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The structure of the immobilized Eversa Transform determines the activity/ stability effects of the biocatalyst metallization

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ABSTRACT

In this paper, the human designed lipase Eversa Transform (ETL) has been immobilized on octyl agarose beads using 4 previously published protocols that provided biocatalysts with very different properties. Then, the biocatalysts were submitted to incubation with 7 different metal cations in Tris or buffer, with the objective of checking if the immobilized enzyme altered its properties after metallization and whether this modification has different qualitative and quantitative values when changing the immobilized enzyme protocol (that is, maintaining enzyme, support and enzyme orientation, only changing the enzyme structure). Enzyme activity versus nitro-phenol butyrate at different pH values using different buffers, enzyme activities versus this substrate and triacetin and *R* or *S* methyl mandelate and the enzyme stability under different conditions were studied. The results showed that the enzyme activity/ pH curve and specificity versus different substrates are drastically changed upon metallization, these changes depending on the presence of Tris or phosphate during mineralization and very interestingly, depending on the biocatalyst that is submitted to this treatment. The same treatment could increase the enzyme activity or stability for one biocatalyst while it could be negative for other biocatalysts.

1. Introduction

Enzyme immobilization is an important step in the preparation of industrial enzyme biocatalysts [1,2]. This is an opportunity to tune the final enzyme features, as a proper immobilization system can increase enzyme stability due to different reasons [3–13] and permit the enzyme purification [14–18]. Moreover, enzyme immobilization may alter enzyme conformation (in fact, it is almost impossible to prevent some random enzyme distortion caused by the enzyme-support interactions) [19] and, thus, different activity, specificity, selectivity and resistance to chemicals and inhibitors may be achieved by using different immobilization protocols [20]. Recently the enzyme immobilization techniques have been proposed to be divided in three categories: the enzymes self-attached to the cells via specific binding domains, their immobilization on pre-existing supports and the generation of *ex-novo* solids [21]. In the last category, nanoflowers stand up. In these nanoflowers, the enzymes become incorporated to the crystalline structure of the metal salts (usually phosphate), which in some instances acquire the form of rose petals (from where the name comes) [22–25]. This immobilization strategy permits, in many instances, to modulate enzyme stability and/ or activity, and, in this sense, becomes a very interesting immobilization strategy [22–29]. However, the primary objective of enzyme immobilization is to facilitate enzyme recovery [1,2] and nanoflowers have two main problems: their nano-size makes it difficult to recover them and their mechanical rigidity causes them to break easily upon handling. This way, nanoflowers recovery in large (over 1000 L) reactors becomes a cumbersome task. Some authors have proposed to trap the nanoflowers in macro-porous entities with better mechanical properties (e.g., calcium alginate beads) or to bind them to magnetic nanoparticles, to facilitate their management [30–40]. Other authors recently proposed the possibility of taking advantage of the favorable effects of enzyme metallization in order to avoid these problems is the incubation in

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solutions of metal phosphate of previously immobilized enzymes, using pre-existing supports with the desired mechanical properties. Moreover, this strategy permits to couple the benefits of enzyme immobilization on preexisting supports with those of the nanoflowers. This strategy has been exemplified with some lipases and has permitted to improve the activities and/or stabilities of immobilized enzymes [41–43]. The researchers have shown that the use of the same immobilization technique but producing different final physical features of the support surface alters the effects of the mineralization on the features of the immobilized enzymes [44]. In this instance, this different effect of the enzyme mineralization on enzyme properties may be caused by the fact that the support surface is different or because the immobilized enzyme molecules presented different structures on the different support surfaces. In fact, it has been shown how the different blocking agents produced different lipases structures [45,46].

In this new research effort, we intend to investigate if the mineralization of the same enzyme immobilized on exactly the same support with the same orientation but bearing different conformations may produce different results or the mineralization effects are independent from the immobilized enzyme conformation. That is, the objective is to analyze if the immobilized enzyme conformation is relevant to determine the effects of the mineralization on enzyme activity, specificity or stability. This possibility has been pointed by some researchers immobilizing the same free enzyme bearing different conformations using the nanoflowers strategy [47].

To reach this goal, we have utilized the artificial lipase called Eversa Transform 2.0 (ETL), designed by Novonesis (formerly known as Novozymes) as an evolution from the lipase from *Thermomyces lanuginosus,* to be used in the synthesis of biodiesel in its free form [48–50]. However, it has been showed that its adequate immobilization greatly improves their features (stability, activity) enabling to expand their applications range [51–57]. Lipases present a catalytic mechanism called interfacial activation that enables them to act on the surface of drops of insoluble triglycerides (its natural substrate) [58–62]; similarly lipases can become immobilized on any hydrophobic support [63]. This immobilization technique also enables to purify (only lipases are fully immobilized on these supports at low ionic strength or even in the presence of moderate concentrations of solvents), stabilize (the open and absorbed open form of the lipase is more stable than the enzyme in conformational equilibrium) and hyperactivate (as the open form of the lipase is fixed) lipases, and all this based on a reversible immobilization protocol that enables the inactivated enzyme release and reuse of the support afterwards [64].

Moreover, it has been shown that the immobilization of some lipases on hydrophobic supports via interfacial activation under different conditions alters their features (activity, specificity and stability) [65,66], and this has been correlated to the existence of different immobilized enzyme conformations [67]. ETL has been one of the enzymes where these different properties depending on the immobilization conditions have been found [68]. In this new research effort, we have immobilized ETL on octyl agarose under conditions where, following the previous results, the changes in enzyme activity/stability suggested different enzyme conformations: 50 mM sodium acetate pH 5 plus 100 mM NaCl, 50 mM sodium acetate pH 5, 50 mM sodium bicarbonate buffer pH 9 and Tris buffer pH 7 plus 10 % dioxane. An enzyme loading high enough to saturate the support surface with enzyme molecules (that is, using an excess of enzyme) was utilized to facilitate the mineralization of the immobilized enzymes using many different metals (calcium, nickel, cobalt, copper, manganese, zinc and magnesium) and phosphate. This way, if the effects of these biocatalysts mineralization were similar for the 4 different biocatalysts, the effect of the enzyme conformation on the modification of the enzyme features by mineralization can be considered negligible, while if the effects of the mineralization greatly differed, it can be considered that the immobilized enzyme conformation is a critical feature to determine the effects of the mineralization. That is, the main objective of the current paper is to show if the effects of the enzyme mineralization on the biocatalyst properties may be related or not to the initial conformation of the mineralized enzymes.

2. Materials and methods

2.1. Materials

4B-CL octyl-Sepharose beads (octyl agarose beads), triacetin and *p*-nitrophenyl butyrate (*p*NPB) were purchased from GE Healthcare-Spain. Novozymes Spain kindly gifted Eversa Transform 2.0 (ETL), a liquid lipase preparation containing approximately 41 mg of protein per mL following Bradford method [69]. $CoCl_2$, $CuCl_2$, $ZnCl_2$, $NiCl_2$, $MgCl_2$, $MnCl_2$ and $CaCl_2$ were purchased from Merck/Sigma-Aldrich (Spain). All other used chemicals and solvents were of analytical grade.

2.2. Methods

2.2.1. Determination of enzyme activities

The results are supplied as averages and standard errors after performing the experiments at least 4 times.

2.2.1.1. Hydrolysis of pNPB. The absorbance increase at 348 nm due to *p*-nitrophenol (*p*NP) release was recorded using a Jasco V-730 spectrophotometer [70]. For each reaction, 50 μL of 20 mM *p*NPB in acetonitrile was added to 2.5 mL of 25 mM sodium phosphate at pH 7.0 and 25 °C. The reaction started by adding 50 μL of enzyme solution or suspension and conducted under magnetic stirring and temperature control for 90 s. In some instances, the pH value in the measurement solution was changed using different 25 mM buffers: sodium acetate (pH 4–6), sodium phosphate (pH 6–8), or sodium bicarbonate (pH 8–9). Activity is expressed as micromoles of *p*NP produced per minute.

2.2.1.2. Hydrolysis of triacetin. A solution of 50 mM sodium acetate containing 50 mM triacetin was prepared, with the pH adjusted to 5. To initialize the reaction, immobilized enzyme was added to reach a final concentration of 0.42 % *w/v*. The suspension was stirred continuously in a roller mixer (Tube Roller MXT6S, Scilogex, CT) at a controlled temperature of 25 °C. The degree of conversion was quantified using a Waters 486 HPLC (Waters, Millford, MA, USA), with detection at 230 nm utilizing a Kromasil C18 column (15 cm × 0.46 cm, EKA Chemicals AB, Bohus, Sweden). The mobile phase consisted of 15 % acetonitrile/85 % Milli-Q water (*v*/v), operating at a flow rate of 1 mL/min. At pH 5, the produced 1,2-diacetin did not suffer acyl migration [71]. The retention times were 18 min for triacetin and 4 min for 1,2-diacetin. Initial reaction rates were determined based on maximal triacetin conversions of 15 % to 20 %. Activity is given as micromoles of produced diacetin per minute.

2.2.2. Immobilization of ETL on octyl agarose beads

Octyl-agarose beads were employed to immobilize ETL through interfacial activation [63], offering 25 mg of enzyme per gram of support (exceeding the loading capacity of the support). Initially, the commercial enzyme solution was diluted in the appropriate volumes of the four different immobilization buffers described in the Introduction section [68]: 50 mM sodium acetate at pH 5, 50 mM sodium acetate /100 mM NaCl at pH 5, 50 mM Tris-Cl plus 10 % dioxane at pH 7, and 50 mM sodium bicarbonate at pH 9. Next, 10 g of support was added to 100 mL of the enzyme solution, and the mixture was continuously stirred at 25 °C using a roller mixer. To track the immobilization process, we measured the enzymatic activities of a solution of the enzyme incubated under the immobilization conditions as a reference, the immobilization suspension, and the supernatant of the immobilization suspension using *p*NPB as described above [72]. After 2 h, the suspensions were filtered, and the immobilized lipase biocatalysts were rinsed thoroughly with distilled water. The biocatalysts were then stored at 4 °C for future use.

The ETL biocatalysts features description may be found in [68]. The purification of lipases after immobilization on octyl-agarose is usually obtained [64].

2.2.3. Modification of immobilized ETL with metals salts

The 4 immobilized ETL biocatalysts were treated with metallic salts following the procedure outlined by Guimarães et al. [70]. For this process, 1 g of the immobilized ETL was suspended in 10 mL of 10 mM sodium phosphate/125 mM NaCl at pH 7.4. After that, 400 μL of a 230 mM solution of the respective metallic chloride was added. Other immobilized lipase samples were incubated in 10 mL of 10 mM Tris/125 mM NaCl at pH 7.4, and then the metal chloride was added. In both instances, the immobilized enzyme modification was carried out at room temperature under gentle stirring for 5 h using an Agimatic-S (JP Selecta) (Barcelona, Spain). After the mineralization treatments, the biocatalysts suspensions were filtered, and the biocatalysts were rinsed five times with 10 volumes of distilled water, and then recovered and stored at 4 °C. Under these conditions, the formation of nanoflowers structures cannot be expected, just interactions between the metallic salts crystals and the enzyme.

2.2.4. Enzyme thermal inactivation at different pH values

Biocatalysts were incubated at pH 5 (10 mM sodium acetate), pH 7 (10 mM sodium phosphate) or pH 7 (10 mM Tris-Cl). The inactivation temperatures were chosen to get a reliable and yet rapid inactivation courses. Residual activity was evaluated periodically using *p*NPB and expressed as a percentage of the initial activity.

