situation makes this case a good model case for our coimmobilization strategy, aiming to reuse the most stable coimmobilized lipase.

3.3. *Release of RML from ethylendiamine activated agarose beads*

To really be able to reuse the immobilized CALB to build a new combi-lipase, it was necessary to find mild conditions where RML could be released from the aminated supports. RML immobilization on the aminated support was mainly due to ion exchange. That way, we tried to release the enzyme from the support using an increasing concentration of ammonium sulfate. This reagent has been shown to even stabilize these lipases, making unnecessary to consider a negative effect of this compound on the activity of immobilized CALB, while sodium phosphate presented negative effects [119,120]. This permitted to release (Fig. 5) about 85% of the enzyme using 500 mM ammonium sulfate. This value was not improved when using 1 M ammonium sulfate. The SDS-PAGE analysis of the biocatalysts revealed very tiny RML proteins bands even using 1 M ammonium sulfate (Fig. 6). The SDS-PAGE shows that RML (of around 31 kDa) is immobilized via ion exchange with one contaminant of the commercial extract of higher molecular size.

This could be caused by the hydrophobicity of the vinyl sulfone arm, which could permit some interactions with the enzyme [116]. For this reason, 1% (v/v) of Triton X-100 was added to the desorption mixture. Fig. 5 shows that now, almost 100% of the RML activity could be released from the support, and Fig. 7 confirms that there are not even

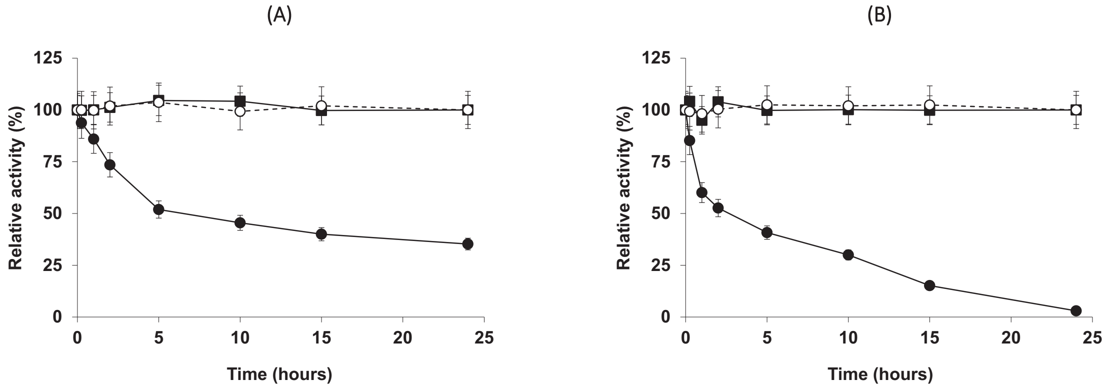

Fig. 1. Immobilization of CALB on vinyl sulfone agarose beads. The immobilization was performed in 10 mM sodium carbonate and 0.3% (v/v) Triton X-100 at 25 °C and pH 10.0. The support/enzyme solution was 1/10 (A) or 1/3 (B). Reference solution: Open circles and dotted line; Suspension: Solid squares and solid line; Supernatant: Solid circles and solid lines. Other specifications are described in Methods.

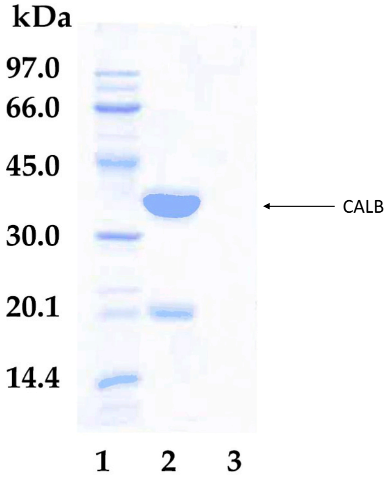

Fig. 2. SDS-PAGE of CALB immobilized in vinyl sulfone agarose beads to check the covalent immobilization. Experiments were performed as described in Methods. Lane 1: LMW markers; Lane 2: Free CALB; Lane 3: Immobilized CALB.

small traces of RML remained on the support (there is not bands of RML on the SDS-PAGE). That way, all the pieces required to develop our strategy seemed to work. Next, we try to build a combi-lipase immobilized biocatalyst where CALB could be reused after RML inactivation.

3.4. Building of the combi-lipase

CALB was immobilized on vinyl sulfone agarose beads (see Fig. 1) for 24 h and then incubated for 72 h, to be finally blocked with 2 M ethylendiamine (this had scarce effect on the enzyme activity, that remained close to 95% of the initially offered to the support). Next, RML was immobilized via anion exchange on the CALB biocatalysts (see Fig. 3). Fig. 8 shows the whole preparation of the combi-biocatalyst, measured using *p*NPB as substrate. Even after hyperactivation, the contribution of RML to the activity of the combi-lipase was lower than that of CALB (50 U per g of biocatalyst versus 30 U per g of biocatalyst, for a total of 80 U per g of biocatalyst). Together with this combi-biocatalyst, a CALB-vinyl sulfone biocatalyst was prepared. Then, a portion of this biocatalyst was inhibited as described in methods and RML was immobilized via ion exchange on it. Both individual biocatalysts were employed as "reference" of the enzyme behavior and submitted exactly to the same protocol than the combi-biocatalyst.

3.5. Reuse of immobilized CALB to build a new combi-biocatalyst after RML inactivation

Next, the combi-biocatalyst was incubated at 53 °C and pH 7.0 (Fig. 9). Initially, the activity decreased quite rapidly, to around 60% after 15 min, and then remained almost unaltered. Focusing the attention on the inactivation of the individual biocatalysts (Fig. 10), 10% of the activity of CALB decreased in 15 min and then remained unaltered.

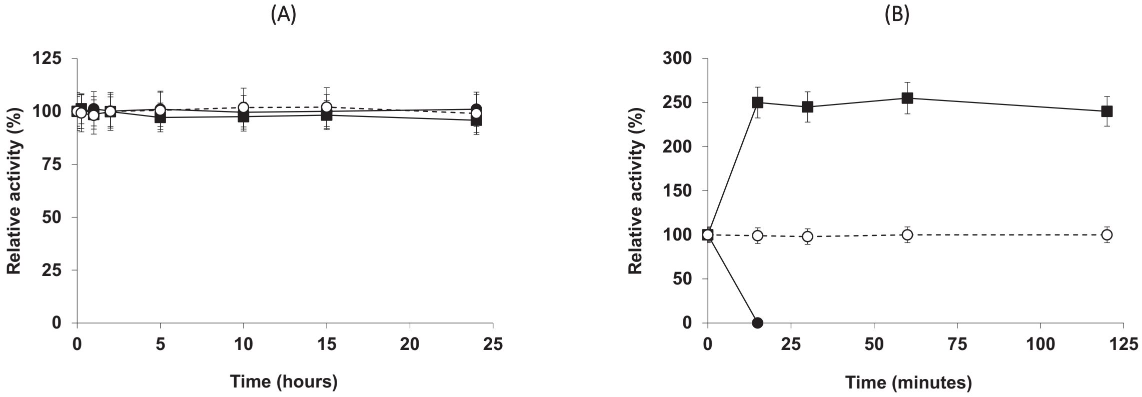

Fig. 3. Immobilization of RML on vinyl sulfone agarose beads (A) or vinyl sulfone agarose beads blocked with ethylendiamine (B). Reference solution: Open circles and dotted line; Suspension: Solid squares and solid line; Supernatant: Solid circles and solid lines. Experiments were carried out as described in Methods. (A): The immobilization was performed in 10 mM sodium carbonate at 25 °C and pH 9.0, using a ratio of support to enzyme solution 1/10. (B): The immobilization was performed in 10 mM sodium phosphate at 25 °C and pH 7.0, using a ratio of support to enzyme solution 1/10.

