



Determination of immobilized lipase stability depends on the substrate and activity determination condition: Stress inactivations and optimal temperature as biocatalysts stability indicators

Thays N. da Rocha^{a,b}, Diego Carballares^a, José R. Guimarães^{a,c},
Javier Rocha-Martin^d, Paulo W. Tardioli^c, Luciana R.B. Gonçalves^b,
Roberto Fernandez-Lafuente^{a,e,*}

^a Departamento de Biotecnología, ICP-CSIC, Campus UAM-CSIC, 28049, Madrid, Spain

^b Federal University of Ceará, Chemical Engineering Department, Campus do Pici, Bloco 709, Fortaleza, CE CEP, 60440-900, Brazil

^c Graduate Program in Chemical Engineering (PPGEQ), Laboratory of Enzyme Technologies (LabEnz), Department of Chemical Engineering, Federal University of São Carlos (DEQ/UFSCar), Rod. Washington Luís, km 235, 13565-905, São Carlos, SP, Brazil

^d Department of Biochemistry and Molecular Biology, Faculty of Biology, Complutense University of Madrid, José Antonio Novais 12, Madrid, 28040, Spain

^e Center of Excellence in Bionanoscience Research, Member of the External Scientific advisory Board, King Abdulaziz University, 21589, Jeddah, Saudi Arabia

ARTICLE INFO

Keywords:

Lipase stability
Lipase optimal temperature
Lipase specificity
Lipase properties tuning

ABSTRACT

Lipases A and B from *Candida antarctica* (CALA and CALB), *Thermomyces lanuginosus* (TLL) and *Candida rugosa* have been immobilized on octyl, octyl-vinyl sulfone (blocked with ethylendiamine) and amino-glutaraldehyde. The biocatalysts exhibited different specificity versus triacetin and p-nitro phenyl butyrate. Optimal activities were determined using triacetin for all biocatalysts, and this ranged from 40 °C for CALA and TLL, to 60 °C for an amino-glutaraldehyde-CRL. The biocatalysts were inactivated at 70 and 75 °C, determining their residual activities at 25 °C or 55 °C. The inactivation courses were very different depending on the substrate; in most cases the biocatalysts maintained more activity during the thermal inactivation using triacetin (except using TLL). When determining the residual activities at 55 °C, the values increased in most cases, reaching high hyperactivation values using CALA (even 23 folds). That way, the “stability” of the different preparations was strongly influenced by the substrate and residual activity determination conditions, and did not agree in most cases with the optimal temperatures.

1. Introduction

One of the points where more emphasis is placed in the preparation of an enzymatic biocatalyst is in enzyme stability (Illanes, 1999; Kadisch et al., 2017; Polizzi et al., 2007). This marks the possibility of enzyme reuse and the range of conditions where the enzyme may be used, and thus, the biocatalyst productivity (Meissner and Woodley, 2022).

When studying the enzyme features, some papers only include the enzyme/activity profile. The enzyme activity typically

* Corresponding author. Departamento de Biotecnología, ICP-CSIC, Campus UAM-CSIC, 28049 Madrid, Spain.
E-mail address: rfl@icp.csic.es (R. Fernandez-Lafuente).

exponentially increases with increased temperature at moderate temperature ranges, but this increase slows down when approaching the optimal temperature (the temperature where the maximum activity is observed under the defined conditions) and then declines when the Temperature is set above optimal temperatures (Daniel and Danson, 2010; Dill et al., 2011; Gillooly et al., 2001; Schnell and Turner, 2004). The activity decreases are usually attributed to changes in the enzyme, that can be a consequence of enzyme denaturation, altered temperature sensitivity of substrate-enzyme or enzyme-product transitions stages alterations (Glowacki et al., 2012), changes in heat capacity of enzyme-bound intermediates (Gürdaş et al., 2012; Keken-Huskey et al., 2016; Yadav and Prakash, 2009), variations in the ionic status at the enzyme active center (Daniel and Danson, 2010) or several of them acting simultaneously (DeLong et al., 2017).

