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Multiple co-interactions of different parameters on the functional properties of immobilized lipases

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ABSTRACT

In order to determine possible co-interactions between enzyme-support effects, and the influence of enzyme-enzyme interactions on their effects on the final enzyme properties, lipase B from *Candida antarctica* was immobilized on different supports, initially immobilized via interfacial activation, at low and saturating enzyme loadings. The used supports were octyl, amino-hexyl-, and the heterofunctional ones obtained by modification with divinyl sulfone, (blocking agents used were ethylenediamine or Gly). The different biocatalysts activities were analyzed using *p*-nitro phenyl butyrate, triacetin and R and S methyl mandelate. The comparison of the biocatalyst as a function of the activity depended on the utilized substrate. In some instances, the effects of the enzyme-enzyme interactions were reflected by the increase in specific enzyme activity (even by a factor over 3). Regarding the stability, the support and the enzyme loading defined this, and all changed when comparing the stabilities of the biocatalysts in phosphate or Tris, where depending on the enzyme loading the most stable biocatalysts could be either one or the other. Fluorescence studies suggested (mainly intensity at the maximal emission wavelength) that the enzymes present different conformations and that the inactivation on Tris and phosphate follows different pathways, and this also depended on enzyme loading.

1. Introduction

The immobilization of lipases on hydrophobic supports is one of the most utilized methods for the production of biocatalysts from these enzymes [1,2], as it permits their one-step immobilization, purification, stabilization and hyperactivation [3]. This lipase immobilization protocol is based on the interfacial activation mechanism of these enzymes, a structural change from a closed form (where the lipase active center is secluded (in most cases) from the medium by a polypeptide chain, called lid) to an open structure (where the lid is shifted, exposing the active center to the medium, together with a large hydrophobic pocket surrounding it) [4–6]. This way, the open form of lipases tends to adsorb on any hydrophobic surface, such as a drop of natural substrates (drops of oils) [7–9], but also to other hydrophobic macromolecules that can be present in the extract [10–12], the open form of other lipase molecule [13–15] or a hydrophobic support [16]. This makes characterization of free lipases complex, as the lipase dimers present altered properties [13–15]. This is another advantage of the immobilization of lipases on hydrophobic supports; all the lipase molecules are immobilized in open and monomeric form, making a fair comparison between different lipases easier [16]. The immobilization of lipases via interfacial activation on hydrophobic supports is reversible, enabling the support reuse after enzyme inactivation [3]. However, this reversibility becomes a problem when using reactants with detergent properties [17], adding high

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concentrations of organic cosolvents or employing high temperatures [18], as the enzyme molecules may release from the support during operation. In fact, the enzymes that are immobilized using this technique may move inside the support particle in certain cases [19]. This may be avoided using heterofunctional supports [18,20–24] or promoting physical [25] or chemical [26,27] intermolecular crosslinking of the immobilized enzyme molecules. Even considering this reversibility in immobilization, the properties of the immobilized enzymes may be altered using different additives during the immobilization step [28,29]. If they are able to induce some conformational changes in the enzyme, these changes are later maintained, and stability, activity, selectivity or specificity of the biocatalysts may be different using the same enzyme and support, just by altering the immobilization conditions [28,29].

The immobilization on these supports is so fast that, if the enzyme loading exceeds that which is necessary to saturate the support surface of enzyme molecules, the immobilized enzyme molecules are packed together, and can interact with each other, leading to changes in enzyme specificity/activity/stability [28,30]. It has also been shown some interactions between the enzyme loading and the inactivation conditions used to determine the enzyme stability. These interactions modify both the quantitative results and the relative stability of the different investigated biocatalysts (changing the relative stability, activity, etc. when changing these biocatalyst parameters) [31]

Recently, our research group has prepared two new heterofunctional supports [32,33]. The first class was the production of amine-hexyl and amine-octyl agarose beads [32]. The enzymes were firstly immobilized on these supports via interfacial activation, as using commercial octyl-agarose beads, but later some ionic exchange between ionic groups located in the enzyme and the support may occur. The full release of the enzyme from these supports required the coupled use of detergent and high ionic strength. However, the stability of the lipases after its immobilization in this new support was lower than that using the standard acyl supports in many instances. This was attributed to the amphipathic character of the amino acyl chains. The other support was the amino-acyl-vinyl sulfone (VS) matrix [33], a support that together with a cationic group and the acyl chain, presented the chemical reactivity of the vinyl sulfone [24,34–38], enabling lipase immobilization via covalent attachment of the enzyme after the first lipase immobilization via interfacial activation. This support is very adequate to give a covalent immobilization of previously adsorbed enzymes, involving all enzyme molecules on the covalent immobilization in different cases [24]. This support will locate three functions in the same spacer arm, in contraposition with the VS groups introduced in octyl-agarose [24], where this possibility does not exist (the octyl group is fixed to the support via ether bonds, and the divinyl sulfone (DVS) reacts with the remaining primary hydroxyl groups in the support, also forming ether bonds). The covalent bonds between the vinyl sulfone moieties of the support and the nucleophiles of the enzyme have proved to fully prevent enzyme release from the biocatalysts under any experimental condition [24]. This tri-functional support permitted to have biocatalysts with a much higher stability than the enzymes immobilized on amino-alkyl supports, in some cases becoming more stable than the octyl-lipase biocatalyst [33].

The previous research was performed using low enzyme loading to better detect the enzyme properties (reducing substrate diffusional matter problems). However, as enzyme-enzyme interactions may alter the final features of the immobilized enzymes, this new research effort try to analyze the possible co-interaction between the enzyme loading and the different variables that can define the enzyme features (spacer arm, buffer). For this goal, in this new research, we have compared the effect of the enzyme loading, using lowly loaded and highly loaded biocatalysts, on supports bearing different spacer arms in the immobilization support. The effect of the protein-protein interactions on enzyme features had been show in other instances, altering the intensity of the effect of the inactivation buffer on enzyme stability, enzyme modification, etc. However, a possible interaction between enzyme loading (protein-protein interaction) and nature of the spacer arm in the acyl group has not been shown. Neither has it been analyzed the possible co-interaction of the enzyme loading, spacer arm and inactivation buffer, showing how the complexity of the events defining the features of an immobilized enzyme is very high, and to the current knowledge, impossible to predict or even be fully understood. As model enzyme, the lipase B from *Candida antarctica* (CALB) was employed [30]. CALB is among the most utilized lipases [39]. It has been utilized in a wide diversity of reactions (oil hydrolysis [40], esterifications [41,42], transesterifications [43,44], interesterifications [45,46], acidolysis [47], amidations [48], polymer production and degradation [49–51], resolution of racemic mixtures [52], enantioselective production of enantiomers from pro-chiral substrates [53], and some promiscuous reactions [54–56]. However, the lid is so small that it does not fully isolate the lipase active center [57] but is still able to become adsorbed on hydrophobic surfaces. The biocatalysts stabilities will be analyzed using two buffers: Tris and sodium phosphate. Sodium phosphate is a buffer that greatly decreases the stability of CALB immobilized via interfacial activation on acyl supports [58]. The VS-blocking agents utilized in this paper were selected from those found to be the best and the worst ones for this enzyme in previous studies [33].