3. Results and discussion

3.1. Mineralization of the biocatalysts

As indicated in the Methods section, the 4 immobilized ETL biocatalysts were incubated in solutions containing Tris or sodium phosphate and then, the metal chlorides were added. In the presence of Tris, the biocatalysts did not take any apparent color when using colored metal salts, while when using phosphate, the biocatalysts acquired an intense color according to the used metal (see Fig. 1S and Figs. 2S–3S using one of these biocatalysts as example). This was an obvious difference: when using phosphate there are crystals of metal phosphate, while using Tris, this did not occur. However, this lack of color is not enough to discard that the metal can interact with the immobilized enzyme molecules and alter their properties. That way, the effects of both modifications were analyzed.

3.2. Effect of the pH on the activity versus pNPB of the different immobilized ETL biocatalysts

Table 1 shows the activity of the different unmodified biocatalyst versus *p*NPB in the pH range 4–9 (out of this range the spontaneous *p*NPB hydrolysis was too significant to get reliable results). Table 1 also shows in some instances the effect of changing the measurement buffers. The highest activity was observed at pH 9 and the lowest at pH 4 in all cases. The most active biocatalysts at pH 9 was that prepared at pH 5 in 50 mM sodium acetate (3057 U/g), followed by the biocatalysts immobilized in 50 mM sodium acetate plus 100 mM NaCl also at pH 5 (1932 U/g), that immobilized in 50 mM Tris plus 10 % dioxane (1489 U/g), being the least active one ETL immobilized at pH 9 (541 U/g). This exemplifies the great differences between the 4 different utilized

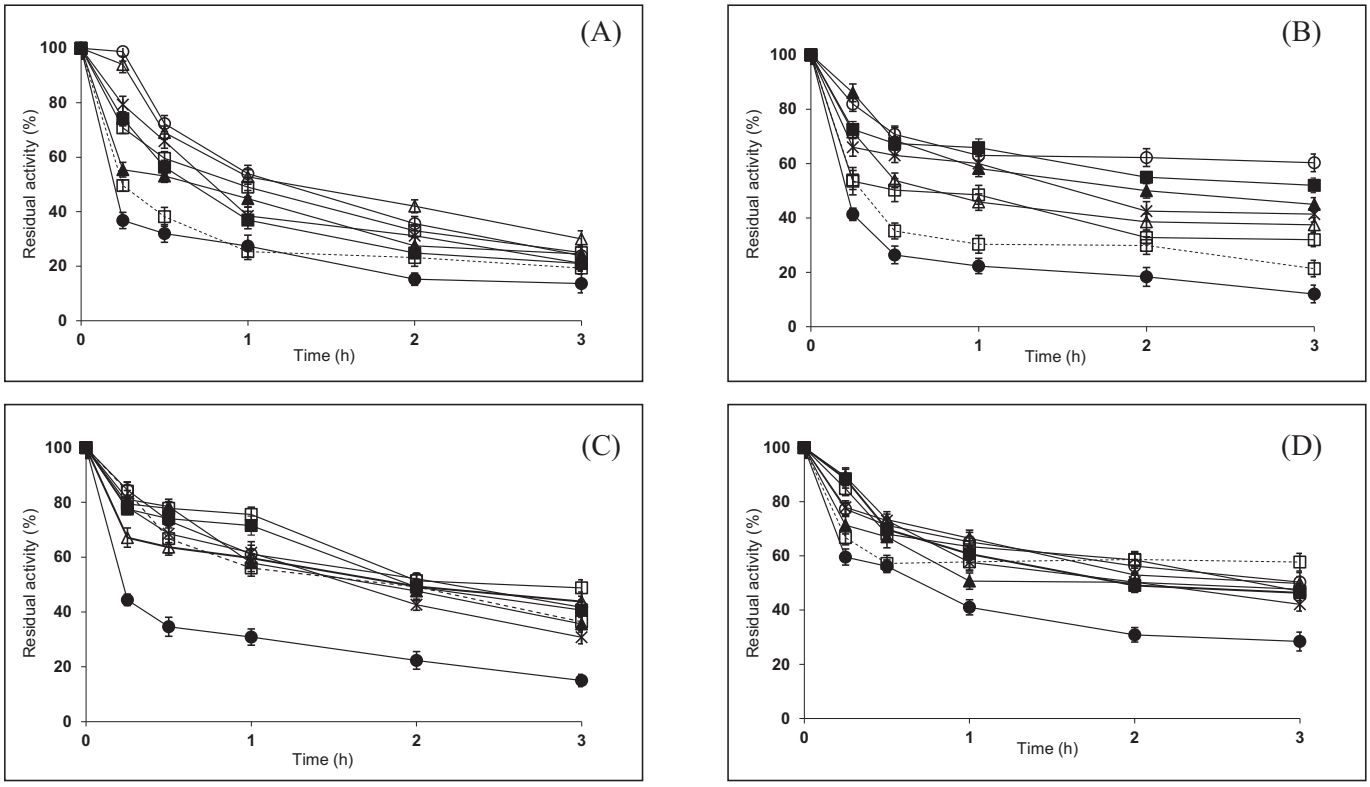

Fig. 1. Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of phosphate. The biocatalysts were inactivated in 25 mM acetate pH 5 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10 % dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9. No mineralized (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

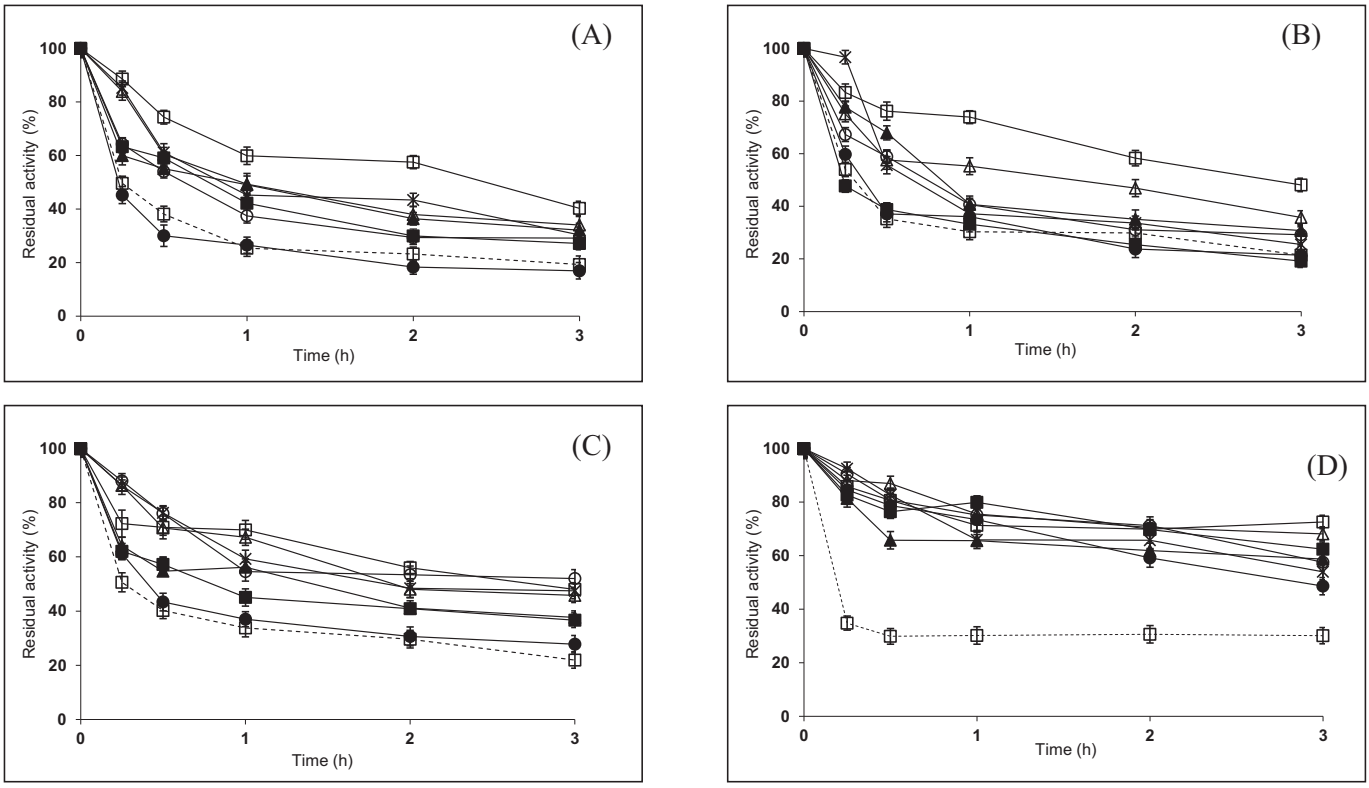

Fig. 2. Inactivation courses of different octyl-ETL biocatalyst modified with different metals in the presence of Tris. The biocatalysts were inactivated in 25 mM acetate pH 5 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10 % dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

biocatalysts [68]. The activities at pH 4 regarding the activities at pH 9 were 8.4 %, 11.3 %, 10.7 % or 26.2 % respectively for the different biocatalysts. At this pH value, even with the different preservation of the enzyme activity, the order of the biocatalysts as a function of their activities were maintained although differences become smaller than at pH 9. Differences in the relative activities could be observed comparing the activities at pH 5. Here the most active enzyme remained the one immobilized at pH 5, but the one immobilized at pH 5 in the presence of NaCl showed the lowest activity, while the enzyme immobilized at pH 7 and pH 9 showed similar activities. In general, at pH values under 7 the enzyme that was immobilized at pH 9 was the least responsible to the changes on the measurement pH values and the enzyme immobilized at pH 5 was the most sensible one.

The change of buffer in the measurements has diverse effects on the activity of the biocatalysts (this activity measurement at two different buffers was performed at pH 6, acetate and phosphate, and pH 8, phosphate and bicarbonate). At pH 6, the change of the buffer presented a negligible effect on the activity of both biocatalyst immobilized at pH 5 and in that immobilized at pH 7 in 10 % dioxane, while the biocatalyst prepared at pH 9 presented 10 % more activity when measuring in acetate. The change of the measuring buffer at pH 8 had more relevant effects, being the activity always higher in bicarbonate, but with diverse intensity: the enzyme immobilized at pH 7 plus 10 % dioxane and the enzyme immobilized at pH 9 were the ones with a smaller activity change, increasing the activity by 14 %–15 %, the enzyme immobilized at pH 5 increased the activity by around 18 %, while the enzyme immobilized at pH 5 plus NaCl increased the activity by 80 %. Again, these data suggested the different properties of the used biocatalysts [68].

3.2.1. *Effect of the pH on the activity versus pNPB of the biocatalyst prepared in 50 mM sodium acetate at pH 5 after mineralization*

Next, we have analyzed the effects on the activity/pH curves of the mineralization of the biocatalyst prepared in 50 mM sodium acetate at pH 5, starting with the biocatalysts mineralized using phosphate (Table 2).