Many papers tend to report the operational enzyme stability under these “optimal” activity conditions, that are those where the enzyme is close to starting to becoming distorted in the activity assay determination time, consequently, usually they show a relatively poor operational enzyme stability, as the whole reaction courses will take much longer times than the activity determination assay. It should be preferable to use suboptimal activity Temperatures to have a much better enzyme operational stability (Grubecki, 2016). Moreover, enzyme optimal temperature is not an objective parameter, as it depends on the experimental conditions and can change from one lab to another just by changes in them. It is expected that the exact pH value in the assay can alter the results, but this is simple to be maintained at similar values from one lab to another. However, the enzyme concentrations used in the activity determination may also be critical to define the optimal temperature, as well as their purity. For example, multimeric enzymes may tend to dissociate during the most drastic assay conditions (Fernandez-Lafuente, 2009), and if this occurs, the enzyme concentration may determine enzyme stability and that way, observed enzyme optimal temperatures. Lipases tend to form lipase-lipase aggregates involving two open forms of the lipases, giving forms with different activity and stability, and this depends on the medium conditions (e.g., ionic strength, presence of detergents or hydrophobic large biomacromolecules or temperature) (Fernández-Lorente et al., 2003; Palomo et al., 2003; Wilson et al., 2006). The substrates/products may have positive/negative effects on enzyme stability (e.g., using hydrogen peroxide, phenols (Fernandez-Lafuente et al., 2000; Grubecki, 2021, 2018a, 2018b; Hernandez et al., 2012)), and that way, their concentrations during the enzyme activity assay may also alter the results. Some buffers or salts have negative or positive effects on enzyme stability, even in a different form for different enzyme preparations (Brahm et al., 2021a, 2021b; Fernandez-Lopez et al., 2015, 2016; Zaak et al., 2017a) and this can also alter the final “optimal” temperature of the enzyme. It may be also expected that the calculated optimal temperature of an enzyme may depend on the duration of the activity assay. At first glance, the longer the enzyme is exposed to high temperature, the longer the heat may induce undesired changes in the enzyme structure. That way, longer assay temperatures can yield lower observed “optimal” temperature. In this sense, the optimal temperature can only be expected to be similar from one lab to another if the measurement conditions and protocols are fully identical. That way, characterization of immobilized enzymes stability may be not a simple task, as they may be affected by many different factors, including the immobilization and the activity determination protocols, (Bolivar et al., 2022; Illanes et al., 1998; Illanes and Wilson, 2003; Wilson and Illanes, 2020).

The complexity of the understanding of the stability of immobilized enzyme preparations can be also related to the diversity of underlying causes to drive to the enzyme inactivation (structural, partition effects, microenvironment, substrate access) (Bolivar et al., 2016; Bommarius and Paye, 2013).

Some other papers only include stress inactivations, typically taking samples under stress inactivation conditions and assaying the residual activities under mild conditions. This way, it is likely that a partially distorted enzyme can regain some of the lost activity by the thermal inactivation during the activity determination assay (Rodrigues et al., 2009b; Rueda et al., 2015; Soler et al., 1997; Suescun et al., 2015), and the final residual activity that is measured may depend on the assay time and substrate concentration.

However, both stress inactivations and optimal temperatures are considered to be good estimations of the enzyme stability, and although somewhat imperfect, they are expected to be correlated; an enzyme exhibiting a higher optimal temperature will be considered a more stable enzyme, and *vice-versa*.

One of the strategies to increase the enzyme stability is enzyme immobilization (Bommarius and Paye, 2013; DiCosimo et al., 2013; Garcia-Galan et al., 2011; Iyer and Ananthanarayan, 2008; Klibanov, 1979; Liese and Hilterhaus, 2013; Mateo et al., 2007; Rodrigues et al., 2021; Sheldon and van Pelt, 2013). Initially designed to permit the enzyme recovery and reuse, nowadays immobilization is expected to improve many functional properties (selectivity, activity, specificity, purity, resistance to inhibitors), being enzyme stabilization one of the main targets (Garcia-Galan et al., 2011; Iyer and Ananthanarayan, 2008; Klibanov, 1979; Mateo et al., 2007; Rodrigues et al., 2021). Recently, the causes of enzyme stabilization following immobilization (involving rigidification of the enzyme structure, generation of microenvironments, prevention of subunit dissociation, etc.) have been reviewed (Rodrigues et al., 2021).