2. Material and methods

2.1. Materials

The liquid formulation of lipase B from *Candida antarctica* (CALB) (24.77 mg protein/mL) was kindly donated by Novozymes (Madrid, Spain). *p*-Nitrophenyl butyrate (*p*-NPB), triacetin and octylamine were acquired from Sigma-Aldrich (Madrid, Spain). Alfa Aesar (Fisher Scientific, Madrid, Spain) supplied *R*- and *S*- methyl mandelate. Octyl-agarose 4BCL was purchased from GE Healthcare, and hexylamine from Thermo Fisher Scientific Spain (Madrid, Spain). Glyoxyl agarose support was produced as previously described [59]. All other used reagents were of analytical grade. Elemental analyses were carried out by CAI Chemical Techniques, Laboratorio de Microanálisis Elemental, Universidad Complutense, using a Leco 932 CHNS combustion microanalyzer.

2.2. Lipase activity using different substrates

2.2.1. pNPB hydrolysis assay

The reaction was carried out by adding 50 μL of soluble or immobilized lipase (enzyme solution or enzyme suspension) to 2.5 mL of 25 mM sodium phosphate at pH 7.0 containing 20 μL of 50 mM *p*-NPB dissolved in acetonitrile. The hydrolytic activity was quantified spectrophotometrically at 348 nm (isosbestic point of *p*NP) [60] using a system thermostatized at 25 °C under magnetic stirring for 90 s. One international unit of activity (U) was defined as the amount of enzyme that hydrolyses 1 μmol of *p*-NPB per minute under the conditions indicated above.

2.2.2. Triacetin hydrolysis assay

The reaction was performed mixing 25–300 mg of wet biocatalysts and 3–9 mL of 50 mM of triacetin prepared in 50 mM of sodium acetate at pH 5.0 and 25 °C under gentle stirring. Under these conditions, 1,2 diacetin acyl migration to 1,3 diacetin is reduced [61]. The reaction progress was determined using a HPLC Kromasil C8 column (15 cm long per 0.46 cm wide) and a UV/VIS detector at 230 nm by quantifying the release of 1,2 diacetin. The mobile phase was a solution of 15 % (*v*/v) ultra-pure water and 85 % acetonitrile (v/v) under flow rate of 1 mL/min. Activity is represented as micromoles of diacetin produced per minute.

2.2.3. R- or S-methyl mandelate hydrolysis assay

The reactions were performed adding 25–300 mg of wet biocatalysts

to 3–9 mL of 50 mM of *R*- or *S*-methyl mandelate in 50 mM of sodium acetate pH 5.0 at 25 °C under continuous stirring. The reactants concentrations were determined using a HPLC with a Kromasil C8 column (15 cm long per 0.46 cm wide) and a UV/VIS detector at 230 nm. The mobile phase was 10 mM ammonium acetate containing 35 % acetonitrile (*v*/v) at pH 2.8 utilizing a flow rate of 1 mL/min. Activity is represented as micromoles of mandelic acid produced per minute.

2.3. Preparation of alkyl-amine supports

They were prepared as previously described [33]. Octylamine agarose support was prepared using 30 g of glyoxyl agarose and 120 mL of 4 M octylamine dissolved in 1,4-dioxane. The mixture was kept under mild stirring for 24 h at 25 °C. After that, 10 mg of sodium borohydride were added per ml of suspension and the suspension was mildly stirred for 2 h. Then, the reduced support was filtered and washed with 600 mL of 50 % (*v*/v) 1,4-dioxane solution, 600 mL of 200 mM sodium carbonate pH 9.0, 600 mL of 200 mM sodium acetate pH 4.0, and finally with an excess deionized water, vacuum dried, and stored at 4 °C [33].

Hexylamine agarose support was obtained using 30 g of glyoxyl agarose and 120 mL of 4 M hexylamine solution pH 10. The suspension was mildly stirred at 25 °C. After 24 h, 10 mg of sodium borohydride was added per milliliter of suspension and stirred for 2 h. Then, the reduced support was filtered, washed with 600 mL of 200 mM sodium carbonate pH 9.0, 600 mL of 200 mM sodium acetate pH 4.0, and finally with excess water, vacuum dried, and stored at 4 °C [33].

2.4. Supports modified with DVS

A mass of 10 g of the desired support (octyl, octylamine, or hexylamine-agarose) was mixed with 200 mL of 333 mM sodium carbonate at pH 11.5 containing 7.5 mL DVS [33,62]. The suspension was gently stirred for 2 h, and then washed with an excess of water, vacuum dried, and stored at 4 °C.

2.5. CALB immobilization

CALB was immobilized using two different enzyme loadings, 1 mg per gram of support (called low load), and 14 mg per gram of support (this guarantees the full coating of the support surface with the lipase) [3,16] (called high load). For immobilization on octyl, octylamine, and hexylamine, the commercial enzyme solution was diluted in 5 mM sodium phosphate at pH 7.0. For immobilization on the supports modified with DVS, the lipase was diluted in 5 mM sodium acetate at pH 5.0 [24,33]. One gram of each support was added to 10 mL of enzyme solution, and submitted to gentle stirring at 25 °C for 2 h. The immobilization courses were monitored by measuring the enzyme activity in the supernatant, suspension, and reference (soluble enzyme), at different time intervals using *p*-NPB as substrate. Then, the biocatalysts were washed with distilled water, vacuum filtered and suspended in 10 mL of 50 mM sodium bicarbonate at pH 8.0, and incubated for 3 h to favor the enzyme-support covalent reaction [63]. Finally, the biocatalysts were washed with distilled water, vacuum filtered, and resuspended in 10 mL of 2 M EDA or Gly adjusted at pH 8.0 for 24 h to block the remaining VS groups in the support [33]. After this time, the covalently immobilized and blocked biocatalysts were washed with abundant water, vacuum filtered, and stored at 4 °C.

2.6. Thermal inactivation of the biocatalysts

The inactivation courses of the different biocatalysts were studied using 25 mM of Tris-HCl or 100 mM sodium phosphate at pH 7.0 (1:10 ratio, *w*/*v*). The temperature was adjusted to have reliable but not overly long inactivation courses, facilitating the comparison between the different biocatalysts [64,65]. The exact inactivation temperature is included in each Figure legend. Periodically, samples of the inactivation suspensions were collected to determine their residual activities using the *p*NPB assay as described in section 2.2.1. Residual activities were determined as the percentage regarding the initial activity. In some instances, when the residual activity of the biocatalysts reached 20 %, the samples were washed, resuspended in 25 mM Tris buffer and stored at 4 °C at least for one week. These samples were submitted to fluorescence studies (see below).