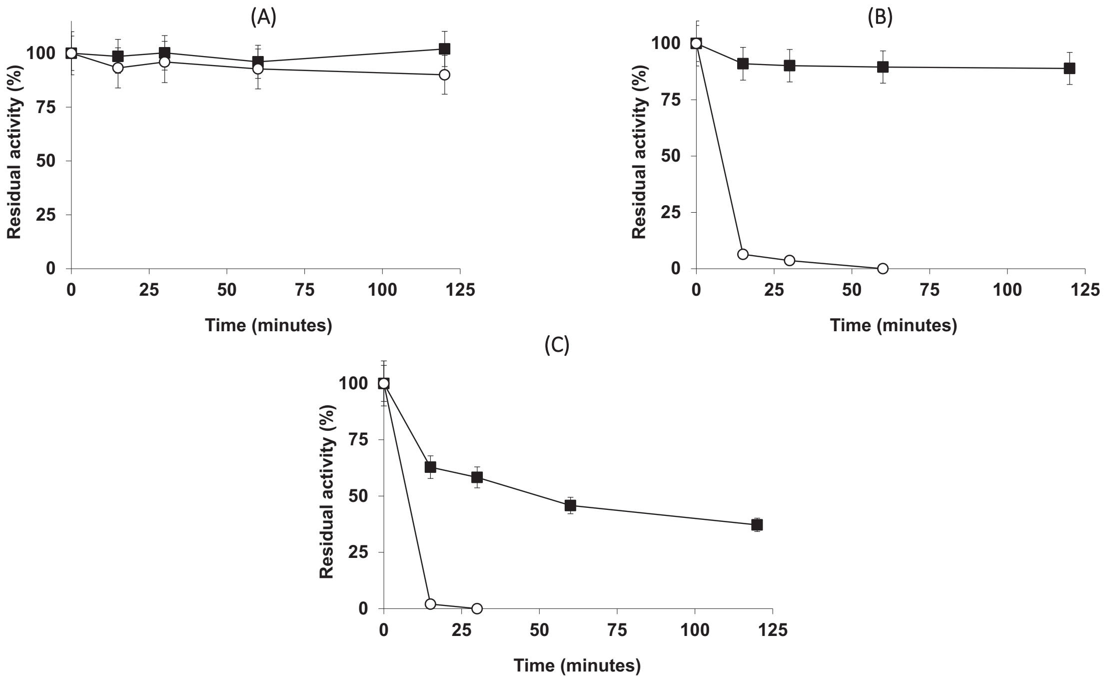

Fig. 4. Thermal inactivation courses of immobilized CALB and RML in 10 mM Tris at pH 7.0 and 50 (A), 53 (B) or 60 (C) °C. Experiments were performed as described in Methods. Solid squares: CALB covalently immobilized on vinyl sulfone, incubated for 72 h and blocked with ethylendiamine. Open circles: RML immobilized on ethylendiamine blocked vinyl sulfone agarose beads.

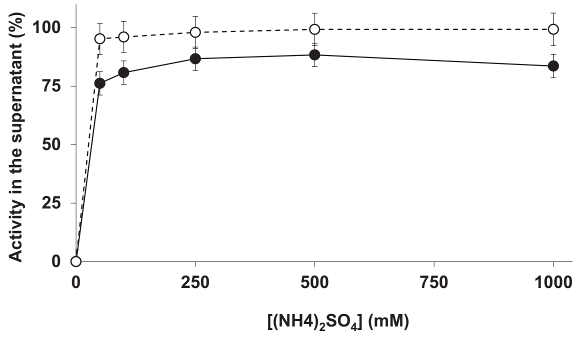

Fig. 5. Desorption of RML from ethylendiamine blocked vinyl sulfone agarose beads. The immobilized enzyme was incubated in growing concentrations of ammonium sulfate [(NH4)$_2$SO$_4$] in the absence (solid circles, solid lines) or presence (open circles, dotted lines) of 1% (v/v) Triton X-100. 100% is the initial activity of the immobilized enzyme. Other specifications are described in Methods.

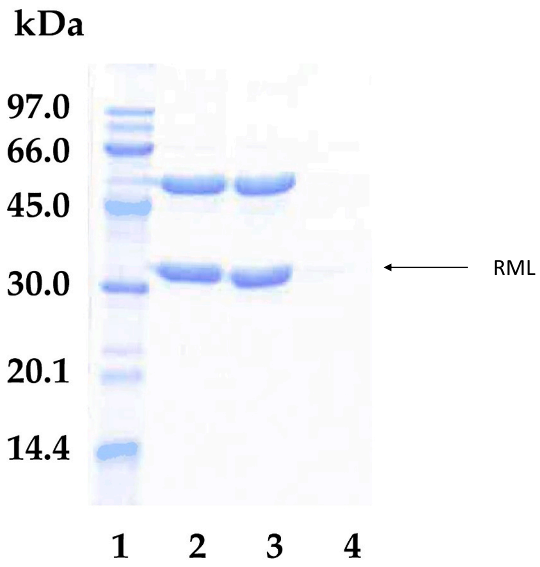

Fig. 6. SDS-PAGE of RML immobilized on ethylendiamine blocked vinyl sulfone agarose beads after incubation and washing with different ammonium sulfate concentration in 10 mM Tris at pH 7.0 and 25 °C. Experiments were carried out as described in Methods. Lane 1: LMW markers; Lane 2: Initial RML biocatalyst; Lane 3: Protein remaining in the biocatalysts after incubation and washing with 10 mM Tris; Lane 4: Protein remaining in the biocatalysts after incubation and washing with 1 M ammonium sulfate.

As expected, more than 90% of the activity of RML was lost after 15 min under that conditions (Fig. 10). After incubation in ammonium sulfate solution and washing to eliminate RML, the CALB biocatalyst recovered almost 100% of the initial activity. This could be just the effect of incubating the biocatalysts under milder conditions, that enable enzyme recovery of the initial conformation (as it has been previously reported) [28].

As expected, the RML biocatalyst activity became 0 (Fig. 10) after incubation and washing, and the activity of the washed combi-biocatalyst fits that of the individual CALB (Figs. 9 and 10). Then, a fresh batch of RML was immobilized on the combi-lipase (both, those having active CALB and inhibited CALB), recovering the initial activity (Figs. 9 and 10). This was repeated for 5 cycles, with very similar results the activity of the combi-biocatalyst and the individual biocatalysts, suggesting that the employed protocol permitted to eliminate all detergent molecules and that the presence of RML molecules have no effect on the stability/activity of immobilized CALB (the used enzyme loading was reduced to prevent effect of enzyme-enzyme interactions) [32,122,123]. The final activity of the CALB biocatalyst after the last cycle was higher than 90% of the initial value (both, using the individual and the recovered combi-lipase biocatalysts) (Figs. 9 and 10), and the last combi-biocatalyst presented an activity fairly similar to the initial one (Fig. 9).

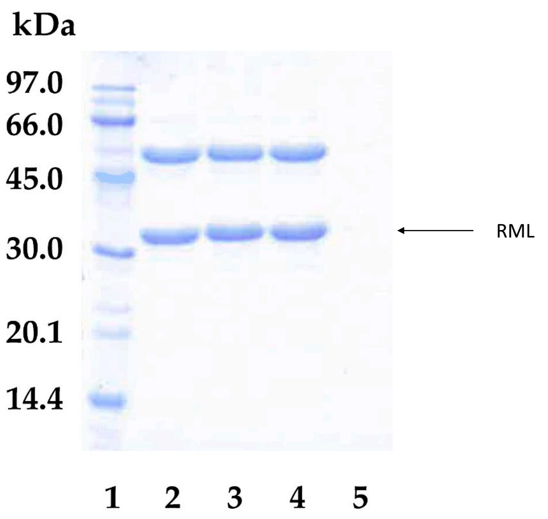

Fig. 7. SDS-PAGE of RML immobilized on ethylendiamine blocked vinyl sulfone agarose beads after incubation and washing with different ammonium sulfate concentrations in 10 mM Tris/ 1% (v/v) Triton X-100 at pH 7.0 and 25 °C. Experiments were carried out as described in Methods. Lane 1: LMW markers; Lane 2: Initial RML biocatalyst; Lane 3: Protein remaining in the biocatalysts after incubation and washing with 10 mM Tris; Lane 4: Protein remaining in the biocatalysts after incubation and washing with 10 mM Tris/ 1% Triton X-100, Lane 5: Protein remaining in the biocatalysts after incubation and washing with 0.5 M ammonium sulfate/ 1% Triton X-100.

