International Journal of Biological Macromolecules 191 (2021) 79–91

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Enzyme-support interactions and inactivation conditions determine *Thermomyces lanuginosus* lipase inactivation pathways: Functional and florescence studies

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ARTICLE INFO

Keywords:
Lipase interfacial activation
Inactivation of enzymes
Enzyme-support interaction
Substrate specificity
Enzyme tuning by immobilization
Enzyme inactivation under different conditions

ABSTRACT

Lipase from *Thermomyces lanuginosus* (TLL) has been covalently immobilized on heterofunctional octyl-vinyl agarose. That way, the covalently immobilized enzymes will have identical orientation. Then, it has blocked using hexyl amine (HEX), ethylenediamine (EDA), Gly and Asp. The initial activity/stability of the different biocatalysts was very different, being the most stable the biocatalyst blocked with Gly. These biocatalysts had been utilized to analyze if the enzyme activity could decrease differently along thermal inactivation courses depending on the utilized substrate (that is, if the enzyme specificity was altered during its inactivation using 4 different substrates to determine the activity), and if this can be altered by the nature of the blocking agent and the inactivation conditions (we use pH 5, 7 and 9). Results show great changes in the enzyme specificity during inactivation (e.g., activity versus triacetin was much more quickly lost than versus the other substrates), and how this was modulated by the immobilization protocol and inactivation conditions. The difference in the changes induced by immobilization and inactivation were confirmed by fluorescence studies. That is, the functional and structural analysis of partially inactivated immobilized enzyme showed that their inactivation pathway is strongly depended on the support features and inactivation conditions.

1. Introduction

Enzymes are biological catalysts with some outstanding properties (e.g., high selectivity, specificity, activity under mild conditions) [1–4] useful in modern chemical industries (from pharmaceutical to food and energy industries) [5–7]. The enzyme catalytic features are determined by the exact conformation of their tridimensional structure. Small changes in the enzyme conformation can greatly alter enzyme properties. For example, enzyme properties may be tuned via immobilization on different supports [8–13] or by immobilization on the same support but under different experimental conditions [14–17].

From this point of view, it should be considered that enzymes have fragile structures, and when used as industrial catalysts, the enzyme activity may decrease. Using immobilized enzymes, this decrease in enzyme activity may be reduced (e.g., via multipoint or multisubunit immobilization), but it will still exist. This decrease in immobilized enzyme activity may be due to some causes not related to changes in the enzyme conformation, such as support pore occlusion, loss of biocatalyst solid due to disaggregation of the support particle, enzyme release from the support, etc. [18]. However, it may be assumed that, at least part of this enzyme deactivation is promoted by changes in the enzyme conformation, and that the new enzyme structure can exhibit not only a

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https://doi.org/10.1016/j.ijbiomac.2021.09.061
Received 16 August 2021; Received in revised form 7 September 2021; Accepted 9 September 2021
Available online 16 September 2021

different catalytic activity, but also a different enzyme selectivity or specificity [8]. That is, using the enzymes in a simple process, like hydrolysis of a monofunctional substrate, the changes in the enzyme will only alter the reaction rate. However, in more complex processes, like a kinetic resolution (mainly in processes where the enzyme specificity is not total), these changes can fully alter the process efficiency. Similarly, all processes that can be determined by the enzyme features will be affected not only in reaction rate but in final yields and product purity (e.g., kinetically controlled synthesis [19], interesterification [20], acidolysis [21–24], etc.). There are few cases where the effect of enzyme inactivation in the enzyme performance on these processes has been studied [25–28].

There are not papers in literature (at least, we have not been able to find any paper in this paper) showing the possibility of the existence of different pathways in the conformational changes involved in enzyme inactivation under different conditions. This should produce different changes in enzyme features (e.g., enzyme selectivity, specificity, response to the medium) when changing the experimental conditions. This lack of studies may be caused because partially inactivated enzymes may aggregate and this makes very complex any kind of systematic analysis, In fact, we have been able to find just one paper in this regard even using immobilized enzymes. Using immobilized trypsin, it was possible to determine that the inactivation pathways may differ depending on the inactivation pH conditions and the degree of enzyme structure rigidification (degree of multipoint covalent attachment) [29]. However, this paper did not correlate these enzyme conformation changes with changes in functional enzyme features, except enzyme activity.

This paper analyzes the situation of the progressive thermal inactivation of immobilized lipase biocatalysts, as they are among the most tunable enzymes [14,30–32]. To this purpose, we have utilized the lipase from *Thermomyces lanuginosus* (TLL), one of the most popular lipases [33]. The enzyme has been immobilized utilizing its capacity to become adsorbed on hydrophobic surfaces via interfacial activation, as this ensures that all immobilized enzyme molecules exhibited the open structure and are in monomeric form [34,35]. As the enzyme may be desorbed from the support during inactivation [36], making the understanding of the inactivation processes more complex, we have employed a heterofunctional support: octyl-vinyl sulfone agarose beads, able to covalently fix the enzyme after the first adsorption via interfacial activation [18,37]. Agarose has been chosen as support because it has the advantage of being an inert support, and the only interactions between immobilized enzymes and support should be those produced by the moieties introduced in the beads by the researcher [38]. Moreover, it is transparent and permits to characterize the immobilized enzyme molecules through UV–vis and fluorescence spectroscopy techniques [16,39]. The octyl layer permits the immobilization of the lipase via interfacial activation [34], and the use of vinyl sulfone groups in the support permits to fulfill a double objective. First, it prevents the enzyme release during the heating, ensuring to maintain the same enzyme loading and discarding the enzyme release as biocatalysts inactivating cause, as 100% of the enzyme molecules are covalently attached to the support [37]. Second, the blocking of the remaining vinyl sulfone groups located in the support with different blocking agents permits to alter the enzyme nano-environment, altering the enzyme –support interactions [37,40–43]. The blocking agents will become immobilized on the supports via very stable covalent bonds (secondary amino in the case of the compounds used in this paper). We have used blocking agents that can react with the support (having primary amino groups) and that will produce very different surfaces: aspartic acid will introduce two anionic groups and one cationic one, ethylenediamine will introduce two cationic groups in a small moiety, hexyl amine will introduce a secondary amino group in the point of attachment to the support, but also a long aliphatic chain that will be the one in contact with the enzyme, and Gly, a very small groups very hydrophilic, having a cationic and a anionic group. Moreover, this different final blocking agents of the vinyl sulfone lipase biocatalysts has permitted to alter the lipase stability, activity and specificity [39,40]. We can formulate the hypothesis that these different enzyme-support interactions may also alter the lipase inactivation pathway, producing different partially inactivated enzyme structures, baring different functional properties. This information may be very relevant to understand the inactivation of immobilized enzymes and may be hard to be obtained using other immobilization methods: in this instance, we have identical enzyme orientation, the exact number of enzyme-support covalent attachments, and the only difference is the physical properties of the support surface obtained after the blocking step (Fig. 1) [39,40]. The activity determination at two different pH values permit to check if the different inactivated immobilized enzyme molecules may have different response in terms of remaining activity under different reaction conditions.

The use of TLL, an enzyme with wide specificity [33], permits to follow its inactivation using very different substrates to check if the losses of enzyme activity are similar whatever the substrate utilized. To this purpose, we have used four different substrates, the chiral methyl mandelate (isomers (*R*)- and (*S*)- were employed, that way, indications on changes of TLL enantiospecificity may also be detected), the synthetic monofunctional *p*-nitrophenyl butyrate (*p*NPB), and the multifunctional triacetin (the produced 1,2 diacetin [44] may be used to synthetize more complex products [45]), the most similar one to the natural substrates of the enzyme (triglycerides).

To prevent protein-protein interactions that could alter the results [16,46] and prevent substrate diffusional limitations that could alter the activity and apparent stability of the immobilized enzymes [47,48], we have utilized an enzyme loading quite below the maximum loading of the support with this enzyme [49].

Thus, the inactivation of differently blocked TLL-octyl-vinyl-agarose beads biocatalysts has been followed using the four substrates cited above under three different inactivation pH values (5, 7, and 9). The alteration of the fluorescence caused by the inactivation has also been recorded to analyze if, together with functional changes, some different structural changes may be found.

2. Materials and methods

2.1. Materials

Lipozyme® TL 100 L, a liquid lipase formulation containing lipase from *Thermomyces lanuginosus* (TLL) (19.57 mg of protein/mL), was kindly donated by Novozymes Spain (Alcobendas, Spain). Octyl-Sepharose® CL-4B beads were purchased from GE Healthcare (Alcobendas, Spain). Triacetin, *p*-nitrophenyl butyrate (*p*-NPB), L-aspartic acid, hexylamine, and ethylenediamine (EDA) were from Sigma Aldrich Spain (Madrid, Spain). Divinyl sulfone (DVS), glycine, (*R*)- and (*S*)-methyl mandelate were from Thermo Fisher scientific Spain (Madrid, Spain). All other reagents and solvents were of analytical grade. Bradford's method [50] was used to quantify the protein concentration employing bovine serum albumin as reference.