During selection of the immobilization protocol to increase enzyme stability, researchers tend to select one of the above methods, expecting that they can be somehow correlated with the other (as in many instances they are) (Siar et al., 2018a, 2018b, 2019). This is even considering that the situation using immobilized enzymes may be more complex than using free enzyme. In situations where substrate diffusion may decrease the enzyme activity (Benítez-Mateos et al., 2020; Bortone et al., 2014; Schmieg et al., 2020; Van Daele et al., 2016; Xiu et al., 2001), it has been described that product formation responds twice as strongly to increased temperature than diffusion or transport matters (Ritchie, 2018).

Thus, in this paper we present the stability features of several of the most used lipases (lipases from *Candida antarctica* (forms A and B, CALA and CALB) (Anderson et al., 1998; Błaszczuk and Kiebasinski, 2020; Domínguez De María et al., 2005; Gotor-Fernández et al., 2006; Monteiro et al., 2021), *Thermomyces lanuginosus* (TLL) (Fernandez-Lafuente, 2010) and *Candida rugosa* (CRL) (Benjamin and Pandey, 1998; Domínguez De María et al., 2006)) immobilized following different protocols on agarose beads (Zucca et al., 2016). As explained above, the use of the free lipases may produce many artifacts in stability studies, that way we have focused our attention to the comparison of the different immobilized lipase biocatalysts.

The immobilization on octyl-agarose beads was one of the selected immobilization methods. It is based on the interfacial activation of lipases (Brzozowski et al., 1991; Grochulski et al., 1993; Martinelle et al., 1995; Verger, 1997) on the hydrophobic surface of the support, giving as result a monomeric lipase form with a stabilized open structure (Manoel et al., 2015; Rodrigues et al., 2019). Other immobilization method was the use of heterofunctional vinyl sulfone-octyl agarose (Albuquerque et al., 2016). This protocol of lipase immobilization starts with the interfacial activation of the enzyme on the octyl layer, but later on, some covalent bonds are established between the enzyme and the support (Arana-Peña et al., 2022). The blocking step introduces new groups on the support surface, the enzyme is now exposed to interactions with the blocking agent and the octyl groups, and this can alter the enzyme performance and even its inactivation pathway (Paiva Souza et al., 2021). Finally, the enzymes were covalently immobilized on glutaraldehyde activated aminated agarose beads. This immobilization method is expected to immobilize the enzyme via ion exchange, followed by the enzyme covalent attachment (Barbosa et al., 2014). Then, the biocatalysts stability under stress conditions was analyzed, using two different substrates and two temperatures (25 °C and 55 °C, where chemical decomposition of the substrate starts to be too high to have a reliable activity determination), and the temperature where the maximum activity is obtained for each biocatalyst was also determined. The use of a temperature over the optimal temperature of most of the biocatalysts in the residual activity determination should reduce the reactivation of the enzyme during the residual activity determination.

2. Materials and methods

2.1. Materials

For this study, we have utilized different commercial liquid lipase formulations: Lipozyme B, (CALB, 7.7 mg of protein per mL), NovoCor® ADL (CALA, 7.68 mg of protein per mL), TLL (20.77 mg of protein lipase per mL). They were kindly provided by Novozymes (Madrid, Spain). CRL in powder formulation (3.2% of protein content), glutaraldehyde (25% (v/v)), ethylenediamine (EDA), *p*-nitrophenyl butyrate (*p*-NPB) and triacetin was acquired from Sigma-Aldrich (Madrid, Spain). Divinyl-sulfone (DVS) was purchased from Thermo Fisher Scientific Spain (Madrid, Spain). Octyl-Sepharose® CL-4B beads were acquired from GE healthcare. Bradford's method was employed for determining the concentration of proteins using bovine serum albumin as standard (Bradford, 1976). Products of analytical grade of other solvents and reagents were employed.

2.2. Methods

2.2.1. Preparation of octyl-vinyl sulfone agarose beads

The octyl-vinyl sulfone agarose support was prepared following the protocol development by Albuquerque et al. (2016) with slight modifications. 10 g of octyl-agarose was added to 200 mL of a solution formed by 0.333 M sodium carbonate buffer and 0.35 M of divinyl sulfone at pH 11.5. This suspension was placed under mild agitation for 2 h at room temperature. Finally, the activated support was vacuum filtered, washed extensively with distilled water and stored at 4–6 °C.