In this instance, the mineralized biocatalysts maintained the maximum activity at pH 9 in all cases, although the shape of curves was significantly altered when comparing the biocatalysts among them and with the reference. That way, although in general a decrease in activity was found after the modification under most conditions, there are some exceptions. The most relevant example was the modification with copper when the activity was determined at pH 9 (increasing the activity of the unmodified biocatalyst by almost 50 %), this modification gave the biocatalyst with the highest activity also at pH 6 in acetate (increasing the activity by 10 %). The modification with Mn^{2+} gave a biocatalyst with the highest activity among all the biocatalysts of this group at pH 5 (increasing the activity of the initial biocatalyst by 8.5 %), while the modification with Co^{2+} produced a slight improvement in the activity at pH 9 (5 %). The least and the most active mineralized biocatalyst depended on the pH and buffer used to measure the activity. The modification with calcium and cobalt produced the most active mineralized biocatalysts if the activity was determined at pH 4, while the least active one was that produced using nickel. At pH 5, the most active biocatalyst was the one modified with manganese, while the active one was that modified with zinc. When determining the activity at pH 6 in acetate, the least active biocatalyst was that mineralized using nickel and the most active was that modified with magnesium. The change of buffer to phosphate (with no effects using the non-mineralized biocatalyst) produced diverse effects on the activity depending on the metal used in the treatment. The treatment with copper and calcium promoted

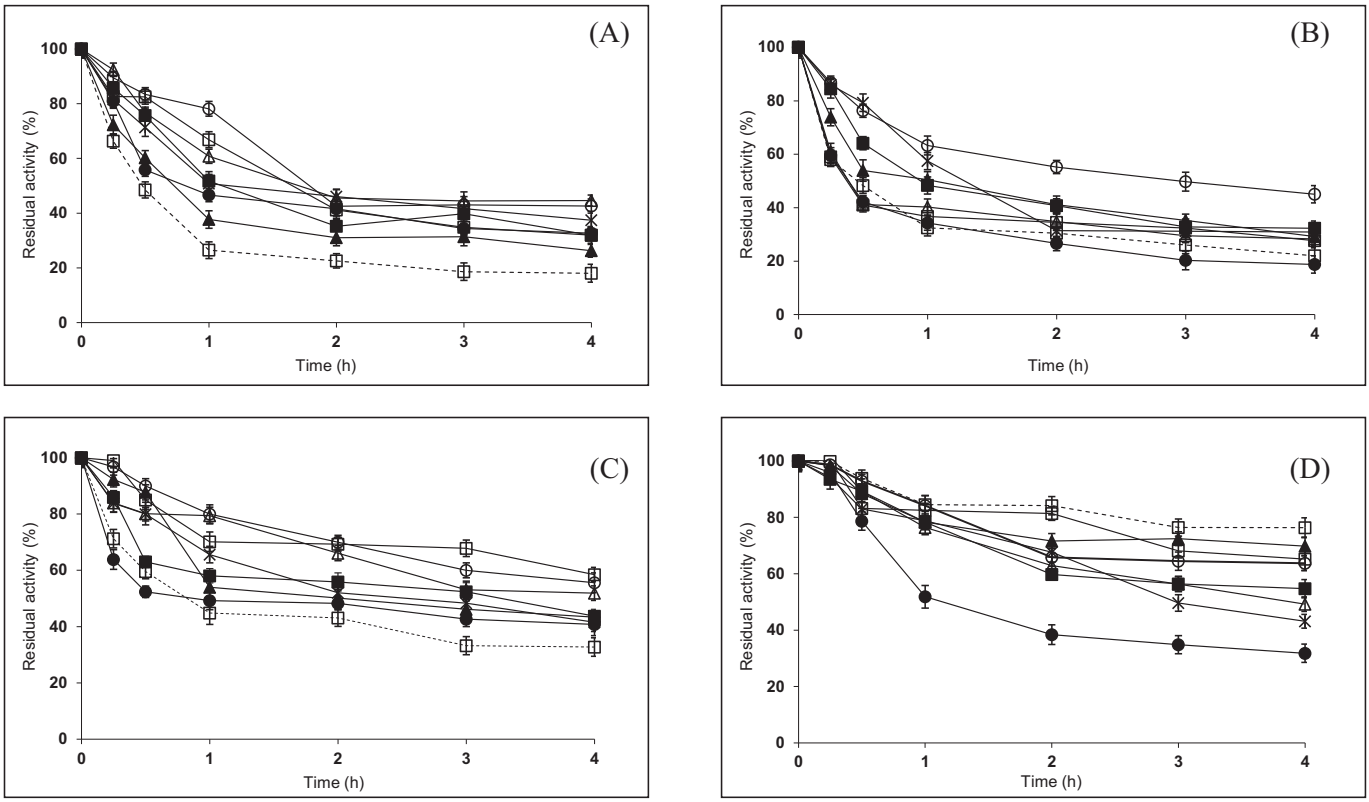

Fig. 3. Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of phosphate. The biocatalysts were inactivated in 25 mM Tris pH 7 and 75 °C. Other specifications are described in Methods. (A octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10 % dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

Table 1
Effect of the pH in the mass *p*NPB activities at 25 °C of different ETL biocatalysts prepared by offering 25 mg of enzyme /g.
Activity is given in micromoles of transformed substrate/ min/ g of biocatalysts. Other specifications are given in Methods section.

Biocatalyst	Reaction conditions							
	25 mM acetate pH 4	25 mM acetate pH 5	25 mM acetate pH 6	25 mM phosphate pH 6	25 mM phosphate pH 7	25 mM phosphate pH 8	25 mM bicarbonate pH 8	25 mM bicarbonate pH 9
50 mM acetate pH 5	256.2 ± 7.8	397.6 ± 9.2	768.1 ± 25.5	768.8 ± 29.7	966.7 ± 38.9	2254 ± 91.1	2654 ± 97.8	3057 ± 101.3
50 mM acetate pH 5 plus 100 mM NaCl	218.1 ± 8.9	275.1 ± 12.8	674.8 ± 17.9	676.5 ± 21.9	830.9 ± 24.9	913.7 ± 54.9	1641 ± 67.9	1932 ± 87.9
50 mM tris pH 7 plus 10 % dio	160.4 ± 6.2	359.7 ± 16.9	475.3 ± 22.8	462.5 ± 19.2	539.3 ± 24.1	1004 ± 48.3	1146 ± 51.5	1489 ± 55.8
50 mM bicarbonate pH 9	141.7 ± 5.0	359.7 ± 16.7	217.2 ± 10.6	197.1 ± 7.2	250.7 ± 10.5	393.7 ± 18.3	451.0 ± 22.7	541.2 ± 25.8

no significant variations, a considerable change was observed for the biocatalyst modified with manganese, which increased the activity by approximately a 50 %. Now, the least active biocatalyst was that modified with magnesium, while the most active was that modified with copper. When determining the activity at pH 7, the treatment with calcium and cobalt produced the most active mineralized biocatalysts, while the least active one was that modified using magnesium (a 2 fold factor was the difference between the activities of these biocatalysts). The lowest decrease in activity at pH 8 in phosphate was obtained by mineralization using copper, cobalt or calcium, while the highest decrease in activity was observed after modification with magnesium. The change from phosphate to bicarbonate at pH 8 produced an increase in all the biocatalysts activity, but the intensity was not the same for all biocatalyst. The biocatalyst with the lowest activity in these conditions remained the one modified with magnesium and the most active was that modified using copper. Finally, at pH 9, the most active biocatalyst was that modified with copper (that even surpassed the activity of the unmodified biocatalyst as stated above), while the modification with magnesium produced the biocatalyst with the lowest activity. Thus, even the response of the mineralized biocatalysts to changes in buffer and pH value was different depending on the metal used in the mineralization.

The incubation of the biocatalysts in the presence of metal chlorides and Tris, even without giving any color, clearly affected the activity of the biocatalysts. Using cobalt, the activity recovery is much lower in this instance (around 50 % than using phosphate). Using zinc, the activity recovery is lower that using cobalt, but the difference with the mineralization in the presence of phosphate is lower (because this biocatalyst exhibited lower activity). Copper gives the highest activity, at pH 9 improving the activity of the unmodified biocatalyst, but the activity is lower than using phosphate, the differences decreasing at pH 4 and 5. Calcium modification decreased the biocatalyst activity compared to the

Table 2
Effect of the pH values in the mass *p*NPB activities at 25 °C of the ETL biocatalyst prepared in acetate at pH 5 after mineralization. The biocatalysts were modified using the conditions described in methods. Activity is given in micromoles of transformed substrate/ min/ g of biocatalysts. Other specifications are described in Methods.

Biocatalyst	Reaction conditions							
	25 mM acetate pH 4	25 mM acetate pH 5	25 mM acetate pH 6	25 mM phosphate pH 6	25 mM phosphate pH 7	25 mM phosphate pH 8	25 mM bicarbonate pH 8	25 mM bicarbonate pH 9
Control	256.2 ± 7.8	397.6 ± 9.2	768.2 ± 25.3	768.8 ± 29.8	966.7 ± 38.9	2254 ± 91.1	2654 ± 97.8	3057 ± 101.4
50 mM acetate pH 5 - cobalt -metal phosphate	208.5 ± 8.2	349.5 ± 7.9	564.6 ± 12.9	604.7 ± 22.6	872.3 ± 32.2	1588 ± 55.9	1995 ± 83.8	3222 ± 120.1
50 mM acetate pH 5 – zinc-metal phosphate	199.2 ± 9.4	272.9 ± 9.7	487.9 ± 17.5	536.1 ± 21.5	720.3 ± 24.4	1222 ± 48.9	1719 ± 79.3	2160 ± 97.7
50 mM acetate pH 5 - copper -metal phosphate	189.9 ± 8.9	343.3 ± 10.1	851.8 ± 35.8	810.1 ± 41.6	888.9 ± 34.0	1597 ± 53.8	2547 ± 101.8	4540 ± 158.9
50 mM acetate pH 5 – calcium -metal phosphate	215.2 ± 10.1	325.2 ± 8.4	686.3 ± 15.7	672.8 ± 23.4	859.8 ± 29.8	1539 ± 81.2	1240 ± 66.9	2673 ± 102.8
50 mM acetate pH 5 - nickel -metal phosphate	173.41 ± 6.4	374.3 ± 10.4	344.9 ± 8.7	588.3 ± 20.8	644.4 ± 21.9	662.5 ± 27.4	729.2 ± 28.9	825.2 ± 27.1
50 mM acetate pH 5 - magnesium - metal phosphate	201.0 ± 8.5	299.1 ± 9.9	325.5 ± 14.9	455.6 ± 18.8	462.6 ± 22.9	402.4 ± 16.3	565.9 ± 14.6	718.4 ± 31.9
50 mM acetate pH 5 - manganese - metal phosphate	198.6 ± 7.3	431.6 ± 19.9	387.7 ± 12.8	738.3 ± 22.9	545.9 ± 13.9	556.7 ± 15.6	797.4 ± 32.5	934.4 ± 55.4
50 mM acetate pH 5 - cobalt - tris	156.7 ± 7.4	230.5 ± 11.8	388.6 ± 14.9	402.7 ± 16.8	493.2 ± 17.6	734.9 ± 30.9	1023 ± 50.4	1695 ± 67.9
50 mM acetate pH 5 - zinc-tris	170.1 ± 8.9	250.5 ± 11.7	461.9 ± 20.6	455.9 ± 18.5	546.6 ± 23.4	801.5 ± 38.9	1653 ± 66.1	2199 ± 89.7
50 mM acetate pH 5 - copper - tris	180.9 ± 8.2	335.7 ± 15.2	659.4 ± 30.2	567.0 ± 24.3	750.5 ± 35.2	932.3 ± 45.7	1748 ± 71.4	3840 ± 54.3
50 mM acetate pH 5 - calcium - tris	198.3 ± 9.2	305.1 ± 13.5	437.7 ± 21.6	466.0 ± 22.6	728.0 ± 34.2	955.6 ± 44.6	1453 ± 65.3	1764 ± 72.7
50 mM acetate pH 5 - nickel - tris	155.0 ± 6.3	224.6 ± 10.6	446.3 ± 21.4	398.3 ± 15.8	459.0 ± 19.6	486.8 ± 22.8	1210 ± 42. 3	1872 ± 74.4
50 mM acetate pH 5 - magnesium - tris	178.0 ± 7.1	242.0 ± 11.5	424.1 ± 20.7	367.4± 16.3	524.4 ± 25.3	622.1 ± 30.2	1145 ± 45.6	2080 ± 86.3
50 mM acetate pH 5 - manganese - tris	172.1 ± 6.2	214.0 ± 10.9	374.9 ± 15.4	376.0 ± 17.4	421.5 ± 19.3	647.2 ± 32.8	910.5 ± 45.6	1255 ± 50.9