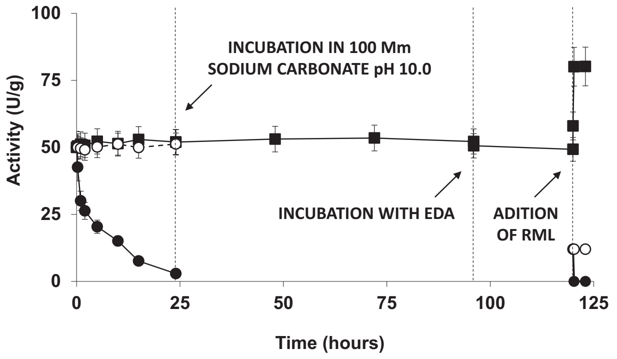

Fig. 8. Preparation of the combi-biocatalysts. Activity evolution in the different steps of the enzyme per enzyme coimmobilization of CALB and RML (including CALB immobilization on vinyl sulfone agarose beads (24 h), long term incubation at pH 10.0 and 25 °C during 72 h, blocking with 2 M of ethylendiamine (EDA) for 24 h and ionic adsorption of RML). Each step is indicated by an arrow. Other specification may be found in Methods. Dotted lines, open circles: Enzymes reference solutions; Solid lines, solid squares: Suspension; Solid lines, solid circles: supernatants.

That way, the CALB could be reused to prepare a new combi-lipase for 5 cycles of RML inactivation, release and re-immobilization, with very high recovered activity.

4. Conclusion

This strategy of enzyme by enzyme immobilization on vinyl sulfone supports may be a good solution for other enzymes that must be coimmobilized and cannot be performed using this protocol. This strategy is summarized in Fig. 11. More interestingly, it becomes an example of the problems generated by the coimmobilization of enzyme presenting very different stability, and it is a way to reuse the most stable enzyme to build new combi-biocatalysts after inactivation of the least stable enzymes.

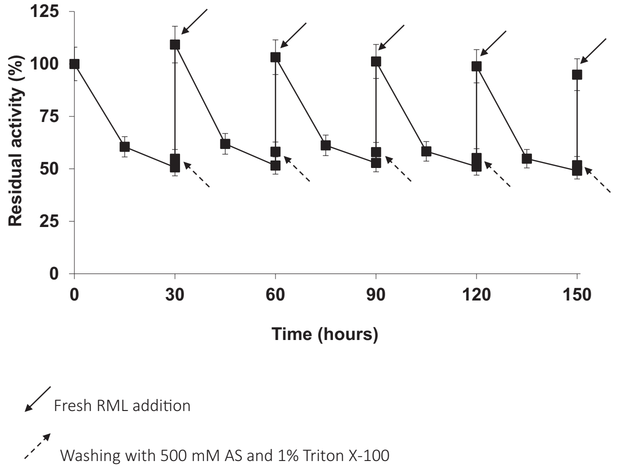

Fig. 9. Reuses of immobilized CALB in the CALB/RML coimmobilized biocatalyst. The figure shows its activity evolution when submitted to diverse cycles of thermal inactivations at 53 °C and pH 7.0, release of RML by incubation and washing with 0.5 M ammonium sulfate (AS)/ 1% (v/v) Triton X-100 in 10 mM Tris at pH 7.0 and 25 °C, washing with water to eliminate the detergent and coimmobilization of a new batch of fresh RML. Each cycle is marked with a dotted arrow. Experiments were carried out as described in Methods.

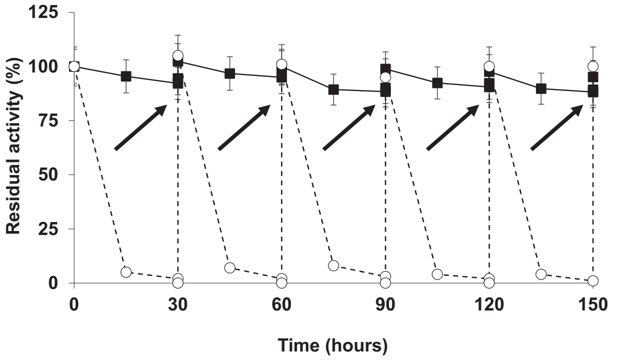

Fig. 10. Effect of the full inactivation and recycling treatments on the activity of individually immobilized biocatalyst of RML and CALB. The figure shows the activity evolution of CALB immobilized in vinyl sulfone (solid line, solid squares) and RML coimmobilized with inhibited CALB (dotted line, open circles). Both were submitted to diverse cycles of thermal inactivations at 53 °C and pH 7.0, incubation and washing with 0.5 M ammonium sulfate/ 1% (v/v) Triton X-100 in 10 mM Tris at pH 7.0 and 25 °C and washing with water to eliminate the detergent. Each cycle is marked with an arrow. In the case of RML, in each cycle a new batch of fresh RML was immobilized. Experiments were carried out as described in Methods.

This paper shows an example of the coimmobilization of two enzymes on vinyl sulfone agarose beads, when one of the enzymes cannot be immobilized on it. To reach this goal, one of the enzymes was covalently immobilized, and after blocking the support with ethylendiamine, and converting the support in an anion exchanger, the other enzyme was immobilized on the support. The covalently immobilized CALB was far more stable than the ionically exchanged RML, that way the combi-lipase biocatalyst should be discarded when 90% of the CALB activity was maintained. The release of the inactivated RML with ammonium sulfate and detergent permitted to recover an immobilized CALB biocatalyst almost identical to the first CALB biocatalysts, and the coimmobilization of a fresh batch of RML permitted to recover an almost identical combi-biocatalyst. Together to a model example of the potential of this support to be use in the solution of the problem of coimmobilization of enzymes with different stability, as commented in introduction, the prepared biocatalyst can have application in the

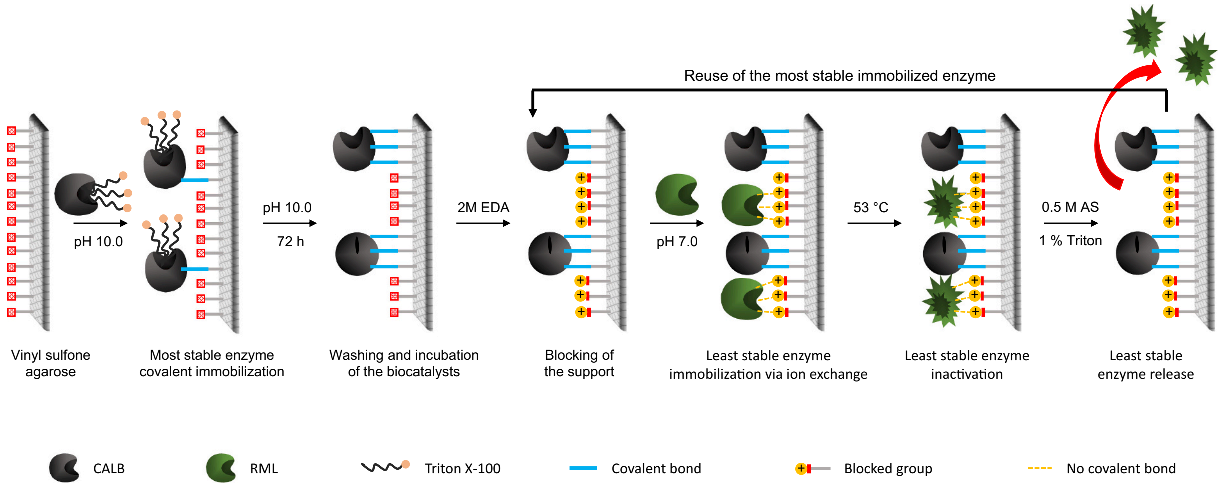

Fig. 11. Schematic representation of the strategy developed in this paper to solve the problem of the coimmobilization of enzymes presenting very different stabilities.