2.2. Methods

All experiments were performed by triplicate, and the results are reported as their mean values and the standard deviation.

2.2.1. Preparation of octyl-vinyl sulfone support (octyl-VS)

The support activation was adapted from a protocol described by Albuquerque et al. [37] with some modifications. 2.5 mL of divinyl sulfone was added to 66.6 mL of 333 mM sodium carbonate at pH 11.5 (to a final concentration of 0.35 M) and stirred with a magnetic stirring bar until the medium turned homogeneous. Then, 10 g of octyl-agarose beads were added and left under gentle stirring for 2 h. Finally, the support was vacuum filtered, washed with abundant distilled water, and stored at 6–8 °C.

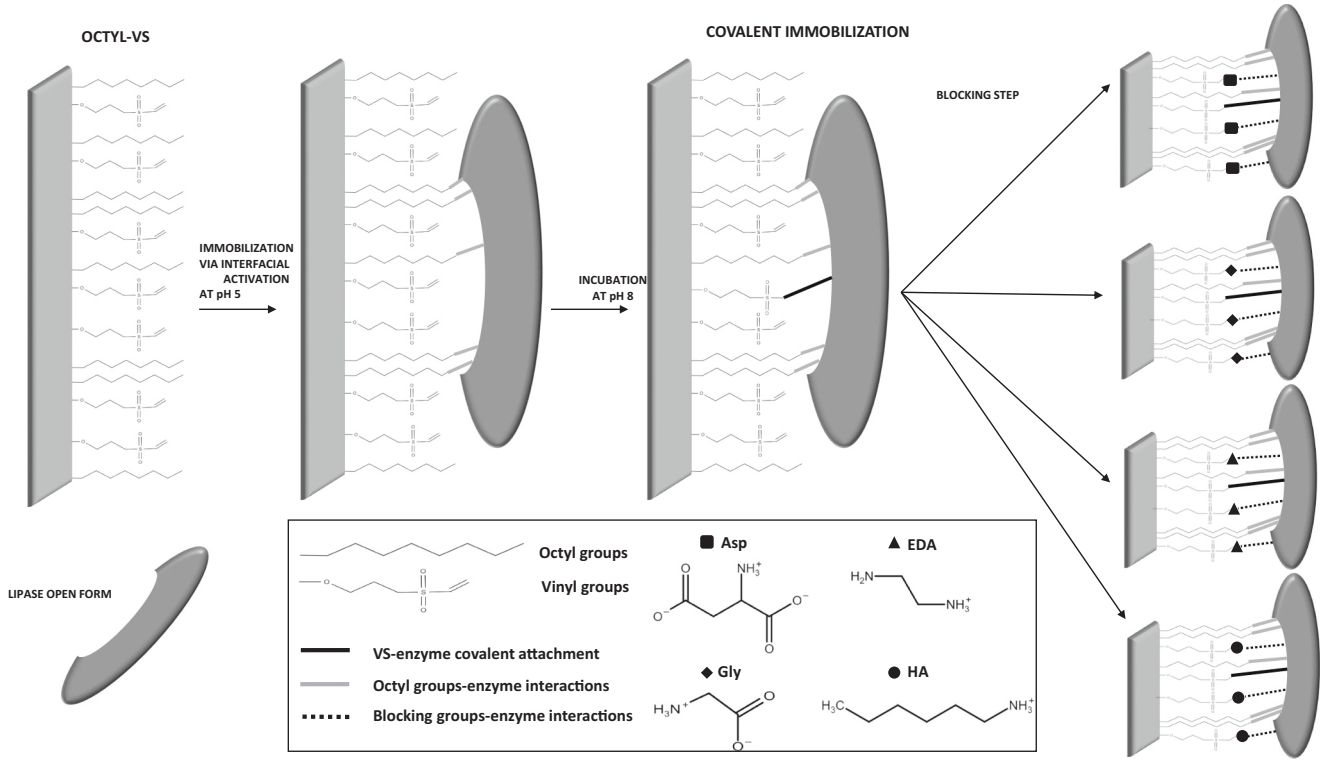

Fig. 1. Scheme of immobilization strategy utilized in this paper: immobilization on VS-supports and further blocking with different reagents. Experiments were performed as described in Methods.

2.2.2. Immobilization of TLL on octyl-VS support

TLL was immobilized on octyl-VS support by interfacial activation as previously described [37], using 2 mg of enzyme per g of wet support (maximum loading of this support with TLL is around 5 mg/mL [46]). The enzymatic solution was diluted in 5 mM sodium acetate at pH 5.0 and 25 °C, and the support was added in a proportion of 1 g/10 mL of the enzyme solution. The immobilization was conducted under gentle mechanical stirring, and the activities of supernatant and suspension were followed using *p*-NPB as substrate. After the immobilization, the immobilized biocatalyst was vacuum filtered, washed with distilled water, and resuspended in 50 mM sodium bicarbonate at pH 8.0 and 25 °C for 4 h, to favor the enzyme-support covalent reaction (maintaining the relation 10 mL of buffer solution per g of support) [37]. Finally, the octyl-VS biocatalyst was blocked with different blocked agents [37], as described below (Fig. 1).

2.2.3. Blocking of the octyl-VS-TLL biocatalysts

As a reaction end point, the enzyme immobilized in octyl-VS was incubated in 2 M of different nucleophiles, ethylenediamine (EDA) and hexylamine (HEX), or some amino acids, such as glycine (Gly) and aspartic acid (Asp), at pH 8.0 at room temperature for 24 h (10 mL of blocking solution per 1 g of biocatalysts) to block the remaining reactive groups [37] (Fig. 1). Finally, the covalently immobilized and blocked biocatalysts were vacuum filtered and washed with an excess of distilled water and stored at 6–8 °C.

2.2.4. Determination of enzyme activity versus different substrates

One enzyme activity unit (U) was defined as μmol of substrate hydrolyzed per minute under the described conditions.

2.2.4.1. Hydrolysis of p-NPB. The enzymatic activity was quantified by continually recording the increase in the absorbance at 348 nm during 90 s, produced by the *p*-nitrophenol that is released (isosbestic point, ε under these conditions is 5150 M^{-1} cm^{-1}) in the hydrolysis of *p*-NPB [51] using a Jasco spectrophotometer (V-730) (Jasco, Madrid, Spain). The reaction was started by adding 50 μL of the sample (free enzyme solution or immobilized enzyme suspension, using a cut tip) in 2.5 mL of 25 mM sodium phosphate at pH 7.0 and 25 °C containing 50 μl of *p*-NPB solution (at a concentration of 50 mM, dissolved in acetonitrile) under magnetic stirring and thermostatization.

2.2.4.2. Hydrolysis of triacetin. The reaction was initialized by adding 0.05–0.1 g of wet biocatalysts (submitted to thermal inactivation or not) to 2–4 mL of 50 mM of triacetin in 50 mM sodium phosphate buffer solution at 25 °C and pH 7.0 under continuous gently stirring using a roller mixer (Tube Roller MXT6S, Scilogex, CT, USA). Under these reaction conditions, the enzyme product, 1,2 diacetin, suffers acyl migration, and a mixture with 1,3 diacetin is obtained [44]. The reaction conversion was measured by HPLC, Jasco UV 15–75 (Jasco, Madrid, Spain), using a Kromasil C18 column (15 cm × 0.46 cm) (Analisis Vinicos, Tomelloso, Spain) with a UV detector at 230 nm, injecting samples of 20 μL. A solution of 15% acetonitrile-85% Milli-Q water at 25 °C was used as a mobile phase with a flow rate of 1 mL/min. The retention times of the compounds were about 4 min for diacetins (both diacetins co-eluted under these conditions) and 18 min for triacetin. Conversions between 15 and 20% were used to calculate the initial reaction rates.

2.2.4.3. Hydrolysis of (R)- or (S)-methyl mandelate. 0.025–0.25 g of wet biocatalysts were added to 0.5–3.5 mL 50 mM solutions of (*R*)- or (*S*)-methyl mandelate in 50 mM of sodium phosphate at pH 7.0 and 25 °C, under gently stirring using a roller mixer (Tube Roller MXT6S, Scilogex, CT, USA). The reaction products were detected in an HPLC, Jasco UV 15–75 (Jasco, Madrid, Spain). The column was an HPLC Kromasil C18 (15 cm × 0.46 cm) (Analisis Vinicos, Tomelloso, Spain) and a solution of 35% acetonitrile/65% aqueous solution of 10 mM of ammonium acetate at pH 2.8 was used as mobile phase with a flow velocity of 1 mL/min. The compounds were determined with UV–VIS detector at 230 nm, by injecting reaction samples of 20 μL and the retention times were about

2.4 min for mandelic acid and 4.2 min for methyl mandelate. Conversions between 15 and 20% were used to calculate the initial reaction rates.