same sample measured using bicarbonate, except at pH 8. The metallization with nickel gave mixed results, activity increase or decrease depending on the measure conditions. The biocatalysts activity is higher than when using phosphate at pH 8 and 9 in the measure buffer, and at pH 6 when the activity is determined in acetate (but not in phosphate), being lower under the other measurement conditions. Using magnesium, the activity is higher now than in the other case at pH 7–9 or at pH 6 using acetate to determine the activity (again, no using phosphate), being lower under the other conditions. Using manganese, the enzyme modification in Tris gave more activity at pH 8 and 9 than if the biocatalyst is modified in phosphate.

The results suggest that the metal, even in the absence of phosphate, is able to modify the enzyme features, usually promoting a more significant decrease in enzyme activity under acidic than at alkaline pH value. The metal modification with and without phosphate not only altered the activity and the effect of the pH, also the effect of the buffer on enzyme activity. This is more clearly visualized at pH 6, since some biocatalysts are more active after modification using phosphate metals in one buffer while other biocatalysts submitted to the same modification are less active in that buffer.

That way, metallization of the enzyme clearly affected the enzyme features using the biocatalyst prepared in 50 mM sodium acetate at pH 5, and the effect depended on the metal nature and the presence or not of phosphate during modification, and the measurement conditions.

3.2.2. Effect of the pH on the activity versus pNPB of the biocatalyst prepared in 50 mM sodium acetate plus 100 mM NaCl at pH 5 after mineralization

The biocatalyst prepared in 50 mM sodium acetate plus 100 mM NaCl at pH 5 was less active than in the previous case (see Table 1). Table 3 shows the results of the biocatalyst mineralization. Using phosphate in the modification, at pH 9 the activity usually increases activity, even by 75 % using Cu^{2+}. Exceptions are the mineralization using nickel and magnesium (the activity decrease to 1/3 or its initial value). In Tris, the effect of the incubation with the metals is negative in the *p*NPB activity of the biocatalyst, becoming manganese the metal that produced the lowest activity (down to 70 %). Copper treatment again produces the best results, with a slight increase in enzyme activity (by around 12 %), but become with only 64 % of the activity of the biocatalyst metallized in the presence of phosphate. Measuring the activity at pH 8 in bicarbonate, the effect of the mineralization on enzyme activity was negative in most cases, using Tris or phosphate, the only biocatalyst that increased the activity was that mineralized in Tris using copper. The least active biocatalyst was that prepared in phosphate using magnesium (around 30 % of the activity of the unmodified biocatalyst) and in Tris using manganese (less than 50 %). However, using phosphate at pH 8 to determine the enzyme activity, only the treatments with nickel or magnesium (to around 39 %) using phosphate as buffer in the mineralization produced a decrease in activity. In this instance, the modification with zinc gave the biocatalysts with the higher activity (increasing the activity almost by 60 %). Under these conditions to determine the enzyme activity, mineralization with Tris also produced a decrease on enzyme activity (only copper given an increment in enzyme activity, by only 5 %). Manganese gave the lowest activity (decreasing the activity to 55 %). Using pH 7 to determine the enzyme activity, the results were mixed. The mineralization using phosphate and zinc, calcium, copper (that gave the highest activity, improving the biocatalyst activity by 55 %), promoted an increase on enzyme activity while using cobalt, nickel, manganese or magnesium (the least active biocatalyst, with less than 45 % of the unmodified biocatalyst) produced a decrease in enzyme activity. At this pH incubation with metals and Tris produced a general decrease in enzyme activity, with manganese again producing

Table 3
Effect of the pH values in the mass *p*NPB activities at 25 °C of the ETL biocatalyst prepared at pH 5 in the presence of 100 mM NaCl after mineralization. The biocatalysts were modified using the conditions described in methods. Activity is given in micromoles of transformed substrate/g of biocatalysts. Other specifications are described in Methods.

Biocatalyst	Reaction conditions							
	25 mM acetate pH 4	25 mM acetate pH 5	25 mM acetate pH 6	25 mM phosphate pH 6	25 mM phosphate pH 7	25 mM phosphate pH 8	25 mM bicarbonate pH 8	25 mM bicarbonate pH 9
Control	218.2 ± 8.9	275.2 ± 13.0	674.8 ± 18.1	676.8 ± 21.9	830.9 ± 24.9	913.8 ± 55.0	1641 ± 68.0	1932 ± 88.0
50 mM acetate pH 5 plus 100 mM NaCl -cobalt - metal phosphate	202.3 ± 9.1	304.9 ± 11.0	331.8 ± 14.1	414.1 ± 11.9	695.3 ± 23.9	1296 ± 63.1	1395 ± 83.1	2120 ± 105.1
50 mM acetate pH 5 plus 100 mM NaCl - zinc - metal phosphate	198.7 ± 8.9	319.2 ± 11.2	409.5 ± 17.0	411.6 ± 21.9	957.3 ± 48.9	1435 ± 72.3	1466 ± 67.0	3071 ± 127.0
50 mM acetate pH 5 plus 100 mM NaCl - copper -metal phosphate	235.1 ± 12.1	611.5 ± 32.9	568.0 ± 22.9	527.3 ± 29.5	1273 ± 59.0	1346 ± 63.3	1446 ± 66.0	3401 ± 116.0
50 mM acetate pH 5 plus 100 mM NaCl - calcium - metal phosphate	216.8 ± 11.0	304.7 ± 13.8	343.4 ± 19.0	441.6 ± 21.0	1052 ± 47.6	1174 ± 51.3	1152 ± 46.1	2570 ± 107.1
50 mM acetate pH 5 plus 100 mM NaCl - nickel - metal phosphate	205.5 ± 10.0	369.8 ± 18.0	330.0 ± 16.0	566.3 ± 22.7	535.3 ± 21.0	621.0 ± 30.7	649.8 ± 33.9	878.6 ± 45.9
50 mM acetate pH 5 plus 100 mM NaCl - magnesium -metal - phosphate	162.1 ± 7.9	245.8 ± 11.5	252.5 ± 10.6	405.2 ± 14.9	363.1 ± 13.8	354.6 ± 15.3	487.6 ± 23.0	639.4 ± 30.0
50 mM acetate pH 5 plus 100 mM NaCl - manganese - metal phosphate	191.9 ± 10.0	332.2 ± 16.3	477.6 ± 23.4	400.3 ± 16.0	727.3 ± 29.8	915.5 ± 50.6	1507 ± 68.6	2225 ± 51.3
50 mM acetate pH 5 plus 100 mM NaCl - cobalt - tris	174.0 ± 8.3	248.8 ± 11.7	347.9± 14.8	397.0 ± 19.5	522.2 ± 23.5	696.3 ± 27.2	1197 ± 47.2	1294 ± 51.5
50 mM acetate pH 5 plus 100 mM NaCl - zinc - tris	185.2 ± 8.3	260.9 ± 12.6	395.0 ± 18.1	413.0 ± 20.5	614.4 ± 28.4	757.5 ± 33.9	1265 ± 53.2	1479 ± 63.9
50 mM acetate pH 5 plus 100 mM NaCl - copper - tris	183.5 ± 9.3	360.2 ± 17.5	524.8 ± 25.4	481.2 ± 19.4	795.8 ± 39.0	972.2 ± 48.6	1799 ± 89.0	2176 ± 104.8
50 mM acetate pH 5 plus 100 mM NaCl - calcium - tris	153.6 ± 7.3	234.3 ± 11.5	401.8 ± 28.4	411.9 ± 20.2	610.7 ± 29.3	720.4 ± 35.3	1087 ± 49.2	1442 ± 70.2
50 mM acetate pH 5 plus 100 mM NaCl – nickel - tris	185.6 ± 8.3	305.8 ± 12.6	402.6 ± 18.5	448.3 ± 20.9	650.7 ± 30.4	867.8 ± 42.5	1335 ± 53.4	1942 ± 77.8
50 mM acetate pH 5 plus 100 mM NaCl - magnesium tris	154.3 ± 6.8	233.3 ± 10.4	355.2 ± 15.4	364.9 ± 16.3	529.8 ± 23.7	707.7 ± 33.9	1092 ± 43.2	1504 ± 70.2
50 mM acetate pH 5 plus 100 mM NaCl - manganese - tris	178.4 ± 7.3	212.8 ± 9.9	275.6 ± 12.8	299.5 ± 13.4	400.4 ± 18.6	503.7 ± 22.6	800.4 ± 38.5	1365 ± 54.7

the biocatalyst with the lowest activity (to least than 50 %) and copper the one with the highest (decreasing the activity by only 5 %). At pH 6 in the presence of phosphate, all biocatalysts incubated in metal and phosphate decreased the activity, with cobalt, zinc, magnesium and manganese giving similar results (to around 60 %), giving the biocatalyst modified with nickel the highest activity (83 %). Using Tris in the mineralization, results were also negative in the *p*NPB activity under these conditions, manganese treatment gave the least active biocatalyst (44 %) and copper the most active (more than 70 %). Changing the buffer for acetate, the effect of the modification was also negative for the enzyme activity using both phosphate or Tris in the mineralization. Copper gave the highest activity when using phosphate in the mineralization (almost 85 %) or Tris (almost 78 %) the least activity biocatalyst being that prepared using magnesium and phosphate (37 %) or Tris and manganese (40 %).

Determination of the enzyme activity at pH 5 showed a different picture. Now, the mineralization in the presence of phosphate increased the enzyme activity (with maximum increase using copper (increasing the activity 2.2-fold), only magnesium treatment decreasing the activity (to almost 90 %). Using Tris in the mineralization, only copper (by 30 %) and nickel (by 11 %) treatment increased enzyme activity, manganese giving the biocatalyst with the lowest activity (more than 75 %). Finally, the determination of the activity at pH 4 shows that the biocatalyst activity is not very affected when using phosphate in the mineralization, being copper the only slightly increasing enzyme activity (by more than 7 %) and magnesium giving the biocatalyst with the lowest activity (to around 75 %). If the modification was performed in Tris, the effect was negative for all biocatalysts, zinc, nickel and copper giving the highest activity (around 85 %) and calcium the lowest (71 %).