production of free fatty acids from oil hydrolysis, or of biodiesel, biolubricants or biosurfactants from oils of free fatty acids, as all of these process involve heterogeneous substrates really composed of many different components, and the enzyme specificity may be a problem for their full conversion.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] K.-E. Jaeger, T. Eggert, Lipases for biotechnology, Curr. Opin. Biotechnol. 13 (4) (2002) 390–397, https://doi.org/10.1016/S0958-1669(02)00341-5.
[2] M.T. Reetz, Lipases as practical biocatalysts, Curr. Opin. Chem. Biol. 6 (2) (2002) 145–150, https://doi.org/10.1016/S1367-5931(02)00297-1.
[3] A.R. Ismail, H. Kashtoh, K.H. Baek, Temperature-resistant and solvent-tolerant lipases as industrial biocatalysts: Biotechnological approaches and applications, Int. J. Biol. Macromol. 187 (2021) 127–142, https://doi.org/10.1016/j.ijbiomac.2021.07.101.
[4] S.H. Hamdan, J. Maiangwa, M.S.M. Ali, Y.M. Normi, S. Sabri, T.C. Leow, Thermostable lipases and their dynamics of improved enzymatic properties, Appl. Microbiol. Biotechnol. 105 (19) (2021) 7069–7094, https://doi.org/10.1007/s00253-021-11520-7.
[5] R. Ferraccioli, Progress on the stereoselective synthesis of chiral molecules based on metal-catalyzed dynamic kinetic resolution of alcohols with lipases, Symmetry (Basel) 13 (9) (2021) 1744, https://doi.org/10.3390/sym13091744.
[6] R.D. Schmid, R. Verger, Lipases: Interfacial enzymes with attractive applications, Ang. Chem. 37 (1998) 1608–1633, https://doi.org/10.1002/(sici)1521-3757(19980619)110:12<1694::aid-ange1694>3.3.co;2-v.
[7] R.R. Sousa, A.S. Silva, R. Fernandez-Lafuente, V.S. Ferreira-Leitão, Solvent-free esterifications mediated by immobilized lipases: a review from thermodynamic and kinetic perspectives, Catal, Sci. Technol. 11 (17) (2021) 5696–5711.
[8] H. Zhao, G.A. Baker, S. Holmes, New eutectic ionic liquids for lipase activation and enzymatic preparation of biodiesel, Org. Biomol. Chem. 9 (2011) 1908–1916, https://doi.org/10.1039/c0ob01011a.
[9] Q. Wang, M. Ge, X. Guo, Z. Li, A. Huang, F.u. Yang, R. Guo, Hydrophobic poly (ionic liquid)s as “two-handed weapons”: Maximizing lipase catalytic efficiency in transesterification of soybean oil toward biodiesel, Appl. Catal. A Gen. 626 (2021) 118350, https://doi.org/10.1016/j.apcata.2021.118350.
[10] P. Lozano, T. De Diego, D. Carrié, M. Vaultier, J.L. Iborra, Over-stabilization of *Candida antarctica* lipase B by ionic liquids in ester synthesis, Biotechnol. Lett. 23 (2001) 1529–1533, https://doi.org/10.1023/A:1011697609756.
[11] D.F. Izquierdo, J.M. Bernal, M.I. Burguete, E. García-Verdugo, P. Lozano, S. V. Luis, An efficient microwave-assisted enzymatic resolution of alcohols using a lipase immobilised on supported ionic liquid-like phases (SILLPs), RSC Adv. 3 (2013) 13123–13126, https://doi.org/10.1039/c3ra42467g.
[12] P. Lozano, T. De Diego, D. Carrié, M. Vaultier, J.L. Iborra, Enzymatic catalysis in ionic liquids and supercritical carbon dioxide, ACS Symp. Ser. 856 (2003) 239–250, https://doi.org/10.1021/bk-2003-0856.ch020.
[13] A. Ghanem, Trends in lipase-catalyzed asymmetric access to enantiomerically pure/enriched compounds, Tetrahedron. 63 (8) (2007) 1721–1754, https://doi.org/10.1016/j.tet.2006.09.110.
[14] M. Kapoor, M.N. Gupta, Lipase promiscuity and its biochemical applications, Process Biochem. 47 (4) (2012) 555–569, https://doi.org/10.1016/j.procbio.2012.01.011.
[15] P. Reis, K. Holmberg, H. Watzke, M.E. Leser, R. Miller, Lipases at interfaces: a review, Adv. Colloid Interface Sci. 147–148 (2009) 237–250, https://doi.org/10.1016/j.cis.2008.06.001.
[16] J.M. Palomo, C. Ortiz, M. Fuentes, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Use of immobilized lipases for lipase purification via specific lipase-lipase interactions, J. Chromatogr. A. 1038 (1-2) (2004) 267–273, https://doi.org/10.1016/j.chroma.2004.03.058.
[17] J.M. Palomo, C. Ortiz, G. Fernández-Lorente, M. Fuentes, J.M. Guisán, R. Fernández-Lafuente, Lipase-lipase interactions as a new tool to immobilize and modulate the lipase properties, Enzyme Microb. Technol. 36 (4) (2005) 447–454, https://doi.org/10.1016/j.enzmictec.2004.09.013.
[18] J.M. Palomo, M.M. Peñas, G. Fernández-Lorente, C. Mateo, A.G. Pisabarro, R. Fernández-Lafuente, L. Ramírez, J.M. Guisán, Solid-phase handling of hydrophobins: Immobilized hydrophobins as a new tool to study lipases, Biomacromolecules 4 (2003) 204–210, https://doi.org/10.1021/bm020071l.
[19] P. Wang, J. He, Y. Sun, M. Reynolds, L.i. Zhang, S. Han, S. Liang, H. Sui, Y. Lin, Display of fungal hydrophobin on the Pichia pastoris cell surface and its influence on *Candida antarctica* lipase B, Appl. Microbiol. Biotechnol. 100 (13) (2016) 5883–5895, https://doi.org/10.1007/s00253-016-7431-x.
[20] E. Subroto, S. Nurhasanah, S. Joni Munarso, Lipase immobilization by adsorption techniques on the hydrophobically modified matrix: a review, Int. J. Eng. Trends Technol. 69 (2021) 49–55. https://doi.org/10.14445/22315381/IJETT-V69I1P208.
[21] N.F. Mokhtar, R.N.Z.R. Raja Noor Zaliha, N.D. Muhd Noor, F. Mohd Shariff, M. S. Mohamad Ali, The immobilization of lipases on porous support by adsorption and hydrophobic interaction method, Catalysts 10 (7) (2020) 744, https://doi.org/10.3390/catal10070744.
[22] R.C. Rodrigues, J.J. Virgen-Ortíz, J.C.S. dos Santos, Á. Berenguer-Murcia, A. R. Alcantara, O. Barbosa, C. Ortiz, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions, Biotechnol. Adv. 37 (5) (2019) 746–770, https://doi.org/10.1016/j.biotechadv.2019.04.003.
[23] P.K. Sharma, G. Vinodh Kumar, J. Kaur, Common methods employed in directed evolution and their application in modification of lipases, Res. J. Pharm. Biol. Chem. Sci. 3 (2012) 183–191.
[24] M. Wang, T. Si, H. Zhao, Biocatalyst development by directed evolution, Bioresour. Technol. 115 (2012) 117–125, https://doi.org/10.1016/j.biortech.2012.01.054.

[25] K. Engström, J. Nyhlén, A.G. Sandström, J.-E. Bäckvall, Directed evolution of an enantioselective lipase with broad substrate scope for hydrolysis of α-substituted esters, J. Am. Chem. Soc. 132 (20) (2010) 7038–7042, https://doi.org/10.1021/ja100593j.

[26] M. Ruiz, M. Galvis, O. Barbosa, C. Ortiz, R. Torres, R. Fernandez-Lafuente, Solid-phase modification with succinic polyethyleneglycol of aminated lipase B from *Candida antarctica*: effect of the immobilization protocol on enzyme catalytic properties, J. Mol. Catal. B Enzym. 87 (2013) 75–82, https://doi.org/10.1016/j.molcatb.2012.10.012.

[27] M. Galvis, O. Barbosa, R. Torres, C. Ortiz, R. Fernandez-Lafuente, Effect of solid-phase chemical modification on the features of the lipase from *Thermomyces lanuginosus*, Process Biochem. 47 (3) (2012) 460–466, https://doi.org/10.1016/j.procbio.2011.12.001.

[28] P.M.P. Souza, D. Carballares, N. Lopez-Carrobles, L.R.B. Gonçalves, F. Lopez-Gallego, S. Rodrigues, R. Fernandez-Lafuente, Enzyme-support interactions and inactivation conditions determine *Thermomyces lanuginosus* lipase inactivation pathways: Functional and florescence studies, Int. J. Biol. Macromol. 191 (2021) 79–91, https://doi.org/10.1016/j.ijbiomac.2021.09.061.