2.7. Fluorescence studies of the different immobilized CALB preparations

The intrinsic fluorescence of the different immobilized CALB biocatalysts was quantified employing a Corning™ 96-Well Solid Black Polystyrene Microplates 96-well plates with flat bottom (Fisher-Scientific, Leicestershire (UK)) equipment. To perform these studies, 100 μL of 1:10 (w:v) suspension of immobilized CALB suspended in 100 mM Tris-HCl at pH 7 were added to each plate-well. The samples were excited at 280 nm with a 2 nm slit and the emission spectrum was recorded at 300–500 nm using a microplate reader (Cytation 5, BioTek® with the software Gen5). The wavelengths that yielded the maximum intensity (λ_{max}) and the maximum fluorescence intensity (I_{max}) were calculated, subtracting the signal of the supports (free of enzyme) to the spectra obtained using the samples with the immobilized CALB. The obtained raw fluorescence data were analyzed using Origin 8 software. Results are the mean of duplicate measures performed in two individually prepared samples (that is, at least 4 data for result were used).

2.8. SDS-PAGE analysis

SDS-PAGE was carried out employing a 12 % polyacrylamide gel and a BioRad Mini-PROTEAN® system. The experiment was performed as described by Laemmli [66] with some modifications. The immobilized biocatalysts or enzyme samples were diluted in 4 % SDS (*w*/*v*) and 10 % mercaptoethanol (*v*/v) (the rupture buffer), and boiled for 8 min. Next, the suspension was centrifuged at 10,000 rpm for 2 min to discard the support and 15 μL aliquots of the supernatants of each sample were applied to the gel. Aliquots of 5 μL molecular standard (LMW-SDS Marker 14.4–97 kDa) was also applied, and was run at 100 V. Finally, gels were stained using Coomassie brilliant blue stain. This treatment is enough to release any enzyme molecule that is only physically adsorbed on the support studies [33]. It has been utilized to conform the covalent immobilization of the enzymes and the possibility of enzyme release in the inactivation processes.

3. Results

3.1. Supports characterization

The oxidation of the glyceryl supports with sodium periodate indicated that the support consumed around 50 micromols per g of support, that correspond to the number of glyoxyl groups generated in the support. The different supports utilized in this paper were characterized via elemental analysis to ensure the correct introduction of the different groups (Table 1). The initial octyl agarose has a very low amount of N and we were unable to detect S. After amination, a large amount of N could be appreciated in the dried material. Assuming that the percentage of agarose remains similar to 4 % in the wet support, around 47 μmol/g of wet support were introduced, in agreement with the amount of initial

Table 1
Elemental analysis of the different supports.

	μmol N/g	μmol S/g
Octyl	58.1 ± 3.8	≥10
Amino-hexyl	1185.1 ± 15.1	≥10
Octyl-VS	59.5 ± 3.8	956.3 ± 8.9
Amino-hexyl-VS	1180.8 ± 13.9	1409.2 ± 17.1

glyoxyl groups. The modification of the octyl-agarose support with DVS introduced around 38 micromols/g of wet support, while the modification of the hexyl-amine agarose gave around 56 micromols/g (this involved the secondary amino groups plus some hydroxyl groups). This will be the supports used to immobilize CALB in this research effort.

3.2. CALB immobilization on the different supports

Fig. 1 shows the immobilization of CALB at low and high loadings (see methods) on octyl agarose. When using the low loaded samples, all enzyme was immobilized in the first measurement, while in the high loading, around 15 % of the offered enzyme remains in the supernatant, and did not immobilize even extending the immobilization for some hours. While the low loaded biocatalyst maintained intact its activity versus *p*NPB during the immobilization, the highly loaded biocatalyst showed a decrease in enzyme activity (expressed activity of the immobilized enzyme decrease to around 70 %). In the case of the low loaded biocatalyst, an increase in enzyme activity could be expected after interfacial activation, but this enzyme has a small lid that did not fully isolate the active center from the medium [57]. In the case of the highly loaded biocatalyst, the decrease in enzyme activity could be explained by the substrate diffusional problems generated with this very high volumetric activity and low substrate concentration, it may be that the enzyme molecules located in the inner area of the support did not receive any substrate [67,68]. The existence of lipase molecule-lipase molecule interactions, that could alter the enzyme features, cannot be fully discarded [30,31]. That way, the highly loaded biocatalyst has under 12 mg enzyme/g of support, and the lowly loaded one presented 1 mg/g.

Using hexyl-amine agarose, the picture is different (Fig. 2). The immobilization of the enzyme is also very fast, with all enzyme immobilized using a low load and reaching the maximum loading using the higher loaded (again around 85 %). However, in this instance the low loaded biocatalyst exhibited a higher activity than the free enzyme, suggesting that some conformational change has been produced in the enzyme that did not occur using octyl-agarose. Using an over-saturating amount of enzyme, again a certain decrease in enzyme activity can be observed but in a smaller way, in this instance the immobilized enzyme showed an expressed activity of more than 80 %.

Similar immobilization rates and immobilization yields were obtained using octyl-VS-agarose to immobilize CALB (Fig. 3), although in this instance a 15 % increase in enzyme activity could be detected using the low loaded biocatalyst (reported in other lipase immobilization using VS activated supports [24,69]) very likely by some distortion of the enzyme structure. The highly loaded biocatalysts also showed around 70 % of the immobilized enzyme activity. The effect of the incubation and blocking may be visualized in Table 2 and will be discussed in the next point.

Finally, the enzyme was immobilized on amino-hexyl-VS agarose (Fig. 4). While the immobilization yield and immobilization rate were similar to the results obtained using the previous supports, the changes in enzyme activity were more significant: the low loaded biocatalyst increased the activity versus *p*NPB to over 160 %, while the highly loaded biocatalyst presented around 80 % of the expected activity. The effect of the incubation and blocking may be visualized in Table 2 and will be discussed in the next point.

The covalent immobilization of the enzymes on the support was confirmed by SDS-PAGE [33], showing that no protein were released after boiling in SDS.

3.3. Activity of the different CALB biocatalysts versus different substrates

Table 2 shows the activities of the different CALB biocatalysts versus *p*NPB, triacetin and *R* and *S* methyl mandelate of the highly loaded biocatalysts. The activity of the lowly loaded biocatalyst, already described in reference [57], are showed in Table 1S for a simple comparison with the highly loaded biocatalysts.

Starting with the activity versus pNPB, octyl-CALB at low loading presented an activity similar to the free enzyme, the highly loaded biocatalyst was around 5.5 folds more active, while it presented a 12-fold more enzyme. These results fit with the decrease in enzyme activity found in Fig. 1, and they may be attributed to some substrate diffusional limitations. Amino hexyl-CALB presented a higher activity than the octyl (and free) biocatalyst, as expected from the Fig. 2, with 4.8-fold more activity for the high loaded biocatalyst (again this may be explained by substrate diffusional matters) [67,68]. Octyl-VS-CALB-Gly and octyl-VS-CALB-EDA presented a lower activity than the octyl agarose, suggesting that the incubation to get the enzyme-support covalent bonds and the blocking step can produce a slight decrease in enzyme activity (higher using Gly as blocking agent). Results differed when studying the amino-hexyl-VS preparations, both gave higher activities than the octyl- or the amino-hexyl-biocatalysts. In fact, they were even more active than the non-incubated and unblocked preparations (by only a 10 %). This showed the large difference of the effect of the enzyme modification promoted in the VS reaction and blocking using octyl or amino-hexyl biocatalysts. The high loaded biocatalysts exhibited around 5-fold more activity than the lowly loaded, as with the other biocatalyst.