Again, metallization of the enzyme clearly affected the enzyme features using this biocatalyst, and the effect depends on the metal nature and the presence or not of phosphate during the modification, and the measure conditions. Although some similarities may be found with the results using the other biocatalyst, also many differences may be observed, suggesting that the effects of the mineralization also depends on the exact conformation of the enzyme,

3.2.3. Effect of the pH on the activity versus pNPB of the biocatalyst prepared in 50 mM Tris plus 10 % dioxane at pH 7 after mineralization

Then, the biocatalyst prepared at pH 7 in 10 % dioxane was submitted to mineralization in phosphate and Tris and the activity/pH curve analyzed (Table 4). The effect of the mineralization in phosphate on the activity at pH 9 is negative using all metals, cobalt is the one providing the highest activity (almost 85 % of the activity of the unmodified biocatalysts) with magnesium being the lowest one (around 45 %). If the modification was performed in Tris, the results were mixed. While using zinc and cupper the activity increased slightly (by 5 %), cobalt presented a low impact, and the other promoted a decrease in enzyme activity, with the biocatalysts incubated in manganese exhibiting the lowest activity at pH 9 (65 % of that of the unmodified

Table 4
Effect of the pH values in the mass *p*NPB activities at 25 °C of the ETL biocatalyst prepared at pH 7 in the presence of 10 % dioxane after mineralization. The biocatalysts were modified using the conditions described in methods. Activity is given in micromoles of transformed substrate/ min/g of biocatalysts. Other specifications are described in Methods.

Biocatalyst	Reaction conditions							
	25 mM acetate pH 4	25 mM acetate pH 5	25 mM acetate pH 6	25 mM phosphate pH 6	25 mM phosphate pH 7	25 mM phosphate pH 8	25 mM bicarbonate pH 8	25 mM bicarbonate pH 9
50 mM tris pH 7 plus 10% dio	160.4 ± 6.2	359.7 ± 16.9	475.3 ± 22.8	462.9 ± 19.2	539.3 ± 24.1	1004 ± 48.3	1146 ± 51.5	1489 ± 55.9
50 mM tris pH 7 plus 10% dio - cobalt- metal phosphate	180.3 ± 7.3	231.1 ± 10.4	266.6 ± 11.9	328.6 ± 14.7	476.8 ± 16.9	708.3 ± 33.2	891.7 ± 33.1	1261 ± 51.8
50 mM tris pH 7 plus 10% dio - zinc- metal phosphate	245.6 ± 11.5	228.4 ± 8.7	363.6 ± 14.9	267.6 ± 13.3	359.7 ±15.8	520.9 ± 26.1	843.8 ± 36.1	1097 ± 50.1
50 mM tris pH 7 plus 10% dio - copper- metal phosphate	190.8 ± 9.1	200.9 ± 8.7	293.5 ± 12.4	382.5 ± 17.1	519.7 ± 22.1	1017 ± 55.8	854.7 ± 40.3	1055 ± 49.1
50 mM tris pH 7 plus 10% dio - calcium- metal phosphate	171.9 ± 8.8	232.4 ±12.9	330.4 ± 12.5	292.2 ± 10.2	365.5 ± 15.3	462.8 ± 24.1	961.5 ± 46.9	1007 ± 44.1
50 mM tris pH 7 plus 10% dio - nickel- metal phosphate	163.2 ± 6.4	205.0 ± 8.1	357.6 ± 16.4	298.7 ± 13.9	411.6 ± 20.1	480.5 ± 20,1	602.7 ± 27.9	852.8 ± 42.1
50 mM tris pH 7 plus 10% dio - magnesium- metal phosphate	160.4 ± 6.9	191.8 ± 8.5	350.4 ± 14.8	252.1 ± 12.5	345.7 ± 14.1	422.6 ± 19.1	502.6 ± 24.4	691.3 ± 34.1
50 mM tris pH 7 plus 10% dio - manganese- metal phosphate	160.4 ± 7.2	212.7 ± 9.2	399.7 ± 18.4	339.4 ± 15.7	450.6 ± 23.8	628.7 ± 30.1	680.1 ± 31.7	933.3 ± 45.1
50 mM tris pH 7 plus 10% dio - cobalt- tris	151.4 ± 6.3	229.1 ± 9.1	307.7 ± 14.9	366.7 ± 13.9	423.0 ± 20.1	602.2 ± 25.3	767.8 ± 30.6	1402 ± 56.9
50 mM tris pH 7 plus 10% dio - zinc- tris	156.1 ± 6.2	219.0 ± 9.3	308.6 ± 15.2	362.3 ± 16.9	512.7 ± 24.7	584.6 ± 40.6	730.5 ± 33.9	1542 ± 70.5
50 mM tris pH 7 plus 10% dio - copper- tris	197.4 ± 8.4	351.4 ±15.3	463.7 ± 20.4	416.3 ± 18.3	664.6 ± 31.5	801.4 ± 33.5	989.7 ± 45.2	1589 ± 63.5
50 mM tris pH 7 plus 10% dio - calcium - tris	153.4 ± 6.7	236.8 ± 10.4	318.8 ± 13.3	317.0 ± 14.5	491.8 ± 24.1	629.4 ± 30.2	745.4 ± 35.2	1211 ± 55.3
50 mM tris pH 7 plus 10% dio - nickel- tris	139.1 ± 5.2	230.2 ± 11.5	399.4 ± 17.3	310.5 ± 14.9	477.3 ± 20.1	583.9 ± 28.4	794.6 ± 35.2	980.4 ± 45.2
50 mM tris pH 7 plus 10% dio - magnesium- tris	161.2 ± 6.9	158.4 ± 7.1	342.1 ± 15.9	356.8 ± 16.2	426.1 ± 20.0	573.6 ± 26.3	728.5 ± 33.1	1267 ± 60.0
50 mM tris pH 7 plus 10% dio - manganese- tris	144.8 ± 5.2	209.7 ± 9.4	283.4 ± 13.3	260.5 ± 12.4	335.1 ± 14.2	445.6 ± 20.7	451.1 ± 19.8	919.3 ± 39.2

biocatalyst). Measuring the activity at pH 8 in bicarbonate, the decrease in activity after mineralization was more prominent, calcium permitted to maintain the highest activity (almost 85 %) while magnesium was again the metal providing the higher losses in activity (to 45 %). If the incubation with the metal was performed in Tris, the situation was similar, a general decrease in enzyme activity was observed, the smallest using copper (slightly over 85 %) and the highest using manganese (just over 35 %). The change of buffer to phosphate at pH 8 in the activity determination change the picture. The modification with phosphate and copper had almost no effect on enzyme activity, but in general, the decrease in enzyme activity was more important than the mineralization in the presence of phosphate than using bicarbonate in the activity determination, although now the lowest activity was exhibited by the biocatalyst modified with magnesium (40 %), with a slightly higher retention of activity percentage than the least active biocatalyst measuring in bicarbonate. The metal modification in Tris also produced a decrease in enzyme activity, copper modification permitted to maintain the highest percentage of activity (80 %) and manganese modification gave the lowest activity (under 45 %). Measuring at pH 7, the mineralization in the presence of phosphate produced a decrease in enzyme activity, the most active biocatalyst remained that incubated with copper, while the least active ones were those incubated with zinc, calcium and magnesium (around 65 %), the most active was that modified with copper (over 95 %). In the presence of Tris during mineralization, the activity decreased also in all cases except when using copper (activity increased by 20 %). The lowest activity was obtained after incubation in manganese (more than 60 %). Measuring the activity at pH 6 in phosphate as buffer, the activity of the biocatalysts decreased after mineralization in both, Tris or phosphate (in a higher extension). In the presence of phosphate during mineralization, the activities of the biocatalysts ranged from 82 % (using copper) to 54 % (using magnesium). Using Tris in the mineralization, the activities ranged from 90 % (using copper) to 56 % (using manganese). The use of acetate at pH 6 maintained the negative effect of the modification with metals in the presence of phosphate. In this instance, the highest activity was observed using the biocatalyst modified with manganese (almost 85 %) while the lowest one was observed using cobalt in the modification (56 %), followed by that modified with copper (that usually gave the highest activities). If metal incubation was performed using Tris, the incubation with copper left the enzyme activity almost unaltered, while manganese produced a decrease in the enzyme activity to 60 %). When determining the enzyme activity at pH 5, only the incubation of this biocatalyst in copper and Tris maintained the initial activity, all the other metals and buffers decreased the activity, to 53 % using phosphate and magnesium and to 44 % using Tris and magnesium. When determining the activity at pH 4 the situation becomes fully different. The incubation of the biocatalyst with phosphate and cobalt, copper, calcium, and mainly zinc (more than 1.5-fol) increase the enzyme activity, while nickel, magnesium and manganese had no effect on enzyme activity. Situation was different if incubating the enzyme in metal and Tris. Using copper, the enzyme activity increased by 23 %, magnesium had no effect and only manganese (by 10 %) and nickel (by less than 15 %) produced a relevant decrease on enzyme activity.

3.2.4. Effect of the pH on the activity versus pNPB of the biocatalyst prepared in 50 mM bicarbonate at pH 9 after mineralization

Finally, we have analyzed the effects of the mineralization on the *p*NPB activity/pH profile of the biocatalyst prepared at pH 9 (Table 5). The mineralization in the presence of phosphate presented a mixed effect when determining the activity at pH 9, the cobalt, zinc, calcium and

Table 5
Effect of the pH values in the mass pNPB activities of the ETL biocatalysts prepared at pH 9 after mineralization. The biocatalysts were modified using the conditions described in methods. Activity is given in micromoles of transformed substrate/ min/ g of biocatalysts. Other specifications are described in Methods.