[29] E. Abreu Silveira, S. Moreno-Perez, A. Basso, S. Serban, R. Pestana-Mamede, P. W. Tardioli, C.S. Farinas, N. Castejon, G. Fernandez-Lorente, J. Rocha-Martin, J. M. Guisan, Biocatalyst engineering of *Thermomyces Lanuginosus* lipase adsorbed on hydrophobic supports: modulation of enzyme properties for ethanolysis of oil in solvent-free systems, J. Biotechnol. 289 (2019) 126–134, https://doi.org/10.1016/j.jbiotec.2018.11.014.

[30] S. Arana-Peña, N.S. Rios, D. Carballares, L.R.B. Gonçalves, R. Fernandez-Lafuente, Immobilization of lipases via interfacial activation on hydrophobic supports: Production of biocatalysts libraries by altering the immobilization conditions, Catal. Today 362 (2021) 130–140, https://doi.org/10.1016/j.cattod.2020.03.059.

[31] J.R. Guimarães, L.P. Miranda, R. Fernandez-lafuente, P.W. Tardioli, Immobilization of eversa ® transform via CLEA technology using waste cooking oil, Molecules 23 (2021) 193.

[32] S. Arana-Peña, N.S. Rios, D. Carballares, C. Mendez-Sanchez, Y. Lokha, L.R. B. Gonçalves, R. Fernandez-Lafuente, Effects of enzyme loading and immobilization conditions on the catalytic features of lipase from *Pseudomonas fluorescens* immobilized on octyl-agarose beads, Front. Bioeng. Biotechnol. 8 (2020), https://doi.org/10.3389/fbioe.2020.00036.

[33] V.G. Tacias-Pascacio, J.J. Virgen-Ortíz, M. Jiménez-Pérez, M. Yates, B. Torrestiana-Sanchez, A. Rosales-Quintero, R. Fernandez-Lafuente, Evaluation of different lipase biocatalysts in the production of biodiesel from used cooking oil: critical role of the immobilization support, Fuel 200 (2017) 1–10, https://doi.org/10.1016/j.fuel.2017.03.054.

[34] V.G. Tacias-Pascacio, S. Peirce, B. Torrestiana-Sanchez, M. Yates, A. Rosales-Quintero, J.J. Virgen-Ortíz, R. Fernandez-Lafuente, Evaluation of different commercial hydrophobic supports for the immobilization of lipases: tuning their stability, activity and specificity, RSC Adv. 6 (102) (2016) 100281–100294.

[35] X. Xu, Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review, Eur. J. Lipid Sci. Technol. 102 (2000) 287–303, https://doi.org/10.1002/(sici)1438-9312(200004)102:4<287::aid-ejlt287>3.3.co;2-h.

[36] A. Bajaj, P. Lohan, P.N. Jha, R. Mehrotra, Biodiesel production through lipase catalyzed transesterification: an overview, J. Mol. Catal. B Enzym. 62 (2010) 9–14, https://doi.org/10.1016/j.molcatb.2009.09.018.

[37] X. Zhao, F. Qi, C. Yuan, W. Du, D. Liu, Lipase-catalyzed process for biodiesel production: enzyme immobilization, process simulation and optimization, Renew. Sustain. Energy Rev. 44 (2015) 182–197, https://doi.org/10.1016/j.rser.2014.12.021.

[38] R.R.C. Monteiro, S. Arana-Peña, T.N. da Rocha, L.P. Miranda, Á. Berenguer-Murcia, P.W. Tardioli, J.C.S. dos Santos, R. Fernandez-Lafuente, Liquid lipase preparations designed for industrial production of biodiesel. Is it really an optimal solution? Renew. Energy 164 (2021) 1566–1587, https://doi.org/10.1016/j.renene.2020.10.071.

[39] A. de Carvalho Lima, L.N. de Torres, P.W. Lima, R. de Tardioli, S. Júnior, Mathematical modeling of enzymatic syntheses of biosurfactants catalyzed by immobilized lipases, React. Kinet. Mech. Catal. 130 (2020) 699–712, https://doi.org/10.1007/s11144-020-01812-w.

[40] M. Enayati, Y. Gong, J.M. Goddard, A. Abbaspourrad, Synthesis and characterization of lactose fatty acid ester biosurfactants using free and immobilized lipases in organic solvents, Food Chem. 266 (2018) 508–513, https://doi.org/10.1016/j.foodchem.2018.06.051.

[41] V.K. Recke, C. Beyrle, M. Gerlitzki, R. Hausmann, C. Syldatk, V. Wray, H. Tokuda, N. Suzuki, S. Lang, Lipase-catalyzed acylation of microbial mannosylerythritol lipids (biosurfactants) and their characterization, Carbohydr. Res. 373 (2013) 82–88, https://doi.org/10.1016/j.carres.2013.03.013.

[42] N.S.A. Wafti, R. Yunus, H.L.N. Lau, T.C.S. Yaw, S.A. Aziz, Immobilized lipase-catalyzed transesterification for synthesis of biolubricant from palm oil methyl ester and trimethylolpropane, Bioprocess Biosyst. Eng. 44 (11) (2021) 2429–2444, https://doi.org/10.1007/s00449-021-02615-6.

[43] K.V. Fernandes, E.D.C. Cavalcanti, E.P. Cipolatti, E.C.G. Aguieiras, M.C.C. Pinto, F.A. Tavares, P.R. da Silva, R. Fernandez-Lafuente, S. Arana-Peña, J.C. Pinto, C.L. B. Assunção, J.A.C. da Silva, D.M.G. Freire, Enzymatic synthesis of biolubricants from by-product of soybean oil processing catalyzed by different biocatalysts of *Candida rugosa* lipase, Catal. Today 362 (2021) 122–129, https://doi.org/10.1016/j.cattod.2020.03.060.

[44] I.C.A. Bolina, R.A.B. Gomes, A.A. Mendes, Biolubricant production from several oleaginous feedstocks using lipases as catalysts: current scenario and future perspectives, Bioenergy Res. 14 (4) (2021) 1039–1057, https://doi.org/10.1007/s12155-020-10242-4.

[45] E.D.C. Cavalcanti, É.C.G. Aguieiras, P.R. da Silva, J.G. Duarte, E.P. Cipolatti, R. Fernandez-Lafuente, J.A.C. da Silva, D.M.G. Freire, Improved production of biolubricants from soybean oil and different polyols via esterification reaction catalyzed by immobilized lipase from *Candida rugosa*, Fuel 215 (2018) 705–713, https://doi.org/10.1016/j.fuel.2017.11.119.

[46] F.A.P. Lage, J.J. Bassi, M.C.C. Corradini, L.M. Todero, J.H.H. Luiz, A.A. Mendes, Preparation of a biocatalyst via physical adsorption of lipase from *Thermomyces lanuginosus* on hydrophobic support to catalyze biolubricant synthesis by esterification reaction in a solvent-free system, Enzyme Microb. Technol. 84 (2016) 56–67, https://doi.org/10.1016/j.enzmictec.2015.12.007.

[47] S. Arana-Peña, D. Carballares, Á. Berenguer-Murcia, A.R. Alcántara, R. C. Rodrigues, R. Fernandez-Lafuente, One pot use of combilipases for full modification of oils and fats: multifunctional and heterogeneous substrates, Catalysts 10 (2020) 605, https://doi.org/10.3390/catal10060605.

[48] C.P. Passos, S. Yilmaz, C.M. Silva, M.A. Coimbra, Enhancement of grape seed oil extraction using a cell wall degrading enzyme cocktail, Food Chem. 115 (2009) 48–53, https://doi.org/10.1016/j.foodchem.2008.11.064.

[49] J.H. Lee, S.B. Kim, S.W. Kang, Y.S. Song, C. Park, S.O. Han, S.W. Kim, Biodiesel production by a mixture of *Candida rugosa* and *Rhizopus oryzae* lipases using a supercritical carbon dioxide process, Bioresour. Technol. 102 (2) (2011) 2105–2108, https://doi.org/10.1016/j.biortech.2010.08.034.