Using triacetin (at 125 higher concentration than using *p*NPB), some difference can be observed. Using this substrate, the highly loaded biocatalyst, that has only 12 times more enzyme, is 20-fold more active than the lowly loaded biocatalyst. That is, the activity per mg of immobilized enzyme is 80 % higher for the highly loaded biocatalyst than for the lowly loaded biocatalyst, as this cannot be explained by limitations of substrate for the enzyme immobilized in the core of the particles, the results should be a consequence of enzyme-enzyme interactions (previously described for lipase highly loaded biocatalysts) [30,31]. The activity using amino-hexyl-CALB is slightly lower using the

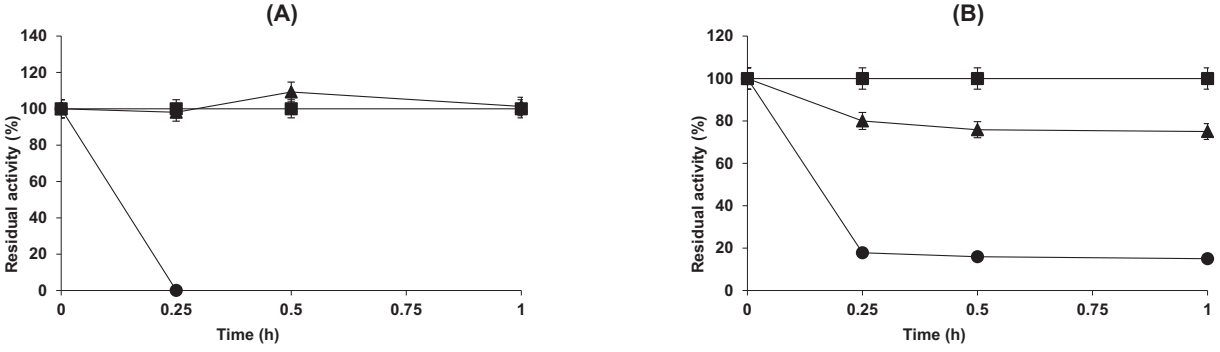

Fig. 1. Immobilization course of CALB on octyl agarose supports using low load (A) and high load (B). The experiments were carried out using 5 mM sodium phosphate at pH 7 and 25 ± 1 °C. Solid square: free enzyme, solid circle: supernatant, solid triangle:

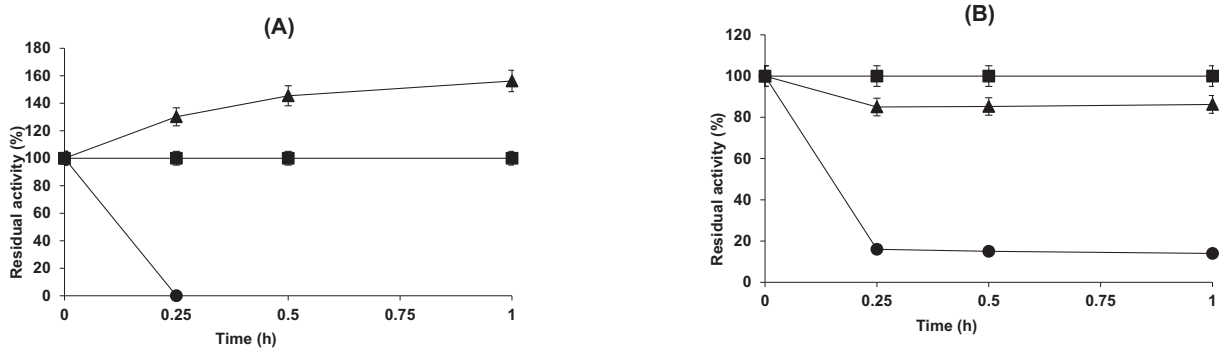

Fig. 2. Immobilization course of CALB on amino-hexyl agarose supports using low load (A) and high load (B). The experiments were carried out using 5 mM sodium phosphate at pH 7 and 25 ± 1 °C. Solid square: free enzyme, solid circle: supernatant, solid triangle: suspension. Further specifications can be found on the materials and methods section.

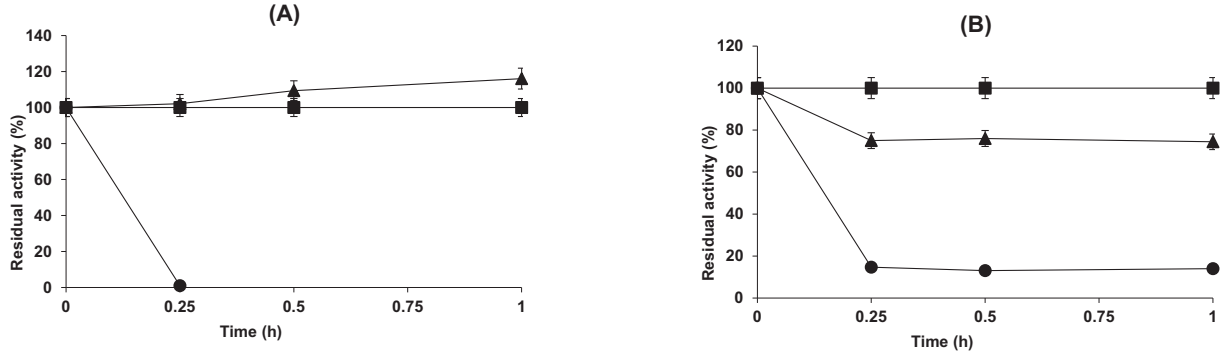

Fig. 3. Immobilization course of CALB on octyl-VS agarose supports using low load (A) and high load (B). The experiments were carried out using 5 mM sodium phosphate at pH 7 and 25 ± 1 °C. Solid square: free enzyme, solid circle: supernatant, solid triangle: suspension. Further specifications can be found on the materials and methods section.

Table 2
Activity (U/g) versus different substrates of CALB immobilized on different supports. ρNPB activity at pH 7.0 and triacetin and methyl mandelate at pH 5.0 and 25 °C. Further specifications can be found in the methodology section. The enzyme loading in the support was 12 mg/g.