2.2.5. Thermal inactivation of the different TLL biocatalysts at different pH values

First, we analyze the inactivation of the biocatalysts at the indicated pH values at different temperatures to have a reasonable inactivation rate, high enough to have the samples in just some days, and slow enough to can take samples under the desired residual activities values. Immobilized TLL was the most stable at pH 5 and the minimal stability was found at pH 9, making necessary to select different temperatures at each pH value. That way, after analyzing inactivation of the biocatalysts in the temperature range 60–75, the following inactivation conditions were selected for this paper.

Biocatalysts were incubated in 50 mM sodium acetate at pH 5.0 and 74 °C, 50 mM Tris HCl at pH 7.0 and 73 °C or 50 mM sodium carbonate at pH 9.0 and 71 °C. Phosphate was avoided at pH 7 by its negative effects on the stabilities of immobilized lipases via interfacial activation [39,52]. Periodically, samples were withdrawn, and their residual activities were measured using the *p*-NPB assay described above. Residual activities were calculated as the percentage of the initial activities. The inactivation continued for the time required for each biocatalyst to maintain only a 25% residual activity, the time was different for each biocatalysts and inactivation condition.

To get samples for the inactivated biocatalysts analysis, the inactivation courses were stopped when their residual activity was around 75%, 50%, or 25% and the partially inactivated biocatalysts were vacuum filtered with distilled water. These biocatalysts were finally washed with 50 mM Tris buffer at pH 7.0, stored at 25 °C for 24 h to enable any reactivation that could interfere in analyzing the inactivation enzyme properties, and then stored at 4 °C to analyze their activities and fluorescence spectra. All biocatalysts were stored exactly under the same conditions that way, if they have different properties, should be due to the inactivation and not to the storing conditions.

2.2.6. Fluorescence studies of the different immobilized TLL preparations

The intrinsic fluorescence of immobilized TLL was measured in Corning™ 96-Well Solid Black Polystyrene Microplates 96-well plates with flat bottom (Fisher-Scientific, Leicestershire (UK)). 100 μL of 1:10 (w:v) suspension of immobilized TLL containing a total of 20 μg of protein in 100 mM Tris-HCl buffer at pH 7 were added to each well. The study was performed by exciting each well at 280 nm with a 2 nm slit and recording the emission spectrum at 300–500 nm using a microplate reader Cytation 5, BioTek® with the software Gen5. The maximum fluorescence intensity (I_{max}) and the wavelength at maximum intensity (λ_{max}) were calculated by subtracting the spectra of the empty carriers to the spectra of the samples with the immobilized TLL. The raw fluorescence data obtained from the fluorometer were analyzed using Origin 8 software.

3. Results

3.1. Activity of the different preparations versus the different substrates

As previously described [37], immobilization yield was 100% in all cases, and after immobilization, all enzyme molecules were covalently attached to the support. The activities of the differently blocked biocatalysts versus the four substrates employed in this paper may be found in Table 1. Using *p*NPB as substrate the biocatalysts offered the highest activity. Only the biocatalysts blocked with HEX presented an activity significantly different, less than 50% than the other preparations. The biocatalysts blocked with EDA were 10% more active than the biocatalyst blocked with Asp or Gly with this substrate.

Triacetin is the second best substrate for these biocatalysts. In this instance, the preparation blocked with HEX is more than 12 fold less activity than the preparation blocked with EDA, which is the most active (around 30% more active than the other two biocatalysts).

Table 1
The activities of the differently immobilized TLL biocatalysts with 50 mM (*R*)- or (*S*)-methyl mandelate (pH 7.0, 25 °C), 50 mM of triacetin (pH 5.0, 25 °C) or 1 mM of *p*NPB (pH 7.0, 25 °C). Experiments were performed as described in Methods.

Activity (U/g of biocatalyst)					
TLL preparations	Blocking agent	*p*NPB	Triacetin	(*R*)-methyl mandelate[a]	(*S*)-methyl mandelate[a]
OC-DVSF-TLL	Asp	197 ± 12	62 ± 4	5.52 ± 0.27	7.32 ± 0.51
	Gly	203 ± 14	67 ± 5	6.07 ± 0.29	6.68 ± 0.40
	EDA	223 ± 16	85 ± 7	6.22 ± 0.32	7.29 ± 0.49
	HEX	88 ± 7	6.9 ± 0.4	2.38 ± 0.13	2.16 ± 0.11

[a] The activity has been multiplied by 100.

Using both isomers of methyl mandelate, the activity of the different TLL biocatalysts was very low, without a relevant preference of the different biocatalysts by one of the isomers (the Asp blocked biocatalyst was a 30% more active using the S isomer, while the preparation blocked with HEX was just a 10% more active with the R isomer). The biocatalysts blocked with EDA and Gly gave the highest activity using the R-isomer, again the one blocked with HEX is the least active (less than 40%). Using the S-isomer, the biocatalysts blocked with EDA and Asp are the most active, shortly followed by the one blocked with Gly.

That way, the starting biocatalysts presented different specificity comparing the activity toward these 4 substrates, suggesting that the different biocatalysts may have a starting different initial structure as results of the different blocking.

3.2. Thermal inactivation of the different TLL biocatalysts at different pH values

Following the protocols described in Methods section, the inactivation temperature was selected for each pH value, until we find conditions where we can have reliable results.

Fig. 2 shows the inactivation courses of the four TLL biocatalysts at pH 5, 7 or 9. After some trials, the selected temperatures were 74 °C at pH 5.0, 73 °C at pH 7.0, and 71 °C at pH 9.0. These temperatures permitted to have reliable inactivation courses for all the biocatalysts at the selected pH values for the most and the least stable preparations.

Differences in enzyme stability depending on the blocking strategy have been previously described [37] and it has been confirmed in this new study. At pH 5 and 9 (Fig. 2A and C), the most stable preparation was that blocked using Gly, followed by the biocatalyst blocked using Asp, EDA and the least stable was that blocked using HEX. Differences in the stability of the Asp blocked biocatalyst compared to the EDA blocked biocatalyst were higher at pH 5 than at pH 9, while Asp blocked preparation increase of difference in stability compared to the two least stale ones was higher at pH 9 than at pH 5. At pH 7 (Fig. 2B), Gly blocked biocatalysts remained as the most stable biocatalyst, and Asp blocked preparation was the next one, while EDA and HEX blocked biocatalysts presented very similar inactivation courses. It seems that Gly, which gives a support with mixed cationic and anionic groups (Fig. 1), is the one that gives the highest stability. To check the intensity of the likely enzyme-supports interactions, no activated agarose beads activated with vinyl sulfone groups (and without octyl nor any additional group than the vinyl sulfone one) were blocked with the different blocking agent used in this paper, and their capacity to adsorb TLL was analyzed. The support blocked with Gly cannot significantly immobilize TLL under the inactivation conditions (not shown results). The blocking agents (EDA and HEX) that produced a cationic nature in the support surface (Fig. 1)