[50] D.H. Lee, J.M. Kim, H.Y. Shin, S.W. Kang, S.W. Kim, Biodiesel production using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases, Biotechnol, Bioprocess Eng. 11 (6) (2006) 522–525, https://doi.org/10.1007/BF02932077.

[51] K. Tongboriboon, B. Cheirsilp, A.H. Kittikun, Mixed lipases for efficient enzymatic synthesis of biodiesel from used palm oil and ethanol in a solvent-free system, J. Mol. Catal. B Enzym. 67 (2010) 52–59, https://doi.org/10.1016/j.molcatb.2010.07.005.

[52] J.S. Alves, N.S. Vieira, A.S. Cunha, A.M. Silva, M.A. Záchia Ayub, R. Fernandez-Lafuente, R.C. Rodrigues, Combi-lipase for heterogeneous substrates: a new approach for hydrolysis of soybean oil using mixtures of biocatalysts, RSC Adv. 4 (14) (2014) 6863–6868, https://doi.org/10.1039/C3RA45969A.

[53] F. Guan, P.u. Peng, G. Wang, T. Yin, Q. Peng, J. Huang, G. Guan, Y. Li, Combination of two lipases more efficiently catalyzes methanolysis of soybean oil for biodiesel production in aqueous medium, Process Biochem. 45 (10) (2010) 1677–1682, https://doi.org/10.1016/j.procbio.2010.06.021.

[54] Y. Liu, Y. Yan, F. Hu, An'na Yao, Z. Wang, F. Wei, Transesterification for biodiesel production catalyzed by combined lipases: optimization and kinetics, AIChE J. 56 (6) (2010) 1659–1665, https://doi.org/10.1002/aic.12090.

[55] S. Cesarini, R. Haller, P. Diaz, P. Nielsen, Combining phospholipases and a liquid lipase for one-step biodiesel production using crude oils, Biotechnol. Biofuels 7 (2014) 29, https://doi.org/10.1186/1754-6834-7-29.

[56] J.H. Lee, D.H. Lee, J.S. Lim, B.H. Um, C. Park, S.W. Kang, S.W. Kim, Optimization of the process for biodiesel production using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases, J. Microbiol. Biotechnol. 18 (2008) 1927–1931.

[57] F. Su, G.-L. Li, Y.-L. Fan, Y.-J. Yan, Enhancing biodiesel production via a synergic effect between immobilized *Rhizopus oryzae* lipase and Novozym 435, Fuel Process. Technol. 137 (2015) 298–304, https://doi.org/10.1016/j.fuproc.2015.03.013.

[58] J.K. Poppe, C.R. Matte, R. Fernandez-Lafuente, R.C. Rodrigues, M.A.Z. Ayub, Transesterification of waste frying oil and soybean oil by combi-lipases under ultrasound-assisted reactions, Appl. Biochem. Biotechnol. 186 (3) (2018) 576–589, https://doi.org/10.1007/s12010-018-2763-x.

[59] N.A. Ibrahim, Z. Guo, X. Xu, Enzymatic interesterification of palm stearin and coconut oil by a dual lipase system, J. Am. Oil Chem. Soc. 85 (1) (2008) 37–45, https://doi.org/10.1007/s11746-007-1157-y.

[60] J. Amoah, S.-H. Ho, S. Hama, A. Yoshida, A. Nakanishi, T. Hasunuma, C. Ogino, A. Kondo, Lipase cocktail for efficient conversion of oils containing phospholipids to biodiesel, Bioresour. Technol. 211 (2016) 224–230, https://doi.org/10.1016/j.biortech.2016.03.062.

[61] J.H. Lee, S.B. Kim, C. Park, B. Tae, S.O. Han, S.W. Kim, Development of batch and continuous processes on biodiesel production in a packed-bed reactor by a mixture of immobilized *Candida rugosa* and *Rhizopus oryzae* lipases, Appl. Biochem. Biotechnol. 161 (1-8) (2010) 365–371, https://doi.org/10.1007/s12010-009-8829-z.

[62] Y.K. Park, G.M. Pastore, M.M. de Almeida, Hydrolysis of soybean oil by a combined lipase system, J. Am. Oil Chem. Soc. 65 (2) (1988) 252–254, https://doi.org/10.1007/BF02636410.

[63] H. Qiao, F. Zhang, W. Guan, J. Zuo, D. Feng, Optimisation of combi-lipases from *Aspergillus niger* for the synergistic and efficient hydrolysis of soybean oil, Anim. Sci. J. 88 (5) (2017) 772–780, https://doi.org/10.1111/asj.12718.

[64] E.C. Toro, D.F. Rodríguez, N. Morales, L.M. García, C.A. Godoy, Novel combi-lipase systems for fatty acid ethyl esters production, Catalysts 9 (2019) 546, https://doi.org/10.3390/catal9060546.

[65] K. Pedro, J. Parreira, I. Correia, C. Henriques, M. Langone, Enzymatic biodiesel synthesis from acid oil using a lipase mixture, Quim. Nova 41 (2017) 284–291. https://doi.org/10.21577/0100-4042.20170180.

[66] C.A. Araki, S.M.P. Marcucci, L.S. da Silva, C.H. Maeda, P.A. Arroyo, G.M. Zanin, Effects of a combination of lipases immobilised on desilicated and thiol-modified ZSM-5 for the synthesis of ethyl esters from macauba pulp oil in a solvent-free system, Appl. Catal. A Gen. 562 (2018) 241–249, https://doi.org/10.1016/j.apcata.2018.06.007.

[67] A. Sánchez-Bayo, V. Morales, R. Rodríguez, G. Vicente, L. Bautista, Biodiesel production (FAEEs) by heterogeneous combi-lipase biocatalysts using wet extracted lipids from microalgae, Catalysts 9 (2019) 296, https://doi.org/10.3390/catal9030296.

[68] L. Zeng, Y. He, L. Jiao, K. Li, Y. Yan, Preparation of biodiesel with liquid synergetic lipases from rapeseed oil deodorizer distillate, Appl. Biochem. Biotechnol. 183 (3) (2017) 778–791, https://doi.org/10.1007/s12010-017-2463-y.

[69] M.D. Ramos, L.P. Miranda, R. Fernandez-Lafuente, W. Kopp, P.W. Tardioli, Improving the yields and reaction rate in the ethanolysis of soybean oil by using mixtures of lipase CLEAs, Molecules 24 (2019) 4392, https://doi.org/10.3390/molecules24234392.

[70] S.K. Karmee, Preparation of biodiesel from nonedible oils using a mixture of used lipases, Energy Sour. Part A Recover. Util. Environ. Eff. 38 (18) (2016) 2727–2733, https://doi.org/10.1080/15567036.2015.1098748.

[71] J.H. Lee, S.B. Kim, H.Y. Yoo, J.H. Lee, S.O. Han, C. Park, S.W. Kim, Co-immobilization of *Candida rugosa* and *Rhyzopus oryzae* lipases and biodiesel production, Korean J. Chem. Eng. 30 (6) (2013) 1335–1338, https://doi.org/10.1007/s11814-013-0058-z.

[72] J.H. Lee, J.H. Lee, D.S. Kim, H.Y. Yoo, C. Park, S.W. Kim, Biodiesel production by lipases co-immobilized on the functionalized activated carbon, Bioresour. Technol. Rep. 7 (2019) 100248, https://doi.org/10.1016/j.biteb.2019.100248.

[73] J.H. Lee, S.B. Kim, H.Y. Yoo, J.H. Lee, C. Park, S.O. Han, S.W. Kim, Kinetic modeling of biodiesel production by mixed immobilized and co-immobilized lipase systems under two pressure conditions, Korean J. Chem. Eng. 30 (6) (2013) 1272–1276, https://doi.org/10.1007/s11814-013-0021-z.

[74] S. Arana-Peña, D. Carballares, R. Morellon-Sterlling, Á. Berenguer-Murcia, A. R. Alcántara, R.C. Rodrigues, R. Fernandez-Lafuente, Enzyme co-immobilization: always the biocatalyst designers' choice…or not? Biotechnol. Adv. 51 (2021) 107584, https://doi.org/10.1016/j.biotechadv.2020.107584.

[75] C. Garcia-Galan, Á. Berenguer-Murcia, R. Fernandez-Lafuente, R.C. Rodrigues, Potential of different enzyme immobilization strategies to improve enzyme performance, Adv. Synth. Catal. 353 (16) (2011) 2885–2904, https://doi.org/10.1002/adsc.201100534.