Biocatalyst	Substrate			
	*p*NPB	Triacetin	(*D*)-Methyl mandelate	(*L*)-Methyl mandelate
Octyl-CALB	36.8 ± 1.8	34.1 ± 1.7	224.5 ± 11.2	26.9 ± 1.1
Amino-Hexyl-CALB	46.8 ± 2.3	58.1 ± 2.8	1212 ± 60.1	67.4 ± 3.3
Octyl-VS-CALB-Glycine	26.7 ± 1.3	30.1 ± 1.5	117.5 ± 5.2	15.5 ± 0.7
Octyl-VS-CALB-EDA	31.3 ± 1.6	60.5 ± 3.1	218.5 ± 11.2	16.2 ± 1.2
Amino-Hexyl-VS-CALB-Glycine	53.9 ± 2.7	111.4 ± 5.6	760.5 ± 37.6	177.4 ± 8.8
Amino-Hexyl-VS-CALB-EDA	55.8 ± 2.8	94.81 ± 4.7	655.5 ± 32.6	148.5 ± 7.5

low loaded biocatalyst (not statistically significant), while the highly loaded biocatalyst became even more active (70 % more active than octyl-CALB). This meant that the activity of the highly loaded biocatalyst per mg of enzyme become more than triple than that of the low loaded biocatalyst. The explanation should be that the enzyme molecules-enzyme molecules interactions produced some conformational alterations that lead to this higher activity versus triacetin [30,31]. Using octyl-VS-CALB-Gly the activity is around half the activity obtained using octyl-VS-CALB-EDA for both highly and lowly loaded biocatalysts. The highly loaded biocatalyst were 18–19 fold more active than the lowly loaded biocatalyst, again confirming the importance of the enzyme-enzyme interactions for the enzyme activity [30,31]. The results also pointed the importance of the blocking agent in the final properties of the biocatalysts [22,70]. The immobilization of CALB on amino-hexyl-VS-agarose produced the most active biocatalysts also using triacetin as substrate, almost doubling the activity using amino-hexyl-agarose. Using the low loaded biocatalyst, the blocking with EDA gave the biocatalyst with the highest activity (by 20 %), but using the highly loaded biocatalyst, the situation reversed, and the blocking with Gly gave slightly higher activity that the biocatalyst blocked with EDA (by less than 20 %). That way, the highly loaded biocatalyst gave 31-fold more activity than the lowly loaded biocatalyst when blocked with Gly and 22-fold when blocked with EDA, in both cases surpassing the 12-fold more enzyme in the support. The highly loaded biocatalyst was the one presenting the highest mass and specific activities of all the studied ones.

The situation was again different using *R*-methyl mandelate to determine the enzyme activity. This substrate permitted to reach the highest activities with all the biocatalysts. Octyl and octyl-VS biocatalyst were the least active ones versus this substrate, the blocking with EDA of the VS biocatalyst improving the activity regarding the octyl for the lowly loaded biocatalyst, while maintaining the activity using the highly loaded biocatalyst. The Gly blocking produced a decrease in enzyme activity, more significant for the highly loaded biocatalyst (that became

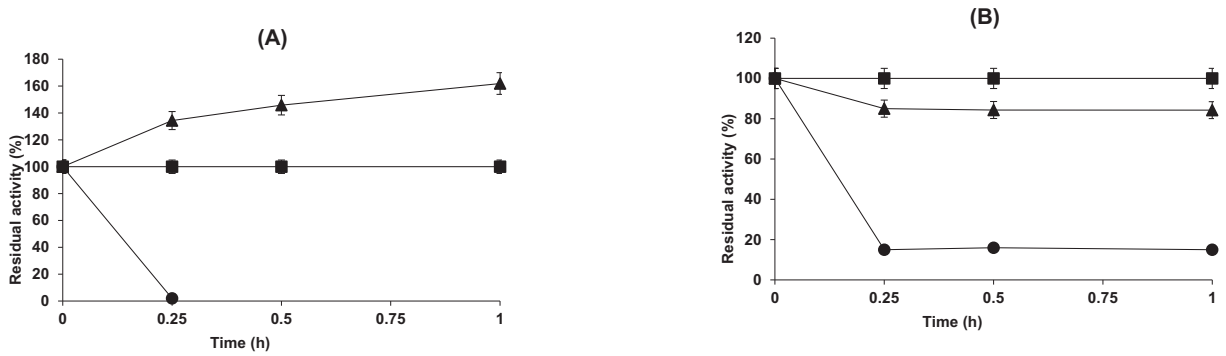

Fig. 4. Immobilization course of CALB on amino-hexyl-VS agarose supports using low load (A) and high load (B). The experiments were carried out using 5 mM sodium phosphate at pH 7 and 25 ± 1 °C. Solid square: free enzyme, solid circle: supernatant, solid triangle: suspension. Further specifications can be found on the materials and methods section.

the highly loaded biocatalysts with the lowest activity among the studied ones). The use of highly loaded biocatalyst increased the volumetric activity by 13, 7 and 9 for octyl-CALB, octyl-VS-CALB-Gly and octyl-VS-CALB EDA respectively, similar or even lower than the excess of enzyme. That way, using this substrate, an increase on the specific activity of the immobilized enzyme was not visualized as when using triacetin. In fact, except using octyl-CALB, the excess of enzyme is lower than the increase in biocatalyst activity. This could be caused because the change in enzyme structure caused by enzyme-enzyme interactions may be positive for triacetin while is negative for *R*-methyl mandelate, at least, using these biocatalysts, or because the higher activity using this substrate is able to promote some substrate diffusion matters inside the biocatalyst particle [67,68].

Results using amino-hexyl to immobilize the enzyme shows a high increase of the activity using both loads when compared to octyl-CALB: almost 4.4-fold for the lowly loaded biocatalyst and 5.4-fold for the highly loaded biocatalyst. The activity of the highly loaded biocatalyst is 16-fold higher than that of the lowly loaded biocatalyst, more than the excess of enzyme, suggesting that in this case the enzyme-enzyme interactions [30,31] had a positive effect on the enzyme activity versus this substrate. The use of amino-hexyl-VS support produced biocatalysts with similar activity to amino-hexyl-agarose when using lowly loaded biocatalysts (slightly higher using EDA as blocking agent and slightly lower using Gly). However, using the highly loaded biocatalysts, the activity decrease compared to the amino-hexyl-CALB, and this is more intense using EDA as blocking agent (to 54 %) than using Gly (63 %). That way, again the specific activity of the enzyme in the highly loaded biocatalyst is smaller than in the lowly loaded biocatalyst.