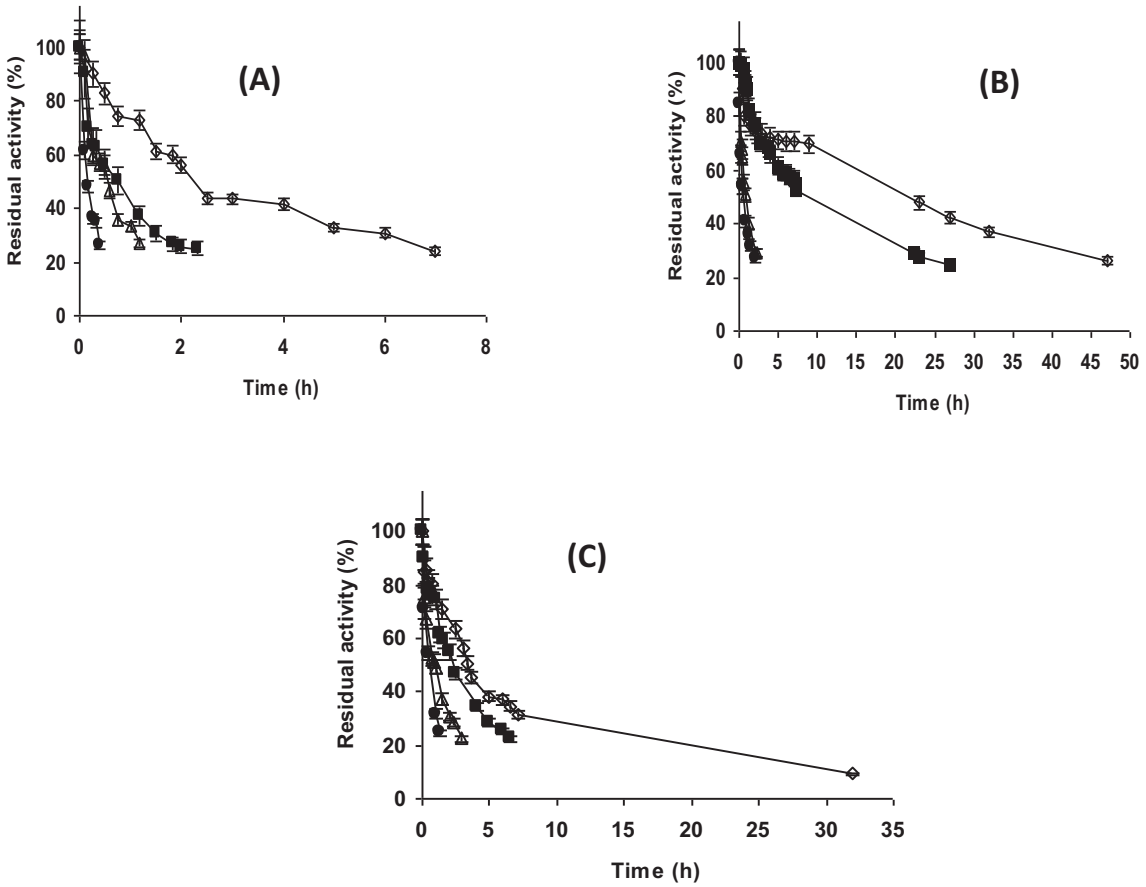

Fig. 2. Inactivation courses of TLL biocatalysts at different pH values. Inactivation conditions were: (A) 50 mM sodium acetate at pH 5.0 and 74 °C. (B) 50 mM Tris HCL at pH 7.0 and 73 °C. (C) 50 mM sodium carbonate at pH 9.0 and 71 °C. Biocatalyst blocked with: solid squares: Asp; empty rhombus: Gly; empty triangles: EDA; solid circles: HEX. Experiments were carried out as described in Methods.

gave the lowest enzyme stability (HEX and EDA). These cationic supports are able to adsorb TLL (isoelectric point is 4.4) under all the inactivation conditions (slower at pH 9) (not shown results). This means that the lipase surface has a tendency to interact with the support surface. Perhaps, these enzyme support interactions may help to maintain erroneous enzyme structures during enzyme inactivation, becoming negative for the enzyme stability [53,54]. Asp blocked support is also a mixed support, but with a anionic nature, as it has 2 anionic groups and one cationic one. The agarose beads blocked with Asp was unable to adsorb TLL under the inactivation conditions in a significant way (less than 10% at pH 5). Differences of Asp blocked stability compared to Gly blocked biocatalyst may be caused by this second anion group or by the larger size of Asp, which facilitates interactions with more groups of the immobilized enzyme than using Gly. In any case, the Asp blocked biocatalyst is the second most stable biocatalyst at the 3 studied pH values. Some reports suggest that a surface of the support with a similar ionic character than the enzyme may be positive for the enzyme stability, because a certain repulsion between the enzyme and the support surface that help to maintain the enzyme conformation [55–59]. However, this is not fully followed in the current enzyme, where Gly is the blocking agent that gave the highest stability on the octyl-vinyl sulfone biocatalyst.

Where these inactivation curves enabled us to get inactivated preparations having different residual activities: 70–80%, 45–55%, and 20–25%, to analyze how the inactivation affects the activity versus the different substrates and analyze its fluorescence spectrum.

3.3. Enzyme reactivation during the 24 h incubation using pNPB as substrate

Table 2 shows the activities of the different samples taken versus *p*NPB just during the inactivation or after 24 h of incubation at pH 7 and 25 °C to permit the enzyme reactivation. This way, we can avoid that the results may be interfered by enzyme reactivations during the enzyme activity determination.

The activity recovered for each biocatalysts is different, and differ depending on the inactivation pH. When the inactivation was performed at pH 5, the Asp biocatalysts is the one that gave the highest final activity in the first two points, (e.g., going from 50 to 71%) but it is overtaken by the preparation blocked with EDA in the last point (this biocatalysts recover from 24 to 57% of activity). It may be expected that the interactions with the support were negative for the recovering of activity and Asp and Gly should be the ones that give the highest activity recovery [55–59], but that was not the case. In inactivation at pH 7, EDA blocked biocatalysts gave the highest activity at all points (e.g., from 28% to 49% in the most inactivated sample). In general, the enzymes inactivated at pH 7 showed a lower activity recovery that the enzymes inactivated at pH 5. When the samples were inactivated at pH 9, the picture was completely different. EDA blocked preparation was the one with the lowest activity recovery (from 22% to 31%), while that blocked with Asp permitted to recover the heist activity using the most inactivated biocatalyst (from 22% to 62%). In the first samples, Gly was the biocatalysts that permitted a highest enzyme activity recovery (e.g., from 45% to 88%).

That way all enzyme biocatalysts could recover part of the lost activity, as has been previously reported [60,61]. The activity recovery

Table 2
Residual activity of different immobilized TLL biocatalysts in the inactivation courses at (pH 5.0, 74 °C), (pH 7.0, 73 °C) and (pH 9.0, 71 °C). The activities were taken during the inactivation and after 24 h of incubation at pH 7.0 and 25 °C using *p*NPB as substrate, as described in Methods section.

	Name of the biocatalyst	Residual activity in inactivations (%)				Residual activity after 24 h (%)			
		Asp	Gly	EDA	HEX	Asp	Gly	EDA	HEX
pH 5	75%	63 ± 3	74 ± 2	59 ± 3	61 ± 3	90 ± 1	88 ± 4	78 ± 3	81 ± 4
	50%	50 ± 2	44 ± 3	46 ± 2	48 ± 2	71 ± 3	68 ± 3	7 ± 3	61 ± 3
	25%	25 ± 1	24 ± 1	27 ± 1	26 ± 1	43 ± 2	36 ± 2	58 ± 3	38 ± 2
pH 7	75%	77 ± 4	71 ± 3	61 ± 3	66 ± 3	77 ± 4	86 ± 4	83 ± 2	71 ± 3
	50%	53 ± 2	48 ± 2	51 ± 3	54 ± 3	51 ± 3	42 ± 2	71 ± 3	51 ± 3
	25%	25 ± 1	26 ± 1	29 ± 1	27 ± 1	32 ± 1	24 ± 2	50 ± 2	25 ± 2
pH 9	75%	61 ± 3	71 ± 3	67 ± 4	71 ± 4	94 ± 4	97 ± 4	84 ± 3	107 ± 4
	50%	47 ± 2	46 ± 2	49 ± 2	54 ± 3	79 ± 3	88 ± 4	75 ± 4	61 ± 3
	25%	23 ± 1	19 ± 1	23 ± 2	25 ± 1	62 ± 3	50 ± 3	32 ± 2	44 ± 3

depends on the surface of the support (as this can alter the enzyme-support interactions) [37,40–43] and on the inactivation conditions (as this can alter the inactivation pathway) [29].

3.4. Evolution of the biocatalysts activity versus different substrates during inactivation alteration of enzyme specificity

Fig. 3 shows the evolution of the TLL biocatalysts activity blocked with Asp versus the 4 substrates. As we use in the X axis the residual activity versus *p*NPB, the comparison between the different biocatalysts activities may be direct, do not depend on the higher or lower stability of the biocatalyst.