[76] P.V. Iyer, L. Ananthanarayan, Enzyme stability and stabilization—Aqueous and non-aqueous environment, Process Biochem. 43 (10) (2008) 1019–1032, https://doi.org/10.1016/j.procbio.2008.06.004.

[77] R.C. Rodrigues, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. Fernández-Lafuente, Modifying enzyme activity and selectivity by immobilization, Chem. Soc. Rev. 42 (15) (2013) 6290–6307.

[78] R.C. Rodrigues, Á. Berenguer-Murcia, D. Carballares, R. Morellon-Sterling, R. Fernandez-Lafuente, Stabilization of enzymes via immobilization: multipoint covalent attachment and other stabilization strategies, Biotechnol. Adv. 52 (2021) 107821, https://doi.org/10.1016/j.biotechadv.2021.107821.

[79] M. Bilal, T. Rasheed, Y. Zhao, H.M.N. Iqbal, J. Cui, "Smart" chemistry and its application in peroxidase immobilization using different support materials, Int. J. Biol. Macromol. 119 (2018) 278–290, https://doi.org/10.1016/j.ijbiomac.2018.07.134.

[80] S. Aggarwal, A. Chakravarty, S. Ikram, A comprehensive review on incredible renewable carriers as promising platforms for enzyme immobilization & thereof strategies, Int. J. Biol. Macromol. 167 (2021) 962–986, https://doi.org/10.1016/j.ijbiomac.2020.11.052.

[81] P.T. Anastas, A. Rodriguez, T.M. de Winter, P. Coish, J.B. Zimmerman, A review of immobilization techniques to improve the stability and bioactivity of lysozyme, Green Chem. Lett. Rev. 14 (2) (2021) 302–338, https://doi.org/10.1080/17518253.2021.1890840.

[82] R.A. Wahab, N. Elias, F. Abdullah, S.K. Ghoshal, On the taught new tricks of enzymes immobilization: an all-inclusive overview, React. Funct. Polym. 152 (2020) 104613, https://doi.org/10.1016/j.reactfunctpolym.2020.104613.

[83] M. Bilal, J. Cui, H.M.N. Iqbal, Tailoring enzyme microenvironment: State-of-the-art strategy to fulfill the quest for efficient bio-catalysis, Int. J. Biol. Macromol. 130 (2019) 186–196, https://doi.org/10.1016/j.ijbiomac.2019.02.141.

[84] C. Bernal, K. Rodríguez, R. Martínez, Integrating enzyme immobilization and protein engineering: an alternative path for the development of novel and improved industrial biocatalysts, Biotechnol. Adv. 36 (5) (2018) 1470–1480, https://doi.org/10.1016/j.biotechadv.2018.06.002.

[85] R.A. Sheldon, S. van Pelt, Enzyme immobilisation in biocatalysis: why, what and how, Chem. Soc. Rev. 42 (15) (2013) 6223–6235, https://doi.org/10.1039/C3CS60075K.

[86] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme Microb. Technol. 40 (6) (2007) 1451–1463, https://doi.org/10.1016/j.enzmictec.2007.01.018.

[87] R.C. Rodrigues, Á. Berenguer-Murcia, R. Fernandez-Lafuente, Coupling chemical modification and immobilization to improve the catalytic performance of enzymes, Adv. Synth. Catal. 353 (2011) 2216–2238, https://doi.org/10.1002/adsc.201100163.

[88] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R.C. Rodrigues, R. Fernandez-Lafuente, Strategies for the one-step immobilization-purification of enzymes as industrial biocatalysts, Biotechnol. Adv. 33 (5) (2015) 435–456, https://doi.org/10.1016/j.biotechadv.2015.03.006.

[89] S. Arana-Peña, C. Mendez-Sanchez, N.S. Rios, C. Ortiz, L.R.B. Gonçalves, R. Fernandez-Lafuente, New applications of glyoxyl-octyl agarose in lipases co-immobilization: strategies to reuse the most stable lipase, Int. J. Biol. Macromol. 131 (2019) 989–997, https://doi.org/10.1016/j.ijbiomac.2019.03.163.

[90] N.S. Rios, S. Arana-Peña, C. Mendez-Sanchez, C. Ortiz, L.R.B. Gonçalves, R. Fernandez-Lafuente, Reuse of lipase from *Pseudomonas fluorescens* via its step-by-step coimmobilization on glyoxyl-octyl agarose beads with least stable lipases, Catalysts 9 (2019) 487, https://doi.org/10.3390/catal9050487.

[91] S. Arana-Peña, D. Carballares, V. Cortés Corberan, R. Fernandez-Lafuente, Multi-combilipases: Co-immobilizing lipases with very different stabilities combining immobilization via interfacial activation and ion exchange. The reuse of the most stable co-immobilized enzymes after inactivation of the least stable ones, Catalysts. 10 (2020) 1207. https://doi.org/10.3390/catal10101207.

[92] H. Zaak, J.F. Kornecki, E.-H. Siar, L. Fernandez-Lopez, V. Cortés Corberán, M. Sassi, R. Fernandez-Lafuente, Coimmobilization of enzymes in bilayers using PEI as a glue to reuse the most stable enzyme: preventing PEI release during inactivated enzyme desorption, Process Biochem. 61 (2017) 95–101, https://doi.org/10.1016/j.procbio.2017.06.014.

[93] S. Peirce, J.J. Virgen-Ortíz, V.G. Tacias-Pascacio, N. Rueda, R. Bartolome-Cabrero, L. Fernandez-Lopez, M.E. Russo, A. Marzocchella, R. Fernandez-Lafuente, Development of simple protocols to solve the problems of enzyme coimmobilization. Application to coimmobilize a lipase and a β-galactosidase, RSC Adv. 6 (66) (2016) 61707–61715, https://doi.org/10.1039/C6RA10906C.

[94] M. Ortega-Muñoz, J. Morales-Sanfrutos, A. Megia-Fernandez, F.J. Lopez-Jaramillo, F. Hernandez-Mateo, F. Santoyo-Gonzalez, Vinyl sulfone functionalized silica: a "ready to use" pre-activated material for immobilization of biomolecules, J. Mater. Chem. 20 (34) (2010) 7189, https://doi.org/10.1039/c0jm00720j.

[95] T.L.d. Albuquerque, N. Rueda, J.C.S. dos Santos, O. Barbosa, C. Ortiz, B. Binay, E. Özdemir, L.R.B. Gonçalves, R. Fernandez-Lafuente, Easy stabilization of interfacially activated lipases using heterofunctional divinyl sulfone activated-octyl agarose beads. Modulation of the immobilized enzymes by altering their nanoenvironment, Process Biochem. 51 (7) (2016) 865–874, https://doi.org/10.1016/j.procbio.2016.04.002.

[96] F.J. Lopez-Jaramillo, M. Ortega-Muñoz, A. Megia-Fernandez, F. Hernandez-Mateo, F. Santoyo-Gonzalez, Vinyl sulfone functionalization: a feasible approach for the study of the lectin-carbohydrate interactions, Bioconjug. Chem. 23 (4) (2012) 846–855, https://doi.org/10.1021/bc200681c.

[97] J. Morales-Sanfrutos, J. Lopez-Jaramillo, M. Ortega-Muñoz, A. Megia-Fernandez, F. Perez-Balderas, F. Hernandez-Mateo, F. Santoyo-Gonzalez, Vinyl sulfone: a versatile function for simple bioconjugation and immobilization, Org. Biomol. Chem. 8 (3) (2010) 667–675.

[98] P. Prikryl, J. Lenfeld, D. Horak, M. Ticha, Z. Kucerova, Magnetic bead cellulose as a suitable support for immobilization of α-chymotrypsin, Appl. Biochem. Biotechnol. 168 (2) (2012) 295–305, https://doi.org/10.1007/s12010-012-9772-y.

[99] K. Labus, A. Turek, J. Liesiene, J. Bryjak, Efficient Agaricus bisporus tyrosinase immobilization on cellulose-based carriers, Biochem. Eng. J. 56 (3) (2011) 232–240, https://doi.org/10.1016/j.bej.2011.07.003.