Using *S*-methyl mandelate, the activity is almost one order the magnitude smaller than using the *R*-isomer. Highly loaded octyl-CALB was 14-fold more active than the lowly loaded biocatalyst. This was the biocatalyst with the lowest activity versus this substrate. The ratio of the activities versus the *R* and *S* isomers was 9 and 8.3 for the lowly and highly loaded biocatalysts, respectively. The use of amino-hexyl-CALB permitted to increase the activity, by almost 30 % using the lowly loaded biocatalyst but by 2.5-fold using the highly loaded biocatalyst (again suggesting very different enzyme properties for both biocatalysts) [30,31]. This made that the activity ratio using both isomers of substrate became 30 using the lowly loaded biocatalyst and 18 using the highly loaded biocatalyst. The highly loaded biocatalyst presented more than 27-fold more activity than the lowly loaded biocatalyst, more than doubling the immobilized enzyme specific activity on this biocatalyst. Both octyl-VS-CALB biocatalyst gave very similar activities using this substrate, slightly increasing the activity of octyl-CALB when using the lowly loaded biocatalysts (by around 15 %), but decreased the activity when using the highly loaded biocatalysts (again suggesting the role of the enzyme-enzyme interactions on the final properties of the biocatalyst) [30,31]. The activities ratio versus *R* and *S* isomers is 7.3 for the lowly loaded amino-hexyl-CALB-Gly and more than 10 for amino-hexyl-CALB-EDA, using the highly loaded biocatalyst the values are 7.3 and 13.7, respectively. Finally, we analyzed the results using amino-hexyl-VS-CALB biocatalysts. The activity increased when compared to that of amino-hexyl-CALB. The blocking with Gly produced a slightly higher activity for both enzyme loads. Using these biocatalysts, activities were 2.6/2.7-fold higher using both loads than the corresponding amino-hexyl-CALB. The blocking with EDA gave biocatalysts 2.1/2.2-fold more active than amino-hexyl-CALB. Both highly loaded biocatalysts were 28-fold more active than the lowly loaded biocatalyst, again giving a higher specific activity for the immobilized enzyme (by more than twofold). This again suggested that the enzyme-enzyme interaction produces some changes that were positive for this substrate [30,31]. The ratios between the reaction rates with both isomers was 4.3/4.4 for both highly loaded biocatalysts (the lowest ones due to the high activity versus the *S* isomer), and 11.4 for the lowly loaded biocatalyst blocked with Gly and 14.5 if blocked with EDA. Again, it looks that there is a great effect of the enzyme loading on the relative activity of the biocatalyst, suggesting that the enzyme-enzyme interactions are very relevant in the final properties of the biocatalysts [30,31]. All these results together show a coupled co-interaction of support, enzyme loading and substrate in the final activity of the immobilized enzyme.

3.4. Stability of the different CALB biocatalysts under different conditions

Fig. 5 shows the inactivations courses of the different biocatalysts at low ionic strength in Tris buffer. The most stable lowly loaded biocatalyst is octyl-VS-CALB-Gly, shortly followed by octyl-CALB, while octyl-VS-CALB-EDA was the second least stable biocatalyst. This has been explained by the negative effect of the amino groups on the support on the stability of CALB [32,33]. In fact, amino-hexyl-CALB is significantly less stable than octyl-CALB, as previously described [32], and it is the least stable biocatalyst of all studied biocatalysts. Amino-hexyl-VS-CALB-Gly was more stable than amino-hexyl-CALB and even more in the case of octyl-VS-CALB-EDA, that has a very similar inactivation course than amino-hexyl-VS-CALB-EDA. Fig. 5B shows the inactivation courses using the highly loaded biocatalysts, where the relative positions of the different biocatalysts differ from the previous case. The most stable biocatalyst remained octyl-VS-CALB-Gly, but now octyl-CALB and amino-hexyl-VS-CALB-Gly are the second most stable biocatalyst, with very similar inactivation courses. Both VS biocatalyst blocked with EDA presented in this case similar stability, while amino-hexyl-CALB stability dropped, the least stable one, far less stable than the other biocatalysts. That way, not only the absolute stabilities buffer comparing high and low loaded biocatalysts, also the relative stability positions are altered, suggesting interactions between effect of the support surface and enzyme loading (causing enzyme-support and enzyme-enzyme interactions, respectively) on the final enzyme stability [22,30,31,70].

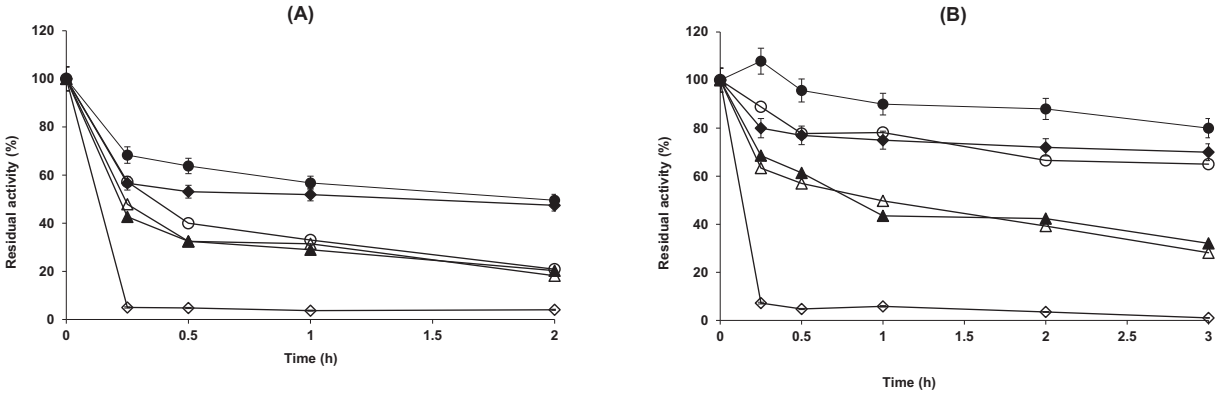

Fig. 5. Thermal inactivation of lowly loaded (A) and highly loaded (B) CALB biocatalysts. The experiments were carried out using 25 mM Tris-HCl at pH 7 and 76 ± 1 °C. Solid diamond: Octyl, Empty diamond: Amino-Hexyl, solid circle: Octyl-VS-Glycine, Empty circle: Amino-Hexyl-VS-Glycine, Full triangle: Octyl-VS-EDA, Empty triangle: Amino-Hexy-VS-EDA. Further specifications can be found on the materials and methods section.

Next, we compared the stabilities using as inactivation medium 100 mM sodium phosphate, a medium that has been described to greatly decrease the stability of the lipases immobilized on hydrophobic supports [58] but not using other supports [71] (Fig. 6). The inactivation temperature was decreased to have reliable inactivation courses. Starting with the low loaded biocatalyst, some changes in the relative positions of the different biocatalysts as a function of the enzyme stability could be detected using the low loaded biocatalysts. The most stable biocatalysts remained octyl-VS-CALB-Gly, but in this case it was followed by amino-hexyl-VS-CALB-Gly and amino-hexyl-VS-CALB-EDA, both surpassing the stability of octyl-CALB, whose inactivation course was closed to that of octyl-VS-CALB-EDA (while this biocatalyst that was clearly less stable than octyl-CALB when inactivated in Tris). Amino-hexyl-CALB remained the least stable biocatalyst. The situation changed again using the highly loaded biocatalysts. Now, the two most stable preparations were both amino-hexyl-VS-CALB-X, the retained activity was higher than using the most stable preparation in the other 3 cases, octyl-VS-CALB-Gly, and octyl-VS-CALB-EDA stability was higher than that of octyl-CALB. Amino-hexyl-CLB remained the least stable preparation, but now the distance with octyl-CALB was smaller.