Biocatalyst	Reaction conditions							
	25 mM acetate pH 4	25 mM acetate pH 5	25 mM acetate pH 6	25 mM phosphate pH 6	25 mM phosphate pH 7	25 mM phosphate pH 8	25 mM bicarbonate pH 8	25 mM bicarbonate pH 9
50mM bicarbonate pH 9	141.7 ± 5.0	359.1 ± 16.3	217.2 ± 10.6	197.1 ± 7.2	250.7 ± 10.5	393.7 ± 18.3	451.1 ± 22.7	541.2 ± 25.1
50mM bicarbonate pH 9- cobalt- metal phosphate	175.0 ± 8.9	176.4 ± 7.1	288.2 ± 12.5	179.5 ± 8.1	244.5 ± 11.5	332.4 ± 15.9	578.6 ± 30.1	639.6 ± 30.1
50mM bicarbonate pH 9- zinc- metal phosphate	176.8 ± 6.1	214.1 ± 9.0	252.2 ± 11.7	185.8 ± 9.1	271.7 ± 13.2	365.2 ± 14.1	647.6 ± 36.7	877.1 ± 41.5
50mM bicarbonate pH 9- copper- metal phosphate	177.2 ± 7.6	185.5 ± 7.1	293.1 ± 13.2	195.8 ± 8.7	337.7 ± 15.1	367.7 ± 16.9	761.8 ± 37.1	912.1 ± 44.1
50mM bicarbonate pH 9- calcium- metal phosphate	187.6 ± 8.6	248.9 ± 11.5	243.1 ± 10.9	209.8 ± 9.9	313.9 ± 15.6	342.7 ± 16.3	119.3 ± 5.7	750.4 ± 30.7
50mM bicarbonate pH 9- nickel- metal phosphate	142.5 ± 6.1	162.3 ± 7.3	227.8 ± 10.9	180.4 ± 8.7	240.3 ± 11.9	255.5 ± 13.9	311.4 ± 15.2	379.2 ± 18.3
50mM bicarbonate pH 9- magnesium- metal phosphate	137.1 ± 5.1	155.2 ± 6.2	231.4 ± 10.9	191.4 ± 8.1	191.5 ± 9.2	291.3 ± 13.6	324.7 ± 14.8	462.4 ± 22.8
50mM bicarbonate pH 9- manganese- metal phosphate	150.4 ± 7.4	164.5 ± 8.2	232.4 ± 11.1	203.6 ± 9.2	271.9 ± 13.9	334.2 ± 15.9	334.5 ± 14.2	457.9 ± 23.2
50mM bicarbonate pH 9- cobalt- tris	133.4 ± 5.3	155.2 ± 7.5	165.3 ± 6.2	160.7 ± 5.2	176.6 ± 8.2	216.7 ± 9.2	291.1 ± 13.2	427.2 ± 20.5
50mM bicarbonate pH 9- zinc- tris	163.7 ± 7.2	166.1 ± 5.2	183.9 ± 8.1	158.9 ± 6.9	198.5 ± 8.5	262.2 ± 12.4	292.9 ± 14.3	437.8 ± 20.1
50mM bicarbonate pH 9- copper- tris	130.9 ± 5.5	164.5 ± 6.3	189.3 ± 7.5	162.7 ± 6.9	183.1 ± 8.0	226.3 ± 10.5	248.4 ± 11.8	432.7 ± 21.3
50mM bicarbonate pH 9- calcium- tris	140.9 ± 5.7	168.4 ± 6.3	193.6 ± 8.5	162.7 ± 7.2	199.4 ± 9.5	269.2 ± 12.9	359.1 ± 17.4	479.8 ± 20.4
50mM bicarbonate pH 9- nickel- tris	147.5 ± 5.9	167.5 ± 8.2	195.6 ± 9.1	154.9 ± 7.1	184.1 ± 7.4	211.1 ± 9.1	291.1 ± 14.2	434.6 ± 20.4
50mM bicarbonate pH 9- magnesium- tris	155.5 ± 7.1	162.3 ± 7.9	185.1 ± 8.1	178.6 ± 8.6	178.6 ± 8.4	222.2 ± 10.3	288.8 ± 13.9	503.3 ± 24.1
50mM bicarbonate pH 9- manganese- tris	135.5 ± 5.3	143.8 ± 6.5	191.3 ± 8.8	153.3 ± 7.1	184.1 ± 9.1	208.7 ± 9.1	291.5 ± 13.7	433.5 ± 20.4

specially copper increased the activity (the last one by almost 70 %), while magnesium, manganese and specially nickel (to 68 %) decreased the activity. When decreasing the pH to 8, the effects were similar in the metals that promoted an increment on enzyme activity, while the 3 that decreased their activity at pH 9, also decreased the activity at pH 8, but with similar activities (ranging from 68 % to 74 %). The change of the buffer during the activity determination to phosphate produced that the mineralized biocatalyst decreased the activity compared to the reference, with the highest activity retention using copper and zinc (around 93 %) and the minimal using nickel (65 %). The activity determination at pH 7 changed the picture. Cobalt and nickel treatments had little effect on enzyme activity, copper treatment increased the activity by almost 35 % while magnesium decreased the activity by almost 25 %. Manganese treatment produced an increased activity (almost by 10 %) in contrast with the previous results. Determining the activity at pH 6 in phosphate, the mineralization produced a slight decrease in activity using cobalt, zinc, nickel (the least active biocatalyst, with a 90 % activity), copper and magnesium almost produced no changes in activity, while manganese and calcium produced slight increases (by around 5 %). The use of acetate at pH 6 as activity determination buffer changed the situation, now all mineralized biocatalysts were more active than the control, the copper treatment giving the highest activity (increasing the activity by 35 %). The activity at pH 5 suffered a decrease in all cases for the mineralized biocatalysts (except for that modified with calcium) when compared with the activity at pH 6, while the control increased the activity. Thus, only the modification with calcium almost maintained the activity intact, while the treatments with nickel, magnesium and manganese gave the lowest activities (lower than 50 %). At pH 4, nickel and magnesium maintained the activity, while all the others increased it (reaching an increase of over 30 % with copper).

If during mineralization the buffer was Tris, the situation was different. Measuring the activity at pH 9 all biocatalysts decreased their activity, ranging from 92 % (using magnesium) to 80 % (using copper, nickel and manganese). At pH 8 in bicarbonate, the activities decreased in a more significant way, moving from 64 % (using cobalt, zinc, nickel, magnesium or manganese) to 55 % (using copper). Calcium treatment was the exception, with more than 7 % increase in the activity under these conditions. The change at pH 8 to phosphate produced a general decrease in the activity of the mineralized biocatalyst, while having almost no effect in the control, with activates in the range 50–55 %, except the biocatalyst treated with zinc, that maintained more than 65 %. The activity at pH 7 also decreased after tis mineralization, with activities ranging from 80 % (using zinc or calcium) to 70 % (using magnesium or cobalt). At pH 6 in phosphate, the mineralization in Tris also produced a decrease in activity for all biocatalyst, the change to acetate slightly reducing in general the decrease in activity. The use of pH 5 even accentuated the decrease in enzyme activity caused by the mineralization (to 40 % for the biocatalyst treated with manganese) to 45 % for the biocatalyst-modified calcium). The activity at pH 4 is the only that gave mixed results, with zinc improving the activity by 15 % or by 10 % using magnesium, while the lowest activities were obtained modifying this biocatalyst with cobalt or manganese (to 93 %).

Taking together the results in all this heading (section 3.2.), it seems that the biocatalysts altered their properties when using phosphate but also using Tris (in some instances with more intensity), but in a dissimilar way. The effects of the mineralization on the enzyme activity depends on the buffer used in the incubation, on the metal nature, but also on the biocatalyst and on the measurement conditions. That is, it looks like these biocatalysts, prepared on the same support and that only differ on the enzyme structure, present a dissimilar response to the mineralization.

3.3. Effect of the metallization on the activity versus triacetin

Using triacetin as substrate, the differences in activity between the 4 initial biocatalysts were not so different as versus *p*NPB (ranging 11 % between the most and the least active biocatalyst) (Table 6). Now, the least active preparations were both prepared at pH 5, being the other two biocatalysts more active and with similar activity (in contradiction with the results using pNPB). These results again pointed that the different enzyme features was quite different.

Table 6
Mass activities versus 50 mM triacetin at pH 5 and 25 °C of different ETL biocatalysts prepared as described in Methods Activity is given in micromoles of transformed substrate/min/ g of biocatalysts. Other specifications are described in Methods.

Biocatalyst	U/g – modified with metal phosphate	U/g – modified with Tris
Octyl-ETL-50 mM acetate pH 5	348.1 ± 13.9	348.1 ± 13.9
Octyl-ETL-50 mM acetate pH 5 – cobalt	327.3 ± 14.8	354.3 ± 20.9
Octyl-ETL-50 mM acetate pH 5 -zinc	340.8 ± 15.2	298.3 ± 16.2
Octyl-ETL-50 mm acetate ph 5 -copper	411.6 ± 19.2	295.3 ± 11.8
Octyl-ETL-50 mM acetate pH 5 -calcium	387.0 ± 16.2	340.8 ± 13.2
Octyl-ETL-50 mM acetate pH 5 -nickel	344.7 ± 13.2	378.7 ± 15.9
Octyl-ETL-50 mM acetate pH 5 -magnesium	367.6 ± 17.3	600.6 ± 24.3
Octyl-ETL-50 mM acetate pH 5 -manganese	204.7 ± 9.8	391.4 ± 18.1
Octyl-ETL-50 mM acetate pH 5 - plus 100 mM NaCl	354.1 ± 16.9	354.1 ± 16.8
Octyl-ETL-50 mM acetate pH 5 plus 100 mM NaCl - cobalt	313.4 ± 13.5	397.1 ± 19.4
Octyl-ETL-50 mM acetate pH 5 plus 100 mM NaCl - zinc	259.6 ± 12.3	417.8 ± 20.1
Octyl-ETL-50 mM acetate pH 5 plus 100 mM NaCl - copper	729.4 ± 34.8	338.7 ± 14.4
Octyl-ETL-50 mM acetate pH 5 plus 100 mM NaCl - calcium	337.3 ± 14.6	388.7 ± 15.1
Octyl-ETL-50 mM acetate pH 5 plus 100 mM NaCl - nickel	331.7 ± 16.3	357.7 ± 16.5
Octyl-ETL-50 mM acetate pH 5 plus 100 mM NaCl - magnesium	303.1 ± 14.3	377.6 ± 15.3
Octyl-ETL-50 mM acetate pH 5 plus 100 mM NaCl - manganese	344.4 ± 15.3	382.4 ± 15.1
Octyl-ETL-50 mM tris pH 7 - plus 10 % dio	386.3 ± 14.8	386.3 ± 14.8
Octyl-ETL-50 mM tris pH 7 plus 10 % dio - cobalt	322.5 ± 13.2	354.4 ± 12.3
Octyl-ETL-50 mM tris pH 7 plus 10 % dio - zinc	300.2 ± 12.4	318.8 ± 11.8
Octyl-ETL-50 mM tris pH 7 plus 10 % dio - copper	371.5 ± 14.5	334.1 ± 13.3
Octyl-ETL-50 mM tris pH 7 plus 10 % dio - calcium	337.8 ± 17.3	311.5 ± 11.6
Octyl-ETL-50 mM tris pH 7 plus 10 % dio - nickel	312.2 ± 13.2	334.8 ± 12.9
Octyl-ETL-50 mM tris pH 7 plus 10 % dio - magnesium	392.1 ± 19.3	248.6 ± 9.9
Octyl-ETL-50 mM tris pH 7 plus 10 % dio - manganese	377.6 ± 14.3	375.3 ± 15.3
Octyl-ETL-50 mM bicarbonate pH 9	386.8 ± 15.4	386.8 ± 15.4
Octyl-ETL-50 mM bicarbonate pH 9 - cobalt	341.2 ± 13.9	246.4 ± 10.1
Octyl-ETL-50 mM bicarbonate pH 9 - zinc	307.9 ± 12.5	385.4 ± 15.7
Octyl-ETL-50 mM bicarbonate pH 9 - copper	416.2 ± 18.3	287.5 ± 9.8
Octyl-ETL-50 mM bicarbonate pH 9 - calcium	253.9 ± 11.4	387.2 ± 14.7
Octyl-ETL-50 mM bicarbonate pH 9 - nickel	392.1 ± 13.2	327.2 ± 13.0
Octyl-ETL-50 mM bicarbonate pH 9 - magnesium	333.8 ± 12.2	316.5 ± 12.6
Octyl-ETL-50 mM bicarbonate pH 9 - manganese	222.1 ± 9.0	325.1 ± 12.9

Next, we have analyzed the effects of the mineralization performed in phosphate and Tris. The effects of these treatments were quite different depending on the used biocatalysts, but they were not as relevant as using *p*NPB.