2.2.2. Preparation of the glutaraldehyde-agarose support

After preparing aminated agarose (Fernandez-Lafuente et al., 1993), the support was activated with glutaraldehyde by incubation in a 10% glutaraldehyde solution at pH 7.0 for 16 h. This permitted to introduce two glutaraldehyde molecules per amino group in the support (Barbosa et al., 2014; Betancor et al., 2006; Monsan, 1978). After, the activated supports were washed with distilled water to remove excess of glutaraldehyde and stored in a fridge.

2.2.3. Enzymes immobilization

The enzyme loadings of the immobilized biocatalysts were; CALB at 1 mg of enzyme/g of support; CALA and CRL at 4 mg of protein/g of support, TLL at 12 mg of enzyme/g of support. The enzyme loading was selected to have reliable activity determinations for all the biocatalysts and substrates, while trying to prevent enzyme-enzyme interactions that could alter the results (Arana-Peña et al., 2020; Fernandez-Lopez et al., 2017; Zaak et al., 2017b). All immobilizations were followed measuring the *p*-NPB activity of suspension, supernatant and a reference of the enzyme under the same conditions but where inert agarose was used.

2.2.3.1. Immobilization of lipases on octyl agarose beads. The lipases solutions were immobilized via interfacial activation on this support (Manoel et al., 2015; Rodrigues et al., 2019, 2021). The enzyme solutions were diluted in 5 mM sodium phosphate buffer at pH 7.0 and 25 °C, and then the octyl agarose support was added in the proportion 1 g of support: 10 mL of enzymatic solution. Immobilization was carried out under mechanical agitation for 2 h, measuring the activity of supernatant, suspension and reference using the *p*-NPB assay described above. When *p*-NPB activity was not detected in the supernatant, the biocatalysts were filtered, washed with distilled water and stored at low temperature (4 °C).

2.2.3.2. Immobilization of lipases on octyl-vinyl sulfone agarose beads. The lipases solutions were diluted in 5 mM sodium acetate at pH 5.0 (to favor the interfacial activation as main cause of enzyme immobilization) and 25 °C and then, octyl-vinyl sulfone agarose support was added in a proportion 1 g of support/10 mL of enzymatic solution (Albuquerque et al., 2016). *p*-NPB assay was utilized to follow the activity of supernatant, suspension and reference (Albuquerque et al., 2016). After, the biocatalysts were washed with distilled water, vacuum filtered and recovered. In order to favor the enzyme-support covalent attachment formation, the biocatalysts were resuspended in 100 mM sodium carbonate at pH 10.0 and 25 °C for 24 h in the same proportion. Afterwards, 10 mL of 2 M ethylenediamine at pH 10.0 and 25 °C was prepared and added in 1 g of biocatalysts for 48 h, to modify the residual vinyl sulfone moieties. Finally, the biocatalysts were washed with distilled water, vacuum filtered and stored in a refrigerator at 4 °C.

2.2.3.3. Immobilization of lipases on amino-glutaraldehyde-agarose beads. All lipases were immobilized on amino-glutaraldehyde-agarose using 1 g of support in 10 mL of enzyme solution prepared in 5 mM sodium phosphate at pH 7.0 and 25 °C for 2 h. The

immobilization was followed measuring *p*-NPB activity of the supernatant, suspension and reference solutions. After immobilization, the biocatalysts were washed with water, vacuum dried and stored at fridge.

2.2.4. Determination of enzyme activity using different substrates

One unit of enzymatic activity (U) was defined as μmol of substrate hydrolyzed by the enzyme per minute and enzyme or biocatalyst amount, under the assay conditions.

2.2.4.1. Hydrolysis of *p*-NPB. 50 μL of 50 mM *p*-NPB dissolved in acetonitrile was added to a cuvette with 2.5 mL of 25 mM sodium phosphate at pH 7.0. To initialize the reaction, 50 μL (free enzyme solution or immobilized enzyme suspension) was added. Lipase activity quantification was performed by determining the rise of the absorbance at 348 nm produced by the release of *p*-NP (isosbestic point of *p*-NP, its ϵ under these conditions is $5150 \text{ M}^{-1} \text{ cm}^{-1}$) for 90 s (Lombardo and Guy, 1981), under regulated temperature (25 °C) and magnetic stirring.