That way, the enzyme-enzyme interactions also have a clear effect on enzyme stability [30,31], and this effect depends on the support and the inactivation buffer. The effect of phosphate seems to greatly depend on enzyme loading and the support nature. These results, coupled to the previous ones, suggest that the immobilized enzyme-enzyme interactions may fully alter the biocatalysts properties.

3.5. Fluorescence analysis of the different biocatalysts

Table 3 shows the florescence of the I_{max} and λ_{max} of the different

Table 3
Fluorescence studies of the different CALB biocatalysts. The experiments were performed as described in methods section.

		I_{max}	λ_{max}
Low loading	Octyl-CALB	3609.7 ± 212.5	313.3 ± 1.2
	Amino-hexyl agarose-CALB	3301.3 ± 14.8	314 ± 0.8
	Octyl-VS-CALB-EDA	4099.3 ± 78.1	314 ± 1.1
	Amino-hexyl agarose-VS-CALB-EDA	3850.3 ± 152.3	314 ± 0.7
	Octyl-VS-CALB-Gly	4093 ± 103	312.7 ± 1.2
	Amino-hexyl agarose-VS-CALB-Gly	3450.3 ± 243	314.7 ± 1.2
High loading	Octyl-CALB	25,395.3 ± 2836.2	314 ± 1.5
	Amino-hexyl agarose-CALB	24,879.3 ± 621.6	314 ± 1.1
	Octyl-VS-CALB-EDA	29,928 ± 1215.6	314 ± 1.2
	Amino-hexyl agarose-VS-CALB-EDA	26,280.3 ± 303.5	314 ± 0.9
	Octyl-VS-CALB-Gly	29,621.3 ± 392.1	313.3 ± 1.2
	Amino-hexyl agarose-VS-CALB-Gly	25,606 ± 159.7	314 ± 1.1

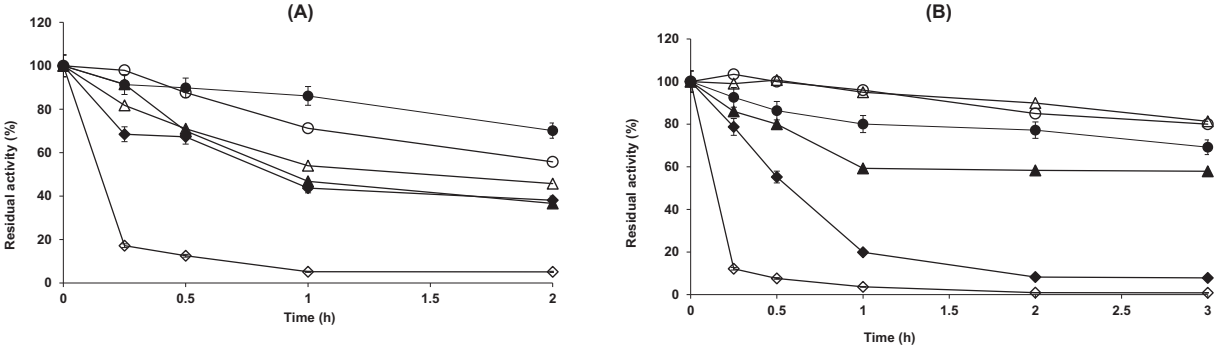

Fig. 6. Thermal inactivation of lowly loaded (A) and highly loaded (B) CALB biocatalysts. The experiments were carried out using 100 mM sodium phosphate at pH 7 and 70 ± 2 °C. Solid diamond: Octyl, Empty diamond: Amino-Hexyl, solid circle: Octyl-VS-Glycine, Empty circle: Amino-Hexyl-VS-Glycine, Full triangle: Octyl-VS-EDA, Empty triangle: Amino-Hexyl-VS-EDA. Further specifications can be found on the materials and methods section.

biocatalysts. Changes of λ_{max} are not so statistically significant for any of the biocatalysts (ranging from 313.3 to 314.7 nm), suggesting that the hydrophobicity of the Trp environment is not significantly altered. This can have some relation to the fact that the support surfaces have hydrophobic moieties, that way the groups environment may not be too different inside the core of the protein or in the medium. The changes in I_{max} are more relevant.

The comparison between highly and lowly loaded bioactalysts may be not fair, as there are some possible artefacts, such the fluorescence quenching at higher surface protein densities, so this cannot be discarded in the explanation of the results. However, the compassion between enzymes biocatalysts at the enzyme same loads may be fairer. Comparing the low loaded biocatalysts, I_{max} shows more differences than λ_{max}, ranging from 3330 for amino-hexyl-CALB to 4100 for amino-hexyl-CALB-EDA. The highly loaded biocatalysts I_{max} ranged from 24,879 for amino-hexyl-CALB to 29,928 for amino-hexyl-CALB-EDA. This, together with the changes in the functional properties of the biocatalysts described in the previous sections, suggested different enzyme structures promoted by the different immobilization protocols.

3.6. Fluorescence analysis of the different biocatalysts after inactivation in Tris and phosphate

The fluorescence spectrum of the different immobilized enzyme biocatalysts inactivated in Tris and phosphate were also analyzed when the residual activity was 20 %, and later incubated for 24 h of incubation in Tris at pH 7 and 4 °C before measurements. This incubation period permitted the enzyme reactivation and eliminate the possibility of comparing enzymes in different reactivation status [24]. The comparison between high and low loaded enzyme may be partially derived from the fluorescence quenching at higher surface protein densities, so this cannot be discarded in the explanation of the results. However, comparison between lowly and highly loaded biocatalysts may be accomplished.

Fig. 7 shows the main results. The non-covalent biocatalysts were discarded, as SDS-PAGE analysis of the biocatalysts showed that the amount of enzyme in the supports were not identical to the initial one (results may be found in reference [33], making any comparison difficult, mainly I_{max} (results not shown).