Using the enzyme immobilized at pH 5 in 50 mM sodium acetate, the incubation in metal and phosphate produced scarce changes in enzyme activity (less than 10 %) except using Cu^{2+}, that increase the activity by 18 %, or Mn^{2+}, that decreased the activity by more than 40 %. The incubation with metal in Tris gave as the most significant results a decrease in activity using Zn^{2+} (87 %) and Cu^{2+} (85 %) (that usually was the metal giving the highest activates using *p*NPB, see section above) and an increase using Mg^{2+} (171 %) (usually among the biocatalyst with the lowest actives using *p*NPB, see section above).

The result was fully different using different biocatalyst. If in the immobilization at pH 5 we added 100 mM NaCl, Co^{2+} (88 %), Zn^{2+} (73 %) and Mg^{2+} (86 %) produced a decrease in activity if added in presence of phosphate, while Cu^{2+}produced an increase in enzyme activity (210 %). The addition of the metal in Tris, produced in most cases biocatalysts with higher activities, but not as high as when using Cu^{2+} in the presence of phosphate: Co^{2+} (112 %), Zn^{2+} (117 %), Ca^{2+} (110 %) or Co^{2+} (108 %). That way, not only it is clear the different results using Tris or phosphate, but the different reaction of the diverse biocatalysts to metallization, the main objective of this paper, suggesting that the initial enzyme structure could be very important for the final effects of the mineralization.

Using the biocatalyst prepared in the presence of 50 mM Tris plus 10 % dioxane at pH 7, the treatments produced a general moderate decrease of enzyme activity except using Mg^{2+} (102 %) or Mn^{2+} (97 %) when the treatment was in the presence of phosphate or Mn^{2+} in Tris (97 %). The most significant decreases in activity when using phosphate were observed using Zn^{2+} (78 %) Ni^{2+} (80 %) or Co^{2+} (83 %). If the treatment was performed using Tris, the lowest activity was found using Mg^{2+} (64 %), Ca^{2+} (80 %) or Zn^{2+} (82 %). Again, results were different depending if crystals were formed or not, and different to the ones found using the other biocatalysts.

Finally, the biocatalyst immobilized at pH 9 when treated with metals decreased the activity except some exceptions. Using phosphate, the activity increased using Cu^{2+} (108 %) and remained unchanged using Ni^{2+}, decreasing the activity in the other cases, being the decrease in activity more significant than that observed using Ca^{2+} (65 %) or using Mn^{2+} 57 %). Using Tris, the activity was maintained using Zn^{2+} and Ca^{2+}, and significantly decreased using Co^{2+} (63 %) and Cu^{2+} (74 %).

That way, it seems evident that the incubation of the enzymes in metals, in Tris or in phosphate, can alter the enzyme features, although in different ways. These changes also differ of that observed using *p*NPB.

Most importantly in the context of our main goal, the effects of both treatments are seriously depended on the enzyme conformation, as the support and the enzyme are the same.

3.4. Inactivation of the different ETL biocatalysts under different conditions

The different biocatalyst were inactivated under diverse pH conditions, that could alter the ionization statutes of the different ionic groups of the enzyme and the metal salts altering the enzyme-salts interactions (pH 5 in acetate and pH 7 in Tris). Moreover, the biocatalysts were inactivated at pH 7 in the presence of sodium phosphate, an anion described to have a negative effect on the octyl-ETL stability [73–76]. It should be remaindered that the enzyme immobilization on octyl-agarose increase the enzyme stability [77]. Furthermore, the commercial preparation of Eversa is quite pure, and this purity was further increased

after immobilization on octyl-agarose [77].

First, we have compared the stabilities of the 4 initial biocatalysts under these 3 inactivation conditions (Fig. 4S). In all cases, the most stable biocatalyst was the biocatalyst prepared at pH 9, although the difference at pH 5 is smaller than at pH 7 in phosphate or Tris (where the differences were maximal). The second most stable catalyst was that prepared at pH 7 in 10 % dioxane (at pH 5 is near to the biocatalyst prepared at pH 9), while both biocatalysts prepared at pH 5 exhibited similar inactivation courses, being the least stable ones. This shows that also in terms of stability the different biocatalysts exhibited different properties, although that prepared at pH 5, which, as discussed above, presented very different activities, presented now similar stabilities.

Fig. 1 shows the results of the inactivation at pH 5 of the 4 different biocatalysts after incubation with the different metals in the presence of phosphate. The effect of the metal treatments on enzyme stability is clear and depends on the initial biocatalyst. Eversa immobilized on 50 mM sodium acetate at pH 5 increased the stability after modification with all metal phosphate salts, except copper, that slightly decreased the stability. The most stable preparation was that obtained after modification with zinc. The situation changes when the treated biocatalyst was Eversa immobilized in 50 mM sodium acetate at pH 5 plus 100 mM NaCl. Now, the stabilizations caused by the mineralization are more relevant, the most stable biocatalyst is that modified with magnesium, while the biocatalyst modified with zinc was in the middle. Copper remained as the metal that produced the biocatalyst with the lowest stability. Eversa immobilized in 50 mM Tris at pH 7 plus 10 % dioxane gave different response to the mineralization, now the mineralization presented a scarce effect on enzyme stability, except copper that produced a series of decreases on enzyme stability. Eversa immobilized on 50 mM sodium bicarbonate at pH 9 was the biocatalyst with smaller changes in enzyme stability, except using copper, which again promoted a destabilization. Fig. 5S shows the comparison of the inactivation courses of the most and least stable metallized biocatalysts compared to the unmodified biocatalysts, where it is easier to appreciate the large differences in the effect of the mineralization of the different biocatalysts that only differs on enzyme conformation (enzyme, support and orientations are identical) [68].

Fig. 2 shows the results of the inactivation at pH 5 of the 4 different biocatalysts after their mineralization by incubation with the different metals in Tris. The incubation of the biocatalysts in Tris/metal solutions offers a fully different picture in some instances in the inactivation courses at pH 5 compared to the biocatalyst previously incubated in metal and phosphate. The negative effect of copper is now minimized, in fact the biocatalyst prepared at pH 9 improved its stability after copper mineralization, and the other biocatalysts presented similar stability after copper mineralization to the unmodified biocatalysts (leaving the copper modified biocatalysts as the one with the lowest stability), while for the other 3 biocatalysts, the stability increased after metal incubation. It remained the least stable mineralized biocatalyst for all biocatalysts except that prepared at pH 5 plus 100 mM NaCl, in this case its stability was between the other biocatalysts stabilities. Fig. 6S illustrates the inactivation profiles of the most and least stable metallized biocatalysts in comparison to the unmodified biocatalysts for an easier visualization.

This shows that the incubation with metals, both in Tris and phosphate, greatly alters enzyme stability, and these effects depended on the initial biocatalyst.

Fig. 3 shows the inactivation in the Tris at pH 7 of the 4 different biocatalysts after incubation with the different metals in the presence of phosphate. Results strongly differ from the inactivation at pH 5. The treatment with copper remained with a negative effect for the biocatalysts prepared at pH 5 plus 100 mM NaCl, and even with much more

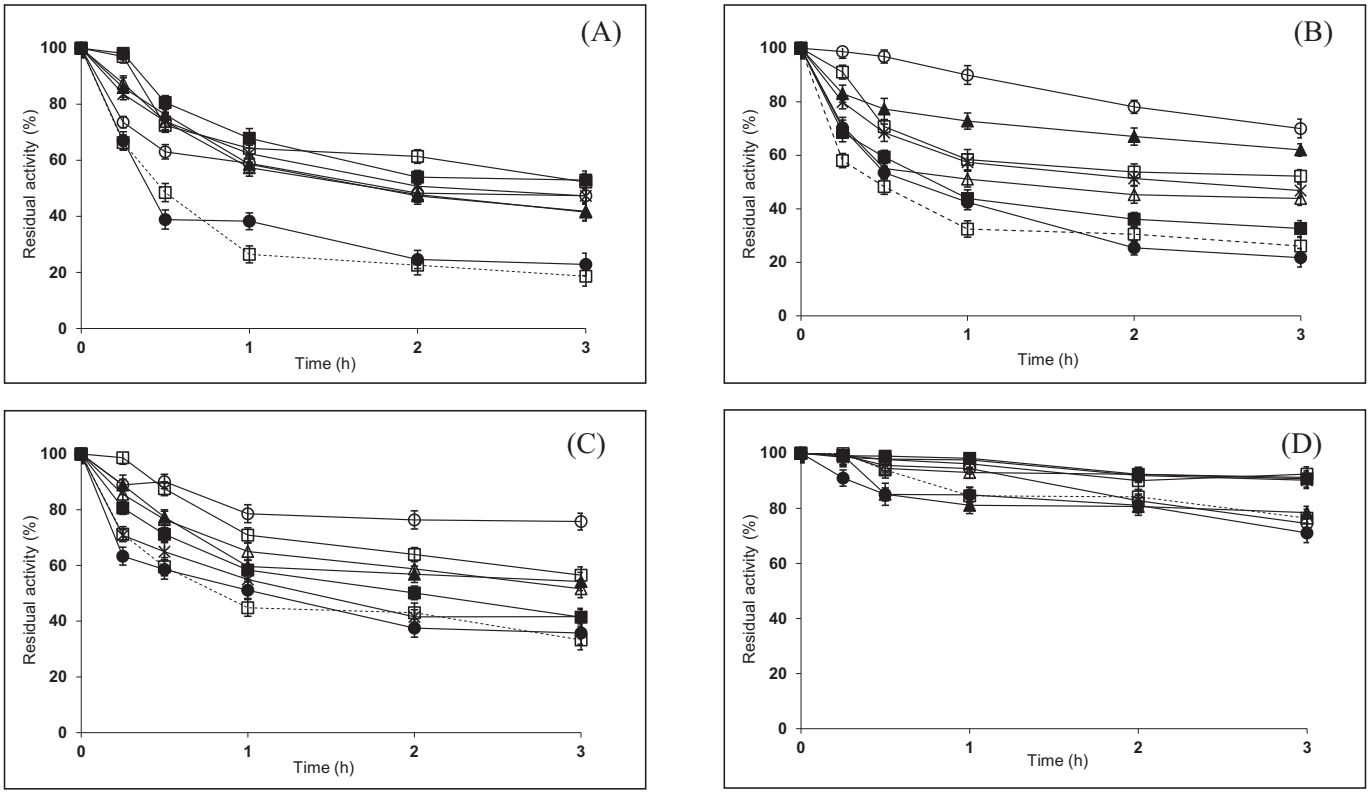

Fig. 4. Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of Tris. The biocatalysts were inactivated in 25 mM Tris pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10 % dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