2.2.4.2. Hydrolysis of triacetin. A mass of 20–90 mg of biocatalyst was added to 5 mL of 50 mM of triacetin in 50 mM sodium acetate buffer solution at pH 5.0, to reduce acyl migration (Hernandez et al., 2011). The reactions were performed at different temperatures: 25, 30, 35, 40, 45, 50, 55, 60 and 70 °C, and the activities were determined through the conversion grades (15–20%) for the production of 1,2 diacetin, where the retention time was 4 min for the products and 18 min for the substrate. The mobile phase was 15/85 acetonitrile- Milli-Q water, with a flow rate of 1 mL/min in a HPLC, Jasco UV 15–75 (Jasco, Madrid, Spain) using a Kromasil C18 column (15 cm $\times$ 0.46 cm) with a UV detector at 230 nm, injecting samples of 20 μL .

2.2.5. Thermal inactivations and recovered activity

0.5 g portions of the biocatalysts were suspended in 4 mL of 10 mM Tris buffer at pH 7.0 in a bath at 70 or 75 °C. Periodically, samples were withdrawn, and their residual activities were measured using the *p*-NPB assay described above considering 100% the initial activity of the preparation. Aiming at a comparison of the enzyme stabilities with the two substrates, periodically, 400 μL aliquots of inactivation biocatalyst were removed at specific times and added in 3 mL of 50 mM of triacetin at 25 and 55 °C in a reactor under magnetic stirring and samples were withdrawn every 10 min to analyze their activities as described above. In parallel 50 μL aliquots of inactivation biocatalyst suspension were taken to measure their *p*-NPB activity at 25 °C.

3. Results

3.1. Immobilization of the different enzymes

Immobilizations of CALA, CALB, TLL and CRL were performed as described in methods. In all cases the immobilization permitted the full immobilization of the offered enzyme activity (results not shown). Table 1 shows the activity of the different biocatalysts using *p*-NPB or triacetin, at 25 °C. We do not show the data using free enzyme as this is strongly dependent on the aggregation of the lipases with other lipase molecules or other components of the extract (Fernández-Lorente et al., 2003; Palomo et al., 2003, 2004, 2005; Wilson et al., 2006), which can alter the enzyme features and make the results very hard to understand.

In the case of CALA, using *p*-NPB to determine the residual activity, the least active biocatalyst was that prepared on amino-glutaraldehyde, which presented half the activity of the other two preparations. The most active preparation was that prepared using octyl-VS (almost 15% more active than octyl-CALA). Using triacetin as substrate, the situation is quite different. Now, the most active preparation is octyl-CALA, 2.5 more active than octyl-VS-CALA and fourfold than amino-glutaraldehyde-CALA (Table 1). All CALA biocatalysts were more active versus *p*-NPB than versus triacetin, but the substrate specificity differed for each biocatalyst.

Using CALB (Table 1), again the most active preparation versus *p*-NPB is octyl-VS-CALB, while the least active one is amino-glutaraldehyde-CALB (55%). Once again, the covalent attachment and blocking steps yielded a more active biocatalyst when preparing octyl-VS than the just interfacially activated enzyme. The situation fully reverts using triacetin, in this instance the most active preparation is amino-glutaraldehyde-CALB, almost doubling the activity of the octyl-CALB; while octyl-VS-CALB presented around 70% of the activity of octyl-CALB. In fact, octyl-VS-CALB was more active versus *p*-NPB than versus triacetin, while amino-glutaraldehyde-CALB was 4.5 times more active versus triacetin, and octyl-CALB was only 1.6 fold more active versus triacetin. The changes in enzyme specificity are far more intense in this instance than using CALA.

Using TLL, the enzyme immobilized on amino-glutaraldehyde was almost fully inactive using both substrates, and it was discarded for further studies. The other two biocatalysts were more active versus *p*-NPB than versus triacetin. Octyl-TLL was more active than octyl-VS-TLL for both substrates, being the difference slightly higher using triacetin.