All the inactivated covalently immobilized biocatalysts presented lower I_{max} than the corresponding biocatalysts. Lowly loaded octyl-VS-CALB-EDA presented a 50 % $I_{max,}$ while λ_{max} increased from 314 to 330.7 nm when inactivated in Tris. If the inactivation was performed in sodium phosphate, the I_{max} decreased a little less, to 53 %, but the λ_{max} decreased to 308 nm. This is the only case where this parameter exhibited this unexpected behaviour for the inactivated biocatalyst, as the enzyme is expected to be more disorganized and that way with a higher exposition to the medium. We can hypotheses that the enzyme inactivated in the presence of phosphate change its conformation in a faster way, but finally the multi anion is able to promote a more compact structure for this partially inactivated biocatalyst. This confirmed that the changes induced by the inactivation in sodium phosphate are different from the changes induced in Tris. Using the highly loaded biocatalyst, I_{max} decreased to 72 % when inactivated in both buffers, and the λ_{max} increased also in a very similar way, to 332 or 330.7 from the initial 314. The values are similar to that found using Tris in the inactivation of lowly loaded biocatalyst, but differs from the results obtained using phosphate, showing the importance of the support enzyme loading in the effect of sodium phosphate. Lowly loaded octyl-VS-Gly decreased the I_{max} to 74 % when inactivated in Tris, and to 72 % when inactivated in phosphate, while the highly loaded biocatalyst decreased to 87 % and to 71 % respectively. Again, an interaction between effect of phosphate and enzyme loading may be found. Regarding the λ_{max}, the lowly loaded biocatalysts increased from 312.7 nm to around 332 nm when inactivated in both buffers. Lowly loaded amino-hexyl-VS-CALB-EDA decreased the I_{max} to 53 % when inactivated in Tris, but down to 68 % when inactivated in phosphate. The highly loaded biocatalyst decreased the I_{max} to 86 % when inactivated in Tris and down to 71 % if it is inactivated in phosphate. Again, different changes may be observed using the highly or lowly activated biocatalysts and Tris or phosphate. λ_{max} increased for the lowly loaded biocatalyst from 314 to 334 nm (in Tris) or 328.7 nm (in phosphate), while for the highly loaded biocatalyst the increase reached 332 nm (in Tris) or to 330.7 (in phosphate). Finally, lowly loaded amino-hexyl-VS-CALB-Gly decreased the I_{max} to 75 % (inactivated in Tris) or to 81 % (when inactivated in phosphate). These are the highest values for the I_{max} of the inactivated lowly loaded biocatalysts. The highly loaded biocatalyst decreased the I_{max} only by 10 % (inactivated in Tris) and to 68 % when inactivated in phosphate. λ_{max} increased for the lowly loaded biocatalysts from 314.4 nm to 332 nm (inactivated in Tris) or 332.7 nm (inactivation in phosphate), while using the highly loaded biocatalysts increased from 314 to 336 nm (in Tris) and 338 (in phosphate).

This way, the structural changes caused by the inactivation that are reflected in the fluorescence spectra differ depending on the support, inactivating buffer and loading, being more significant the changes in I_{max} than the changes in $\lambda_{max.}$

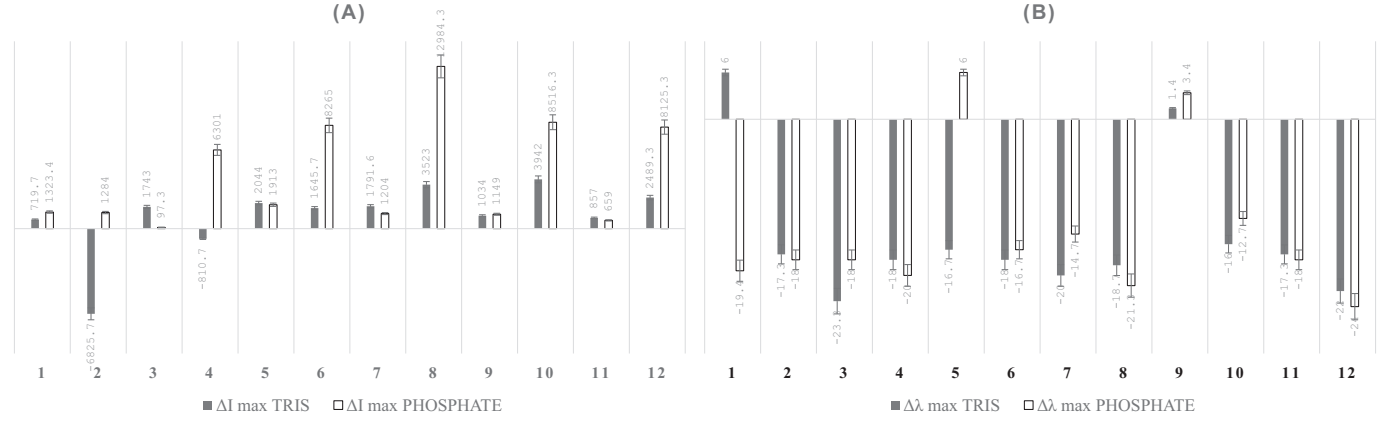

Fig. 7. Variance in fluorescence intensity (I_m) and wavelength (λ_m) of different biocatalysts when inactivated in 25 mM Tris-HCl (■) and 100 mM sodium phosphate (□) buffers at pH 7 and 25 °C. 1: Octyl-CALB low load,2: Octyl-CALB high load,3: Amino-hexyl agarose-CALB low load,4: Amino-hexyl agarose-CALB high load,5: Octyl-VS-CALB-EDA low load, 6: Octyl-VS-CALB-EDA high load,7: Amino-hexyl agarose-VS-CALB-EDA low load,8: Amino-hexyl agarose-VS-CALB-EDA high load,9: Octyl-VS-CALB-Glycine low load,10:Octyl-VS-CALB-Glycine high load,11: Amino-hexyl agarose-VS-CALB-Glycine low load,12: Amino-hexyl agarose-VS-CALB-Glycine high load.

4. Conclusions

The whole set of results presented in this paper shows the great relevance of the support features to determine the properties of immobilized CALB. In terms of activity, the substrate utilized to determine the activity can determine its relative position among the other biocatalysts, as the immobilization using different supports produce different enzyme structures (as shown by the fluorescence studies) and change the enzyme specificity. Another critical parameter that defines the specific activity in the immobilized enzyme is the loading of the support. When saturating the support with CALB molecules, in many instances the specific activity of the immobilized enzyme surpasses that of the lowly loaded biocatalysts. In fact, while a reduction of this value is expected due to the generation of substrate diffusion limitations using biocatalysts with high volumetric activity, the reverse result is observed, suggesting that enzyme-enzyme interactions may be positive for enzyme activity using certain substrates, but not other ones. Regarding the stability, again an interaction between support nature and enzyme loading may be found, becoming very relevant the negative effect of phosphate ions on the stability of CALB immobilized via interfacial activation. This is very different using the highly or the lowly loaded biocatalysts, altering the most stabile biocatalysts in each case.

That way, the effect of enzyme-enzyme interactions, enzyme-support interaction, and medium interactions seems to counteract each other. This suggests that there are many factors leading to the final immobilized features, and that the selection of the correct biocatalysts cannot be extrapolated from partial results using conditions and substrate different to the ones to be utilized in the final process. The use of different immobilization protocols and enzyme loading seems to be able to affect the enzymes structure in different fashions, making the immobilized enzyme to present specific catalytic features. The causes that can explain this are far to be fully understood at present.

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CRediT authorship contribution statement

Pedro Abellanas-Perez: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Diandra de Andrades:** Writing – original draft, Investigation, Formal analysis. **Andrés R. Alcantara:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Fernando Lopez-Gallego:** Writing – original draft, Investigation, Formal analysis, Data curation. **Javier Rocha-Martin:** Writing – original draft, Formal analysis. **Maria de Lourdes Teixeira de Moraes Polizeli:** Writing – review & editing, Formal analysis. **Roberto Fernandez-Lafuente:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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Data availability

No data was used for the research described in the article.

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