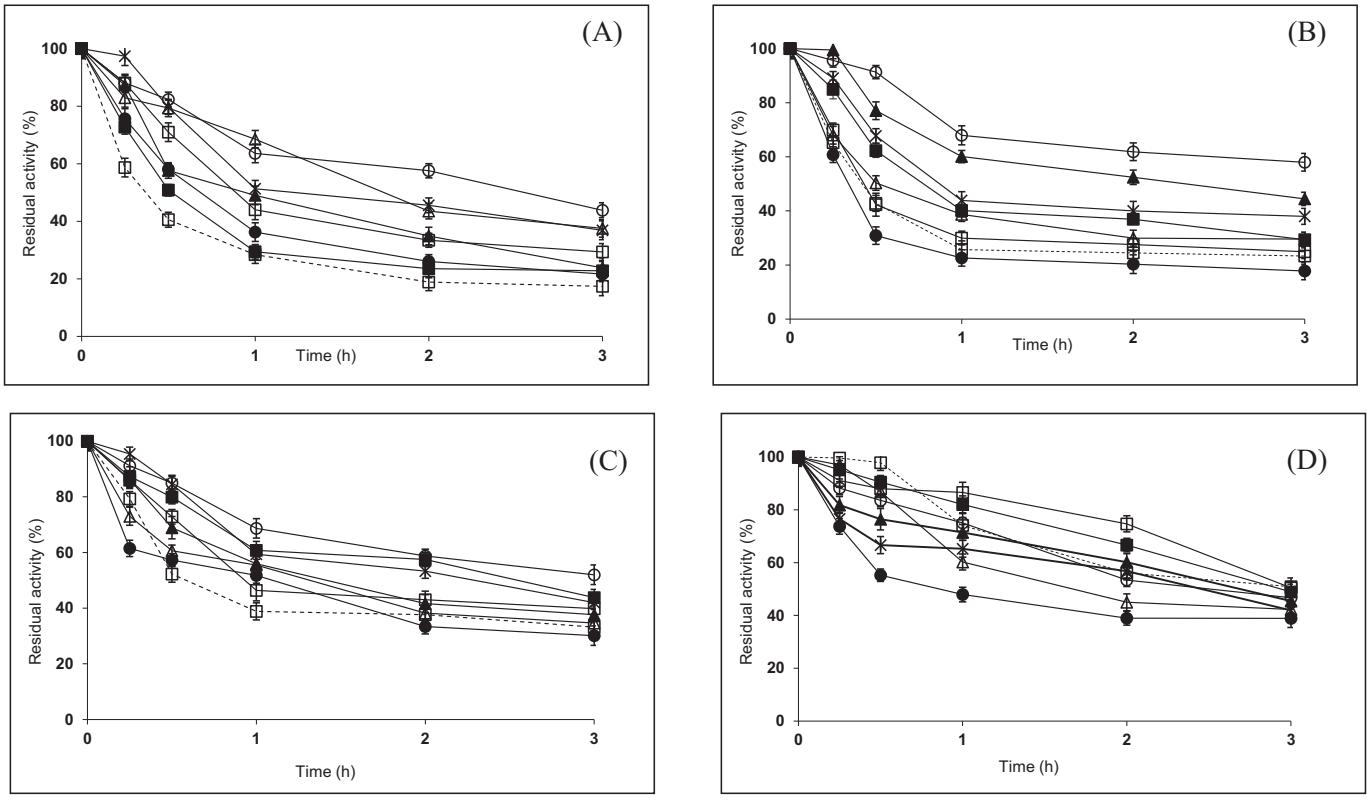

Fig. 5. Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of phosphate. The biocatalysts were inactivated in 25 mM phosphate at pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10 % dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

clarity for the biocatalyst immobilized at pH 9. The biocatalyst prepared at pH 5 improved its stability after copper and phosphate incubation, while the nickel treatment provided the lowest stability. That way, the most stable mineralized biocatalyst depended on the used biocatalyst. Eversa immobilized in 50 mM sodium acetate at pH 5 was stabilized by all metallization treatments, with the highest stability after modification using zinc, the biocatalyst prepared at pH 5 in the presence of 100 mM NaCl offers a similar picture, except for copper, that produced a destabilization. The biocatalyst prepared at pH 7 plus 10 % dioxane is also stabilized by all treatments, this time magnesium and manganese treatments providing the highest stability. The case of the biocatalyst prepared at pH 9 is fully different when compared to the other biocatalysts: all modified preparations decreased enzyme stability, with nickel offering the lowest decrease in enzyme stability and copper the lowest stability. Fig. 7S offers the comparison between the most and least stability biocatalysts for an easy visualization.

The incubation of the biocatalyst in metals/Tris solutions produces some differences on the final stability of the biocatalysts inactivated in Tris at pH 7 (Fig. 4). The modification with copper is again the treatment that produces the biocatalysts with the lowest stability, but the stability is similar to that of the unmodified biocatalyst. Magnesium treatment provides the highest stabilities for the biocatalyst immobilized at pH 5 plus 100 mM NaCl and that prepared at pH 7 plus 10 % dioxane. However, the mineralization of the biocatalyst immobilized at pH 9 did not show a great impact on its stability. The effect of the metal modification is fully different for this biocatalyst using Tris or phosphate (where the modification promoted strongly negative effects, Fig. 3) during metallization. Fig. 8S provides a comparison between the most and least stable biocatalysts, facilitating a clearer visualization of their stability differences.

Again, the metal incubation of the biocatalysts in Tris or phosphate induced great changes in enzyme stability, and, very interestingly in the context of the main objective of this research, this depended on the biocatalysts.

Finally, the enzyme stability at pH 7 in the presence of phosphate was analyzed. The previous incubation of the biocatalysts on metal/phosphate produced in general an increase on enzyme stability, with copper giving the lowest stabilities (in some instances even lower than the no modified enzyme) (Fig. 5). The exception is the biocatalysts prepared at pH 9, where many modifications led to a decrease in enzyme stability. Magnesium is the treatment that usually promoted the highest increase on stability, except for the biocatalyst prepared at pH 9, that is manganese. The treatment with metals in Tris (Fig. 6) offers some changes with the previous case. Nickel treatment enhanced the stability of the biocatalyst prepared at pH 5, whereas copper treatment had a detrimental effect, resulting in the lowest stability among all tested conditions. For the biocatalyst prepared at pH 5 plus 100 mM NaCl, an improvement in stability was observed following treatments with all metal treatments, with manganese exhibiting the most pronounced stabilizing effect. In the case of the biocatalyst prepared in Tris buffer pH 7 plus 10 % dioxane, the highest stability was maintained following magnesium treatment. However, copper ion treatment resulted in increased stability compared to its effect on the biocatalyst prepared in the presence of metal phosphate. Furthermore, treatment with all metal salts led to enhanced stability of the biocatalyst prepared at pH 9. Figs. 9S—10S offers the comparison between the most and least stability biocatalysts for an easy visualization.

The results are a surprise, as the phosphate plays a negative role on the stability of lipases (and Eversa) immobilized on octyl agarose, but here, in some instances, positive effects may be found [77]. This suggests

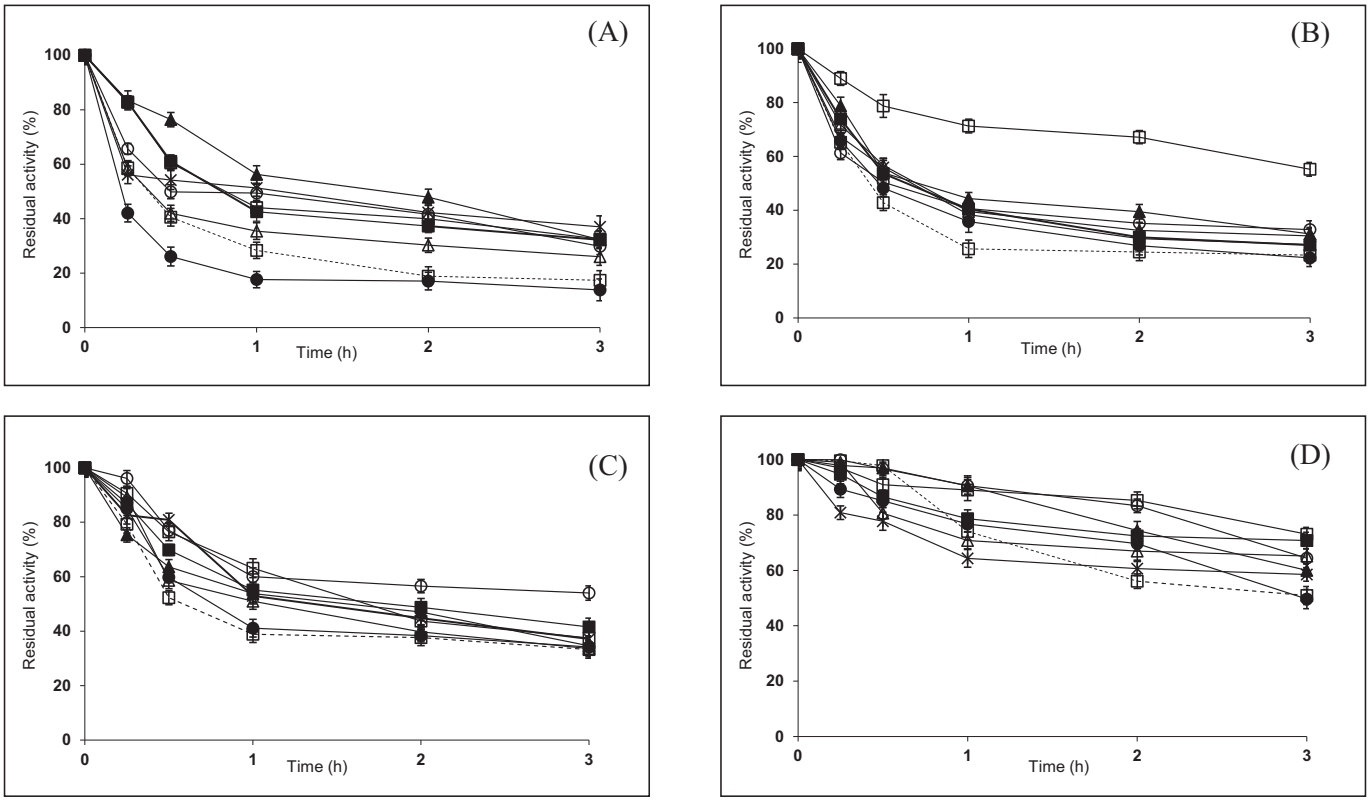

Fig. 6. Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of Tris. The biocatalysts were inactivated in 25 mM phosphate at pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10 % dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

that the crystals are stable on the inactivation conditions and the phosphate anions are no able to interact in free form with the immobile enzyme.

4. Conclusion

The results presented in this paper confirm the great effect of the metal incubation on the properties of immobilized lipases, in this instance Eversa transform. It has been established that the incubation with phosphate and the metals provide different changes to the incubation in Tris and metals (in some cases improving the activity or decreasing the enzyme activity if the metal was used with Tris or phosphate). These changes extend to activity/pH profile, enzyme substrate specificity (including enantiospecificity) and activity, or enzyme stability. Very interestingly, in this new research effort it has been confirmed that the enzyme structure has significant effect on the mineralization effects on the biocatalysts features (using Tris or phosphate), as results were very different using the 4 biocatalysts, prepared using the same enzyme and support and where the only enzyme structure differed. This means that using free enzymes to prepared enzyme-nanoflowers, the conditions of preparation may be critical for the final properties of the biocatalysts, as this not only depends on the crystal formation, but also on the enzyme features.

These results clearly exemplify how the optimal biocatalyst obtained by mineralization of immobilized lipases should be performed exactly on the enzyme formulation that will be used in the reaction, and evaluated under the operation conditions where the biocatalyst will be utilized.

CRediT authorship contribution statement

Leonardo de Souza: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Guilherme J. Sabi:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Pedro Abellanas-Perez:** Writing – review & editing, Investigation, Formal analysis. **Adriano A. Mendes:** Writing – review & editing, Conceptualization. **Paulo W. Tardioli:** Writing – review & editing, Conceptualization. **Javier Rocha-Martin:** Writing – review & editing, Validation, Formal analysis, Data curation, Conceptualization. **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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.148803.

Data availability

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

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