At pH 5 (Fig. 3A), the retained percentage of activity versus *p*NPB decreased slower than with both mandelic acid methyl esters (that decreased its activity in a very similar fashion), being the activity versus triacetin the one that is lost in a more rapid fashion. The biocatalyst inactivation at pH 7 (Fig. 3B) offers a quite different picture. Now, the enzyme residual activity versus (*R*)-methyl mandelate is the one that is better maintained, shortly followed by the residual activity versus the (*S*)-isomer and versus *p*NPB. The retained activity versus triacetin remained as the one that the most rapidly disappeared. Using the biocatalysts inactivated at pH 9 (Fig. 3C) the situation is very similar to that at pH 5, the residual activity is better maintained versus *p*NPB and worse versus triacetin, while the relative activity maintained using the mandelate esters are in between. At the 3 levels of inactivation detected using the activity versus *p*NPB, the retained activity versus triacetin decreased quite similarly, slightly slower at pH 9 than at pH 5 and slightly more rapidly at pH 7. With (*R*)-methyl mandelate, the biocatalyst residual activity at pH 7 decreased significantly slower than at the other two pH values, while using (*S*)-methyl mandelate as substrate, the

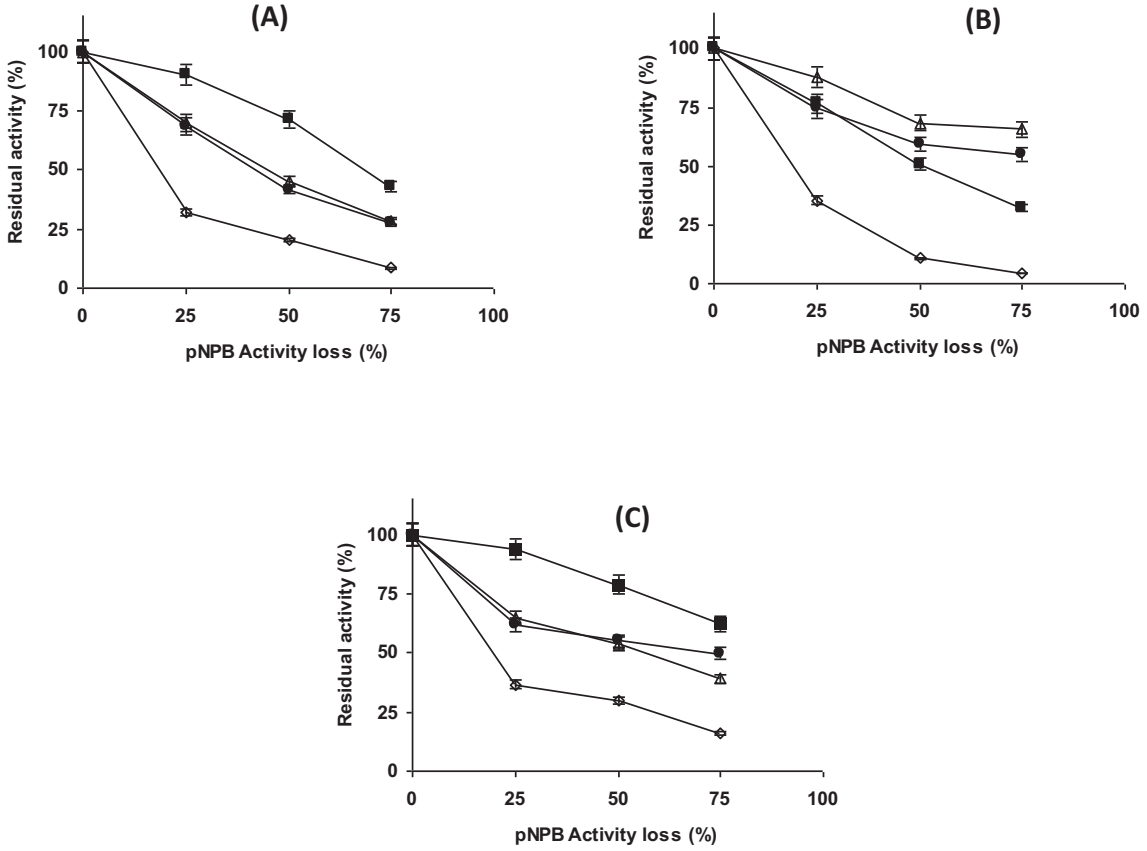

Fig. 3. Effect of the inactivation pH on the residual activity versus different substrates of TLL biocatalysts activity blocked with aspartic acid. Inactivation was performed at (A) pH 5.0 and 74 °C (B) pH 7.0 and 73 °C and (C) pH 9.0 and 71 °C. The substrates use were: solid squares: *p*NPB; empty rhombus: triacetin; empty triangles: (*R*) - methyl mandelate; solid circles: (*S*) - methyl mandelate; as substrates. Other specifications may be found in Methods section.

enzyme relative activity decreases more rapidly at pH 5 and in a similar way at the other two pH values.

Fig. 4 shows the evolution of the retained activity using the biocatalysts blocked with Gly. At pH 5 (Fig. 4A), the residual activity is better maintained using *p*NPB as substrate, and the worst retained activity recovery is observed using triacetin as substrate, while the residual activity decreased in a similar way using both isomers of methyl mandelate. At pH 7 (Fig. 4B), after the first inactivation, where the retained activity versus *p*NPB and both methyl mandelate esters are in the order of 80%, the biocatalyst activity is better maintained using the mandelic esters, and slightly better using the (*R*)-isomer. Triacetin is again the substrate where the retained activity is more rapidly lost. In fact, when still 75% of the initial activity versus (*R*)-methyl mandelate was maintained, only 5% was maintained using triacetin as substrate. At pH 9 (Fig. 4C), the situation was similar to that at pH 5, but with a higher activity loss in the first levels of inactivation when using both isomers of mandelic acid as substrates.

The effect of the pH analyzing the residual activities versus triacetin was similar to that found using the Asp blocked biocatalysts. Using (*R*)-methyl mandelate, the activity retained when the biocatalyst was inactivated to 25% of the activity versus *p*NPB was still 70% when the inactivation was performed at pH 7, and only around 25–30% if performed at pH 5 or 9. Using the (*S*)-isomer, the differences in activity recovery caused by the inactivation pH in retained activities are not so evident.

The results obtained from the different inactivated biocatalysts blocked with EDA may be found in Fig. 5. At pH 5 (Fig. 5A), the highest activity recovery was found using *p*NPB and (*R*)-methyl mandelate, being the residual activity versus triacetin again the one that more rapidly decreased. At pH 7 (Fig. 5B), the retained activity versus triacetin was significantly more rapidly lost than versus the other substrates, which was quite similar. At pH 9 (Fig. 5C), the residual activities versus *p*NPB and (*S*)-methyl mandelate were the ones that kept the highest values, with a higher value using (*S*)-methyl mandelate in the biocatalysts with the lowest retained activity (62% versus 32%). The activity using triacetin remained the most rapidly lost by the TLL biocatalyst, but in this instance it is not too far from the residual activity versus (*R*)-methyl mandelate. In this instance, the enzyme enantiospecificity is strongly affected, as the activity versus (*S*)-methyl mandelate was maintained almost twice better than versus the (*R*)-isomer. This made this biocatalyst, after inactivation, to have a clear preference for the S-isomer.

Focusing on the effect of the pH, the retained activity of this biocatalyst versus (*R*)-methyl mandelate was more quickly lost at pH 9 than at pH 5 or 7, while, using (*S*)-methyl mandelate, it was at pH 5 where the activity decreased the most. At pH 9, this activity was better preserved. For triacetin, differences were not very relevant in the most inactivated biocatalysts. The biocatalysts extracted when maintained 50% of the *p*NPB activity offered some differences in residual activities versus triacetin, 32% at pH 9, and around 20% at pH 5 and 7.

Fig. 6 shows the last biocatalysts, obtained from the inactivation of the most unstable one using *p*NPB as substrate, the one blocked with HEX. At pH 5 (Fig. 6A), the activity recovered using (*S*)-methyl mandelate as substrate is the highest, followed by that recovered using *p*NPB, and triacetin remains as the substrate that gives the lowest activity recovery. At pH 7 (Fig. 6B), the recovered activity using both methyl mandelate isomers become the highest ones, while that using triacetin and *p*NPB, the inactivated biocatalyst recovered activity become quite similar. At pH 9 (Fig. 6C), again, the activity recovery using triacetin was the lowest, and the one using (*S*)-methyl mandelate the highest, being those using *p*NPB and (*R*)-methyl mandelate similar (except in the less inactivated biocatalysts point, where the activity versus *p*NPB even slightly increased compared to the initial one).