Finally, using CRL, all biocatalysts were much more active versus *p*-NPB than versus triacetin. Using *p*-NPB, octyl-VS-CRL was the most active biocatalyst, followed by amino-glutaraldehyde-CRL (around 75%) and the least active was octyl-CRL (around 60%).

Table 1

Activities of the different biocatalyst in the hydrolysis of 1 mM *p*-NPB at pH 7.0 and 50 mM triacetin at pH 5.0 and 25 °C. Experiments were conducted as described in Methods.

Activity (U/g)									
CALA (4 mg/g)			CALB (1 mg/g)		TLL (12 mg/g)		CRL (4 mg/g)		
Biocatalyst	<i>p</i> -NPB	Triacetin	<i>p</i> -NPB	Triacetin	<i>p</i> -NPB	Triacetin	<i>p</i> -NPB	Triacetin	
Octyl	87.57	25.06	23.68	38.57	162.14	91.03	78.15	9.08	
Glutaraldehyde	45.19	6.38	16.85	76.71	*	*	97	3.3	
Octyl-VS	98.26	10.63	29.22	26.18	124.27	50.22	128.53	7.02	

However, this last one was the most active against triacetin, followed by octyl-VS- CRL (over 75%) and amino-glutaraldehyde-CRL (over 35%).

These results agree with the changes on enzyme specificity and activity caused by the immobilization protocol and reported in many different instances (Ahmmed, S. Z.; Gomes, J.; Sreekrishnan, 2008; Arana-Peña et al., 2020; Chaubey et al., 2006; Dos Santos et al., 2015b; Du et al., 2009; Guimarães et al., 2021; Lokha et al., 2020; Paiva Souza et al., 2021; Sabbani et al., 2006; Takaç and Bakkal, 2007). It suggests the conformational changes induced by the covalent bonds and the interactions with the amino groups in the support during the blocking step (Albuquerque et al., 2016; dos Santos et al., 2015; Dos Santos et al., 2015a, 2015b; Paiva Souza et al., 2021) usually produced a somehow more active enzyme form that the just interfacially activate enzyme for *p*-NPB, while these enzyme forms are usually less effective in triacetin hydrolysis. Glutaraldehyde, as it does not immobilize the open form of the enzyme like in the case of octyl-agarose (Manoel et al., 2015), is usually the least active biocatalyst (exceptions are CRL versus *p*-NPB, and notably CALB versus triacetin), but this is clearer in the case of triacetin than in the case of *p*-NPB. That suggests that may be convenient to check the enzyme activity of the immobilized preparations with the target substrate and not only with the synthetic colorimetric one before taking decisions related to the suitability of a specific biocatalyst for a specific process related to the enzyme activity.

3.2. Determination of the effect of the temperature on the activity and stability the different preparations of CALA

Fig. 1 shows the effect of the temperature on the activity versus triacetin of all the CALA biocatalysts described above. *p*-NPB was discarded for this experiment due to the rapid chemical decomposition of this substrate at high temperature, that prevented to perform reliable activity determinations. Fig. 1 shows that, in all cases, maximum activities were found at 40 °C, with relatively similar shapes until reaching this temperature: from 25 °C to 40 °C the activity increased from 5.9 (for octyl-VS-CALA) to 6.4 folds (for octyl-CALA). The decrease in activity at 50 °C was sharper using octyl-CALA (decreasing almost threefold), and milder using amino-glutaraldehyde-CALA (1.8 folds) or octyl-VS-CALA (less than 1.6).

However, when the biocatalysts were incubated at 55 °C, no activity inactivation was detected after 4 h for any of the biocatalysts (results not shown).