[100] A.L. Medina-Castillo, J. Morales-Sanfrutos, A. Megia-Fernandez, J.F. Fernandez-Sanchez, F. Santoyo-Gonzalez, A. Fernandez-Gutierrez, Novel synthetic route for covalent coupling of biomolecules on super-paramagnetic hybrid nanoparticles, J. Polym. Sci. Part A Polym. Chem. 50 (19) (2012) 3944–3953, https://doi.org/10.1002/pola.26203.

[101] H. Zaak, M. Sassi, R. Fernandez-Lafuente, A new heterofunctional amino-vinyl sulfone support to immobilize enzymes: application to the stabilization of β-galactosidase from Aspergillus oryzae, Process Biochem. 64 (2018) 200–205, https://doi.org/10.1016/j.procbio.2017.09.020.

[102] P. Santos-Moriano, L. Monsalve-Ledesma, M. Ortega-Muñoz, L. Fernandez-Arrojo, A.O. Ballesteros, F. Santoyo-Gonzalez, F.J. Plou, Vinyl sulfone-activated silica for efficient covalent immobilization of alkaline unstable enzymes: Application to levansucrase for fructooligosaccharide synthesis, RSC Adv. 6 (69) (2016) 64175–64181.

[103] J.C.S. dos Santos, N. Rueda, O. Barbosa, J.F. Fernández-Sánchez, A.L. Medina-Castillo, T. Ramón-Márquez, M.C. Arias-Martos, M.C. Millán-Linares, J. Pedroche, M.D.M. Yust, L.R.B. Gonçalves, R. Fernandez-Lafuente, Characterization of supports activated with divinyl sulfone as a tool to immobilize and stabilize enzymes via multipoint covalent attachment. Application to chymotrypsin, RSC Adv. 5 (27) (2015) 20639–20649.

[104] J.C.S. Dos Santos, N. Rueda, O. Barbosa, M.D.C. Millán-Linares, J. Pedroche, M. Del Mar Yuste, L.R.B. Gonçalves, R. Fernandez-Lafuente, Bovine trypsin immobilization on agarose activated with divinylsulfone: Improved activity and stability via multipoint covalent attachment, J. Mol. Catal. B Enzym. 117 (2015) 38–44, https://doi.org/10.1016/j.molcatb.2015.04.008.

[105] R. Morellon-Sterling, D. Carballares, S. Arana-Peña, E.-H. Siar, S.A. Braham, R. Fernandez-Lafuente, Advantages of supports activated with divinyl sulfone in enzyme coimmobilization: Possibility of multipoint covalent immobilization of the most stable enzyme and immobilization via ion exchange of the least stable enzyme, ACS Sustain. Chem. Eng. 9 (22) (2021) 7508–7518, https://doi.org/10.1021/acssuschemeng.1c01065.

[106] E.M. Anderson, K.M. Larsson, O. Kirk, One biocatalyst - many applications: The use of *Candida antarctica* B-lipase in organic synthesis, Biocatal. Biotransform. 16 (3) (1998) 181–204, https://doi.org/10.3109/10242429809003198.

[107] C. Ortiz, M.L. Ferreira, O. Barbosa, J.C.S. dos Santos, R.C. Rodrigues, Á. Berenguer-Murcia, L.E. Briand, R. Fernandez-Lafuente, Novozym 435: the "perfect" lipase immobilized biocatalyst? Catal Sci. Technol. 9 (10) (2019) 2380–2420.

[108] V. Gotor-Fernández, E. Busto, V. Gotor, *Candida antarctica* lipase B: an ideal biocatalyst for the preparation of nitrogenated organic compounds, Adv. Synth. Catal. 348 (7-8) (2006) 797–812, https://doi.org/10.1002/adsc.200606057.

[109] S.W. Tsai, Enantiopreference of *Candida antarctica* lipase B toward carboxylic acids: Substrate models and enantioselectivity thereof, J. Mol. Catal. B Enzym. 127 (2016) 98–116, https://doi.org/10.1016/j.molcatb.2014.07.010.

[110] R.N. Lima, C.S. dos Anjos, E.V.M. Orozco, A.L.M. Porto, Versatility of *Candida Antarctica* lipase in the amide bond formation applied in organic synthesis and biotechnological, Mol. Catal. 466 (2019) 75–105, https://doi.org/10.1016/j.mcat.2019.01.007.

[111] R.C. Rodrigues, R. Fernandez-Lafuente, Lipase from *Rhizomucor miehei* as an industrial biocatalyst in chemical process, J. Mol. Catal. B Enzym. 64 (1-2) (2010) 1–22, https://doi.org/10.1016/j.molcatb.2010.02.003.

[112] R.C. Rodrigues, R. Fernandez-Lafuente, Lipase from *Rhizomucor miehei* as a biocatalyst in fats and oils modification, J. Mol. Catal. B Enzym. 66 (1-2) (2010) 15–32, https://doi.org/10.1016/j.molcatb.2010.03.008.

[113] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1-2) (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.

[114] D. Lombardo, O. Guy, Effect of alcohols on the hydrolysis catalyzed by human pancreatic carboxylic-ester hydrolase, Biochim. Biophys. Acta (BBA)- Enzymol. 657 (2) (1981) 425–437, https://doi.org/10.1016/0005-2744(81)90328-4.

[115] J. Boudrant, J.M. Woodley, R. Fernandez-Lafuente, Parameters necessary to define an immobilized enzyme preparation, Process Biochem. 90 (2020) 66–80, https://doi.org/10.1016/j.procbio.2019.11.026.

[116] J.C.S. dos Santos, N. Rueda, A. Sanchez, R. Villalonga, L.R.B. Gonçalves, R. Fernandez-Lafuente, Versatility of divinylsulfone supports permits the tuning of CALB properties during its immobilization, RSC Adv. 5 (45) (2015) 35801–35810.

[117] J.C.S. dos Santos, N. Rueda, R. Torres, O. Barbosa, L.R.B. Gonçalves, R. Fernandez-Lafuente, Evaluation of divinylsulfone activated agarose to immobilize lipases and to tune their catalytic properties, Process Biochem. 50 (6) (2015) 918–927, https://doi.org/10.1016/j.procbio.2015.03.018.

[118] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (5259) (1970) 680–685, https://doi.org/10.1038/227680a0.

[119] J.F. Kornecki, D. Carballares, R. Morellon-Sterling, E.H. Siar, S. Kashefi, M. Chafiaa, S. Arana-Peña, N.S. Rios, L.R.B. Gonçalves, R. Fernandez-Lafuente, Influence of phosphate anions on the stability of immobilized enzymes. Effect of enzyme nature, immobilization protocol and inactivation conditions, Process Biochem. 95 (2020) 288–296, https://doi.org/10.1016/j.procbio.2020.02.025.

[120] H. Zaak, L. Fernandez-Lopez, S. Velasco-Lozano, M.T. Alcaraz-Fructuoso, M. Sassi, F. Lopez-Gallego, R. Fernandez-Lafuente, Effect of high salt concentrations on the stability of immobilized lipases: dramatic deleterious effects of phosphate anions, Process Biochem. 62 (2017) 128–134, https://doi.org/10.1016/j.procbio.2017.07.018.

[121] E.A. Manoel, J.C.S. dos Santos, D.M.G. Freire, N. Rueda, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports involves the open form of the enzyme, Enzyme Microb. Technol. 71 (2015) 53–57, https://doi.org/10.1016/j.enzmictec.2015.02.001.

[122] L. Fernandez-Lopez, S.G. Pedrero, N. Lopez-Carrobles, B.C. Gorines, J.J. Virgen-Ortíz, R. Fernandez-Lafuente, Effect of protein load on stability of immobilized enzymes, Enzyme Microb. Technol. 98 (2017) 18–25, https://doi.org/10.1016/j.enzmictec.2016.12.002.

[123] H. Zaak, E.-H. Siar, J.F. Kornecki, L. Fernandez-Lopez, S.G. Pedrero, J.J. Virgen-Ortíz, R. Fernandez-Lafuente, Effect of immobilization rate and enzyme crowding on enzyme stability under different conditions. The case of lipase from *Thermomyces lanuginosus* immobilized on octyl agarose beads, Process Biochem. 56 (2017) 117–123, https://doi.org/10.1016/j.procbio.2017.02.024.