Analyzing the effect of the pH on the activity decrease using the

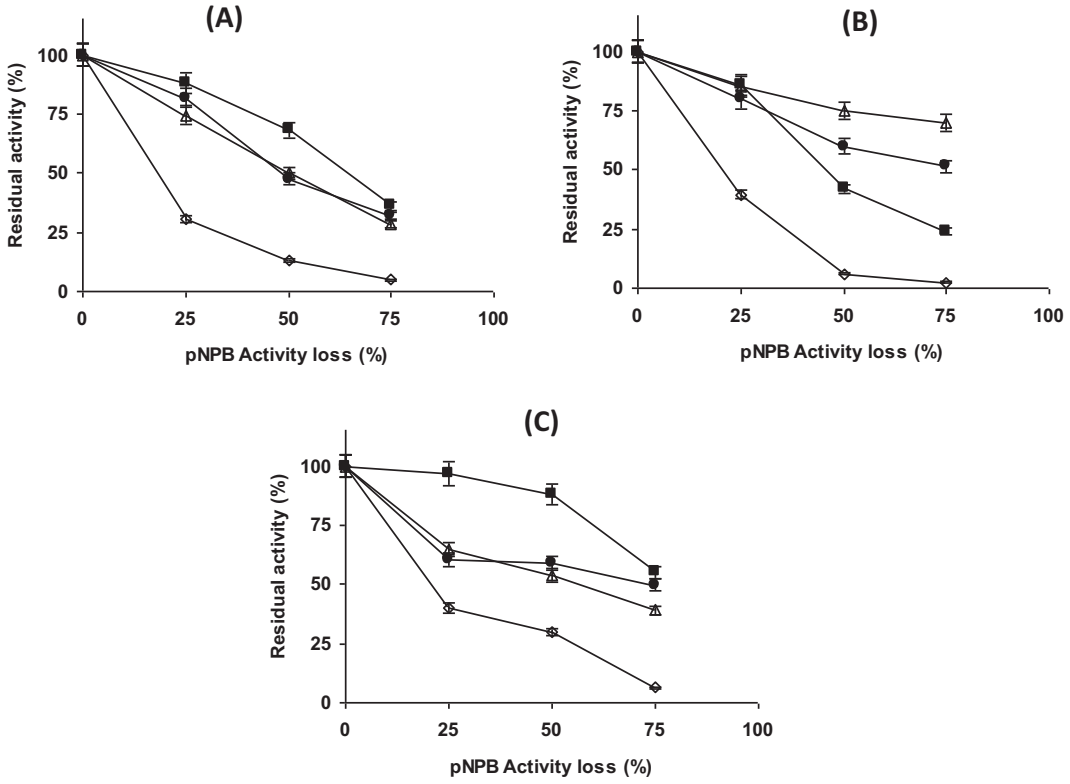

Fig. 4. Effect of the inactivation pH on the residual activity versus different substrates of TLL biocatalysts activity blocked with glycine. Inactivation was performed at (A) pH 5.0 and 74 °C (B) pH 7.0 and 73 °C and (C) pH 9.0 and 71 °C. The substrates use were: solid squares: *p*NPB; empty rhombus: triacetin; empty triangles: (*R*) - methyl mandelate; solid circles: (*S*) - methyl mandelate; as substrates. Other specifications may be found in Methods section.

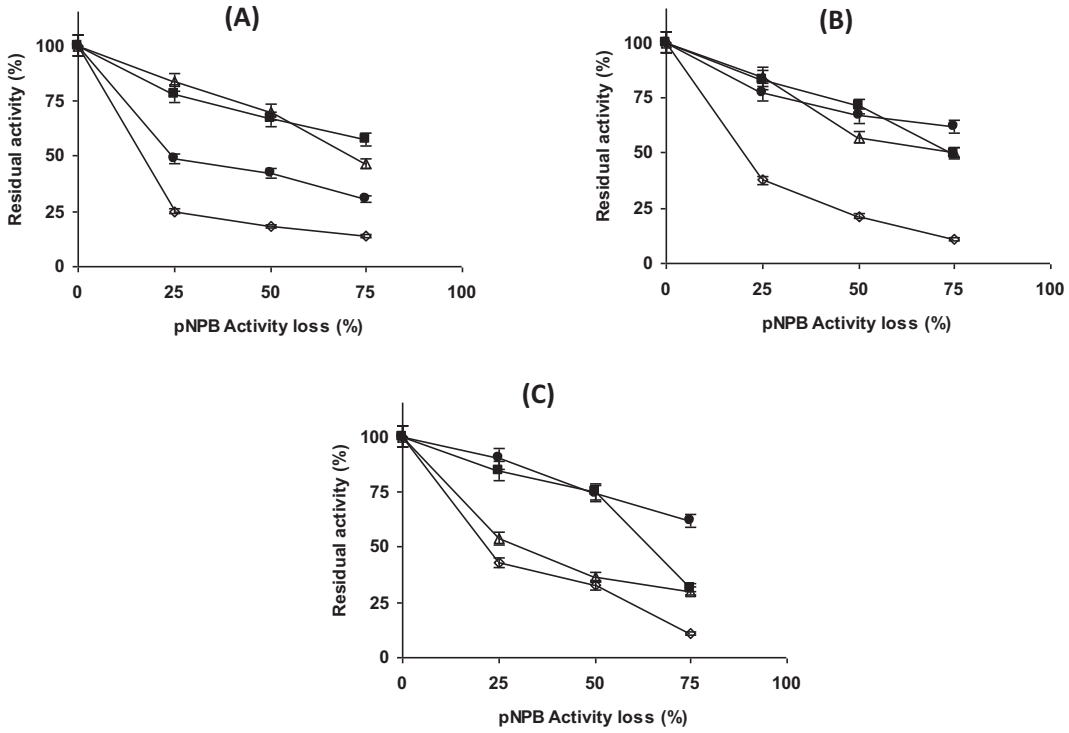

Fig. 5. Effect of the inactivation pH on the residual activity versus different substrates of TLL biocatalysts activity blocked with ethylenediamine. Inactivation was performed at (A) pH 5.0 and 74 °C (B) pH 7.0 and 73 °C and (C) pH 9.0 and 71 °C. The substrates use were: solid squares: *p*NPB; empty rhombus: triacetin; empty triangles: (*R*) - methyl mandelate; solid circles: (*S*) - methyl mandelate; as substrates. Other specifications may be found in Methods section.

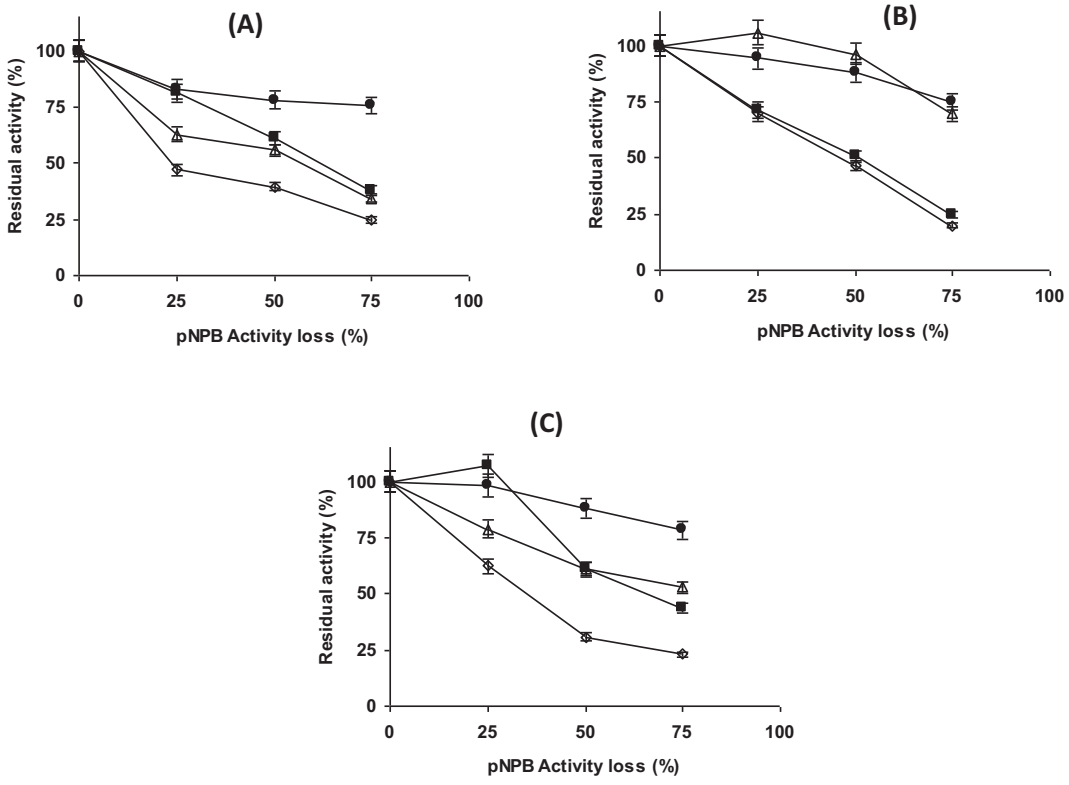

Fig. 6. Effect of the inactivation pH on the residual activity versus different substrates of TLL biocatalysts activity blocked with hexyl amine. Inactivation was performed at (A) pH 5.0 and 74 °C (B) pH 7.0 and 73 °C and (C) pH 9.0 and 71 °C. The substrates use were: solid squares: *p*NPB; empty rhombus: triacetin; empty triangles: (*R*) - methyl mandelate; solid circles: (*S*) - methyl mandelate; as substrates. Other specifications may be found in Methods section.

different substrates, with triacetin it is clear that at pH 7 the residual activity decrease when the inactivation time was prolonged is the most intense while using (*R*)-methyl mandelate is the least intense. Using (*S*)-methyl mandelate, the enzyme residual activity versus is very high at all studied pH values (using the most inactivated enzyme preparations, the observed activity ranged from 75 to 80% at pH 5 and 9, to almost 90% at pH 9).