That way, we incubated the biocatalysts at 75 °C (Fig. 2). If the activity is followed using *p*-NPB at 25 °C, the only biocatalyst that retained full activity after 4 h of incubation is amino-glutaraldehyde-CALA, being the octyl-VS-CALA the least stable preparation. The situation using triacetin is different, an initial increased activity is detected using amino-glutaraldehyde-CALA (more than doubling the initial activity) and octyl- CALA preparation (increasing the activity by one third), that later started to slowly decrease. Octyl-VS-CALA decreased its activity from the start of the inactivation, even more rapidly than when using *p*-NPB to determine the residual enzyme activity. However, the situation became fully different if the activity determination is performed at 55 °C. Octyl-CALA multiple the initial activity by more than 16 folds after 2 h, and retained that activity after 4 h. Amino-glutaraldehyde-CALA also increased the initial activity but “only” by a 5 fold factor after 2 h and decreased after 4 h. Even the enzyme immobilized on VS-octyl initially increased the activity (by 3.5 fold). That way, not only the quantitative stability of the biocatalysts is influenced by the activity determination conditions, but also the qualitative values are different, as in some cases amino-glutaraldehyde-CALA is the most resistant and in other cases, octyl-CALA could be considered the most stable one. It is also evident a discrepancy with the 40 °C found for the optimal temperature, as the incubation at 75 °C increased the activity detected at these temperatures. That way, determining the activity at 55 °C, for this enzyme the residual activity during the incubation at high temperatures was higher in all cases than using 25 °C, in opposition to the initial hypothesis.

To analyze if this trend was also found at other temperatures, the biocatalysts were incubated at 70 °C, a temperature also far above the optimal one (Fig. 3). At this temperature, octyl-CALA progressively increased its activity versus triacetin when determining the residual activity at 25 °C to almost doubling the initial activity value after 4 h, while the activity using *p*-NPB as substrate slightly decreased after 4 h. Using octyl-VS-CALA, the increase in activity versus triacetin was similar (2.5 folds after 4 h), while the activity versus *p*-NPB decreased to less than 80% after this time. Finally, using the glutaraldehyde preparation, a smaller increase in enzyme activity was found in the hydrolysis of triacetin (less than 1.6 fold after 4 h), while the decrease in enzyme activity versus *p*-NPB was

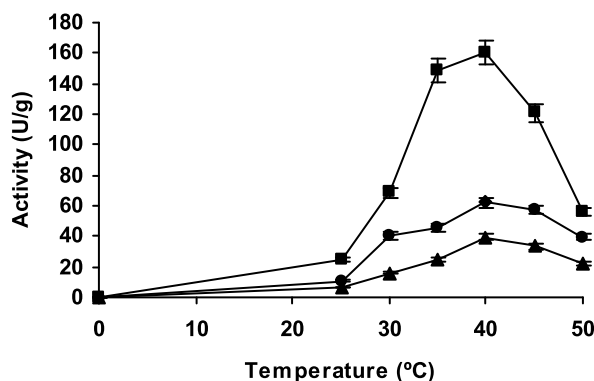


Fig. 1. Activities of the different immobilized CALA (4 mg/g) biocatalysts with 50 mM triacetin at pH 5.0 at different temperatures. Squares: Octyl-CALA; triangles: Glutaraldehyde-CALA and circles: Octyl-VS-CALA. Experiments were performed as described in Methods.

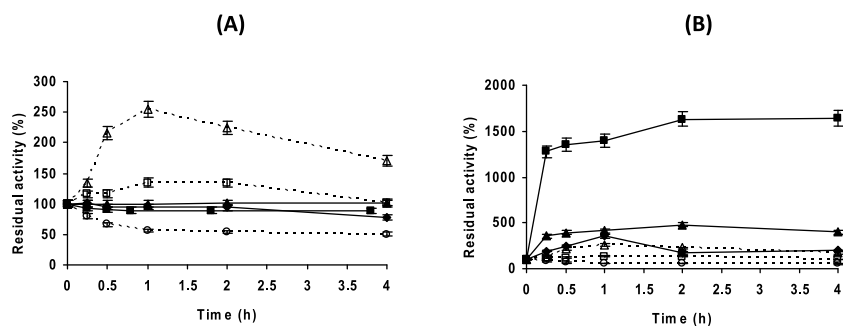


Fig. 2. Activity values of different CALA biocatalysts in the hydrolysis of 1 mM *p*-NPB or 50 mM triacetin. The samples were withdrawn during the inactivation courses performed at pH 7.0 and 75 °C. (A) Hydrolysis at 25 °C of *p*-NPB (pH 7.0) and triacetin (pH 5.0). Solid symbols: *p*-NPB and empty symbols: Triacetin. (B) Hydrolysis of triacetin at pH 5.0. Solid symbols: 55 °C and empty symbols: 25 °C. squares: Octyl biocatalysts; triangles: Glutaraldehyde biocatalysts; circles: Octyl-DVSF biocatalysts. Other specifications are described in Methods section.