Comparing the different biocatalysts with the same substrate under the same inactivation conditions also provide some information. To facilitate this comparison, the figures are included in supporting information (Figs. 1S–3S).

Using triacetin as substrate to determine the enzyme activity at pH 5 (Fig. 1Sa), only the biocatalysts blocked with HEX show a significant difference in the enzyme activity retention, giving the highest activity recovery. If the substrates employed to determine the activity is (*R*)-methyl mandelate (Fig. 1Sb), the highest percentage of initial activity is recovered by the preparation blocked using EDA (47% while the other preparations maintained around 30%), while using (*S*)-methyl mandelate (Fig. 1Sc) is the preparation blocked with HEX (the most inactivated preparation retained 75% of the initial activity). The preparation blocked with EDA is the one that recovered less activity with this substrate in the intermedium points, but the 3 preparations have similar activity using the most inactivated biocatalysts (around 30%).

At pH 7 (Fig. 2S), the preparation blocked with HEX is the one that retained the highest initial activity versus triacetin (19% using the most inactivated biocatalyst) (Fig. 2Sa), followed by that blocked using EDA, being the ones that retain the lowest percentage of the initial activity those blocked using Gly or Asp (under 5%). Using (*R*)-methyl mandelate (Fig. 2Sb), the highest percentage of initial activity is maintained by the biocatalysts that are blocked using HEX. However, the value is similar to that of the preparation blocked with Asp if looking only at the most inactivated preparation (around 70%), being the preparation blocked with EDA, which retained a lower percentage of activity. Using as substrate (*S*)-methyl mandelate (Fig. 2Sc), only the preparations blocked using HEX differs on the retention of activity 88% for the most inactivated preparation when compared to the other preparations (that ranged between 52 and 60%).

Finally, at pH 9 (Fig. 3S), using triacetin (Fig. 3Sa), differences are only relevant using HEX, mainly in the first inactivation point, where the activity retention is clearly higher. Using (*R*)-methyl mandelate (Fig. 3Sb), the preparation blocked using HEX is the one that retained more activity (over 50% in the most inactivated preparation), followed by that blocked with Asp (almost 40% of activity retention) and the other two preparations presented around 30% of the initial activity. Using (*S*)-methyl mandelate (Fig. 3Sc), the highest activity retention is shown by that preparation blocked with HEX (almost 80%), followed by that blocked with EDA (over 60%) and the other two preparations (under 50%).

The results presented above show several facts. First, the loss of activity when the enzyme is submitted to thermal inactivation strongly depends on the substrate utilized in the activity determination. This means that during the inactivation, the enzyme specificity is changing, and this is very important when the enzyme is to be utilized in some kinetic resolution of a chiral mixture, as when the enzyme is becoming inactivated, the activity versus one of the isomers may decrease more rapidly that the activity versus the other isomer.

A proof of the changes in the enzyme specificity during its thermal inactivation is very visible focusing on the activity versus triacetin. The activity versus triacetin decreases more rapidly than when using the other substrates for all biocatalysts and inactivation conditions, in some instances the enzyme activity versus this substrate became almost 0 while with some of the other substrates, it remained over 70%. Regarding the enantiospecificity versus the isomers of mandelic acid, in some instances, the activity decreases similarly with both isomers. In that cases, the enantiospecificity will be unaltered during the enzyme inactivation, However, in other cases, it decreased more rapidly versus the (*R*)-isomer or versus the (*S*)-isomer, then the enzyme enantiospecificity will be changing during the process altering the biocatalyst performance in these kind of processes.

Moreover, the inactivation pH alters how the activity of a specific biocatalyst versus each substrate is lost; suggesting that the conformational changes produced during the inactivation may be different, depending on the inactivation pH. The paper shows examples when a biocatalyst lost more rapidly the activity versus a specific substrate at one pH and that occurred with other substrate at other pH value. For example, using the preparation blocked with Asp (Fig. 3), the activity is better maintained at pH 5 and 9 using *p*NPB, but at pH 7, the higher residual activity after the inactivation is using (*R*)-methyl mandelate. Using EDA as a blocking agent (Fig. 5), at pH 5 the highest activity recovery is obtained using *p*NPB or (*R*)-methyl mandelate, while at pH 9 the activity decrease using (*R*)-methyl mandelate is the second worse.

Similarly, the changes in activity versus the different substrates are not similar under a fixed pH value if changing the blocking agent.

That way, we suggest that the conformational changes induced by the interaction between the enzyme and the support, under the different inactivation conditions, produce different enzyme conformations. To investigate of really some different enzyme conformations are generated during the inactivation of the different preparations, we have decided to perform Trp fluorescence spectra of the different biocatalysts.

3.5. Fluorescence studies of the different TLL biocatalysts

It should be remarked that all samples have been in Tris pH 7 for at least one week before performing these studies, and then have been performed using identical conditions. That way, differences should be derived from structural changes induced by the blocking agent or the inactivation conditions. First, we have studied the effect of the blocking agent nature on the structure of the immobilized TLL biocatalysts by measuring their intrinsic protein fluorescence spectra (Fig. 4S). The analysis of such spectra reveals that using Asp, EDA and Gly, the λ_{max} of immobilized TLL was roughly 328 nm, while using HEX as blocking agent, the λ_{max} of the immobilized TLL red-shifted 4 nm (Fig. 7A). On the contrary, we observed that I_{max} has a stronger dependence on the type of blocking agent (Fig. 7B). The blocking with amines such as HEX and EDA promoted a significant decay if the I_{max} was compared to that one observed for the immobilized TLL blocked with the amino acids Gly or Asp. These fluorescence spectra indicate that the immobilized TLL undergo large structural distortion when is blocked with HEX, explaining the significantly lower recovered activity of the final immobilized biocatalyst (Table 1).

During the inactivation experiments shown in Fig. 2, we withdrawn samples at different residual activity percentages (100, 75, 50 and 25%) to further analyzed their intrinsic fluorescence. Figs. 8 (λ_{max}) and 9 (I_{max}) evidence the different structural distortions undergone by the immobilized TLL blocked with different reagents and inactivated under different pH conditions. When the biocatalysts were inactivated at neutral pH value, we observed a red-shifted of λ_{max} when the biocatalysts were blocked with Asp and Gly during the inactivation course (Fig. 8). On the contrary, the λ_{max} of the biocatalyst blocked with HEX remained almost unaltered regardless of the inactivation degree. Similar insights were observed when the immobilized biocatalysts were inactivated at pH 9. However, at pH 5 the λ_{max} was invariable during the inactivation course in all cases (Fig. 8). When we analyzed the I_{max}, we found out that blocking with HEX promoted the most significant enzyme distortion (lower I_{max} values) of all tested immobilized biocatalyst at pH 7 and 5 but not at pH 9. These data suggest that the conformation of TLL is significantly less packed when the HEX group is highly positive charged (pH 5–7) than when it is more neutral (pH 9) according to the HEX pKa (around 10.7). These results agree with the functionality of these immobilized biocatalyst. Fig. 2 shows how HEX blocking makes an immobilized biocatalyst less stable than Gly blocking under pH 5 and 7 inactivation conditions, however those stability differences were

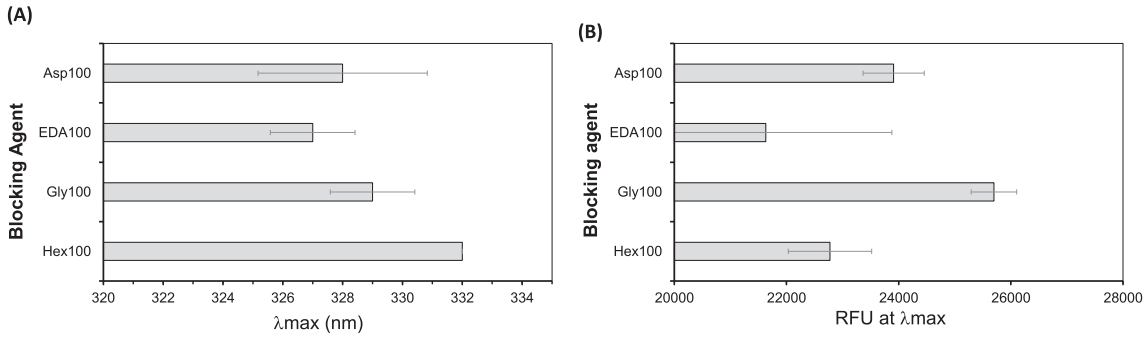

Fig. 7. Intrinsic protein fluorescent of TLL biocatalysts blocked with different blocking reagents. (A) Wavelength at the maximum fluorescence intensity (λ_{max}). (B) Fluorescence intensity at λmax when simples were excited at 280 nm. Spectra can be found in supporting information (Fig. 4S). RFU = relative fluorescence units.

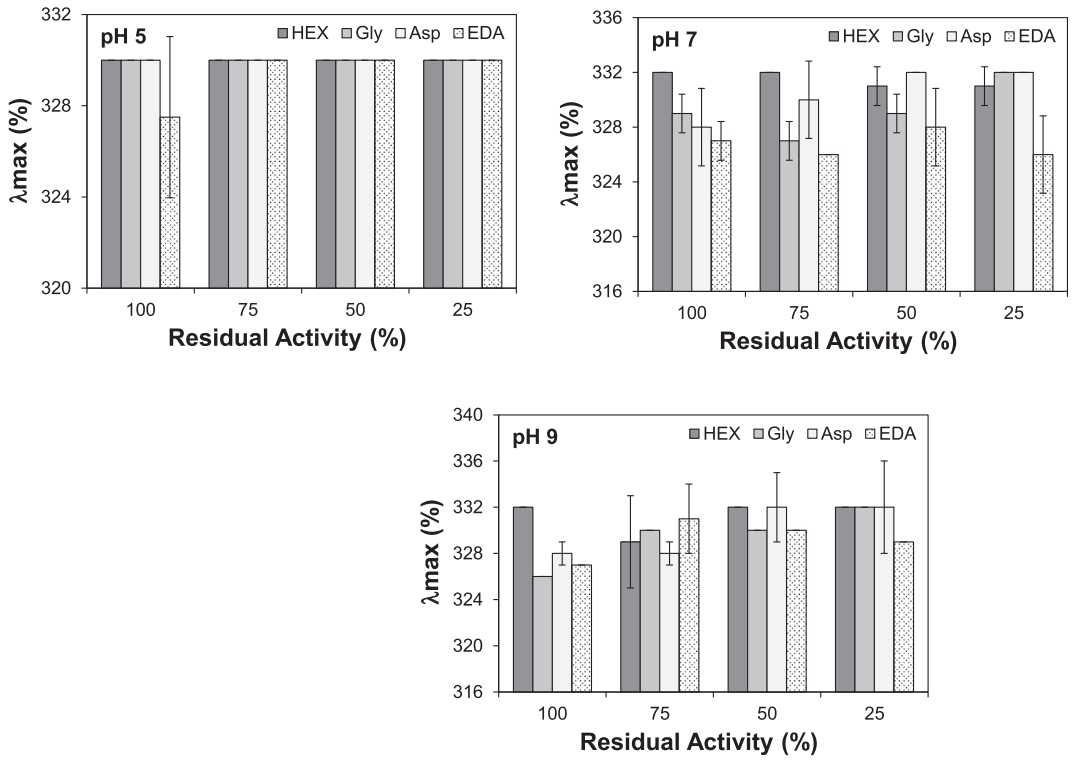

Fig. 8. Intrinsic fluorescence (λmax) of immobilized TLL biocatalyst blocked with different reagents and inactivated at different pH values. The 100 number indicate that they are the fully active biocatalysts. From the intrinsic fluorescence spectra,(λmax) were plotted for the different TLL at different inactivation degrees. The residual activity means the activity exhibited by the TLL biocatalyst after being incubated at certain time at different pHs (Fig. 2).

mitigated at pH 9. Accordingly, the fluorescence spectra of the immobilized enzyme biocatalysts inactivated at neutral pH show that the structural distortions of TLL blocked with HEX were more drastic (low I_{max} values and red-shifted λ_{max}) during the inactivation course than those ones observed using Gly blocked biocatalysts. At alkaline pH, this structural differences between HEX and Gly blocked preparations were less notorious in agreement with the more similar stabilities exhibited by the TLL immobilized biocatalyst blocked with those tow blocking reagents (Fig. 2).

That way, the fluorescence studies confirm that the initial structures of the differently blocked biocatalysts are different, and that both, inactivation pH and blocking agent, conditioned the path-way of the enzyme inactivation.

4. Conclusions

The results presented in this paper show the complexity of the enzyme inactivation. It has been clearly shown that the inactivation of an immobilized enzyme is strongly depended on the possibility of establishing different enzyme-support interactions (as the enzyme has exactly the same orientation and the same number of enzyme-support bonds) and the inactivation conditions. In this paper, as a first proof of concept approach, we have focused on the influence of the pH on the thermal inactivation of the immobilized enzymes, but it will be also very interesting to check if the presence of other inactivating agents, such as organic solvents, ionic liquids, or the inactivation in other conditions (high ionic strength, presence of stabilizers/destabilizers compounds, etc.) may also lead to different inactivation pathways, depending on the surface properties. Functionally, it has been shown how the enzyme specificity is greatly altered during the inactivation, (activity decrease quicker with some substrates than with other substrates), and these changes depend on the nature of the blocking agent and inactivation pH value. Similarly, it has been shown by florescence analysis that the initial enzyme conformation was already depended on the blocking

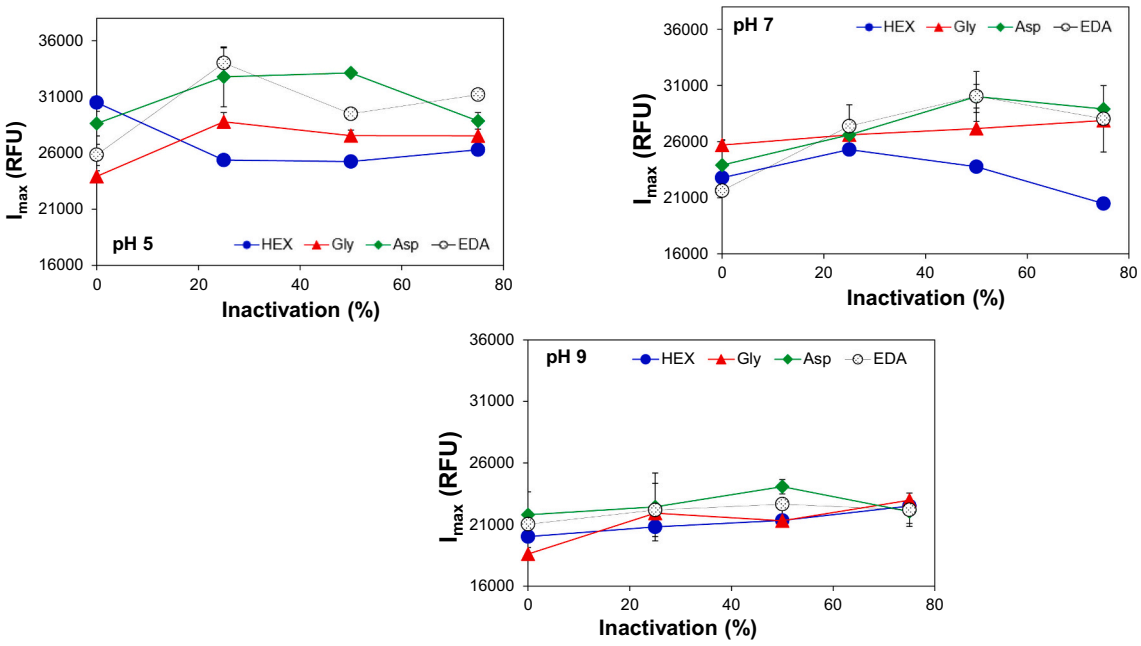

Fig. 9. Intrinsic fluorescence (I_{max} at λmax) were plotted for the different TLL at different inactivation degrees. The inactivation percentage is the activity lost by the different TLL biocatalyst after being incubated at certain time at different pHs. RFU = relative fluorescence units.

agent and that the changes motivated by the inactivation conditions were also determined by the surface of the support and the inactivation pH.

This is the first time that this is shown by functional and structural studies, and open new questions when a biocatalyst is designed and evaluated, as the obtained values may be different depending on the utilized substrate. From the results in this paper, preferably, it should be better to check the biocatalyst stability under the operational conditions and using the target substrate.

CRediT authorship contribution statement

In this paper, Priscila M. Paiva Souza, Diego Carballares and Nerea Lopez-Carrobles prepared the biocatalysts and analyzed their functional behavior during inactivation, Fernando Lopez-Gallego performed the florescence studies, Sueli Rodrigues and supervised the experiments. Roberto Fernandez-Lafuente designed and supervised the experiments. All authors contributed to the writing of the paper.

Acknowledgments

We gratefully recognize the financial support from Ministerio de Ciencia e Innovación-Spanish Government (project number CTQ2017-86170-R). This study was also financed by the Brazilian agencies: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (Funcap) – Grant PR2-0101-00012.01.00/15. DC gratefully thank Ministerio de Ciencia e Innovación for a FPI fellowship. The help and suggestions from Dr. Ángel Berenguer (Departamento de Química Inorgánica, Universidad de Alicante) are gratefully recognized.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2021.09.061.

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