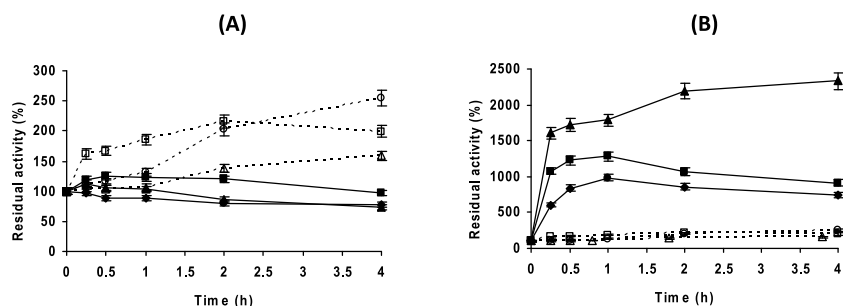


Fig. 3. Activity values of different CALA biocatalysts in the hydrolysis of 1 mM *p*-NPB or 50 mM triacetin. The samples were withdrawn during the inactivation courses performed at pH 7.0 and 70 °C. (A) Hydrolysis at 25 °C of *p*-NPB (pH 7.0) and triacetin (pH 5.0). Solid symbols: *p*-NPB and empty symbols: Triacetin. (B) Hydrolysis of triacetin at pH 5.0. Solid symbols: 55 °C and empty symbols: 25 °C. squares: Octyl biocatalysts; triangles: Glutaraldehyde biocatalysts; circles: Octyl-DVSF biocatalysts. Other specifications are described in Methods section.

around 30% after 4 h.

The inactivation determining the activity versus triacetin at 55 °C is shown in Fig. 3. This temperature was also far higher than the optimal temperature for these biocatalysts, 40 °C (Fig. 1). The activity of all CALA preparations increased over time. Octyl-CALA reached an activity almost twelvefold (similar to that found at 75 °C) and octyl-VS-CALA tenfold higher than the initial one (much higher than the observed at 75 °C), and then decreased, but after 4 h they still are highly hyperactivated (900 or 750%, respectively). The case of the amino-glutaraldehyde-CALA was even more surprising, the activity versus triacetin determined at 55 °C increased in a continuous way to reach a value of more than 2300%, reaching this value after 4 h (when incubated at 75 °C this hyperactivation value was much smaller for this biocatalyst).

This increase in enzyme activity should be correlated to the generation at higher temperatures of immobilized enzyme forms more adequate to hydrolyze triacetin than the initial form, which occurs in a more favorable way for the enzyme that is immobilized in

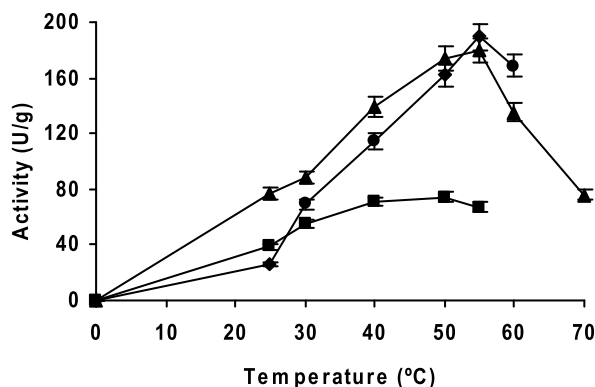


Fig. 4. Activities of the different immobilized CALB (1 mg/g) biocatalysts with 50 mM triacetin (pH 5.0) at different temperatures. Squares: Octyl-CALB; triangles: Glutaraldehyde-CALB and circles: Octyl-VS-CALB. Experiments were performed as described in Methods.

amino-glutaraldehyde.

In any case, the results of this set of experiments makes defining which is the most stable preparation of CALA very complex, and this problem is accentuated if the activity is determined using triacetin at 55 °C, where the enzyme activity significantly increased. Using amino-glutaraldehyde-CALA, this could be related to the favoring of the open form of the lipase at high temperatures (that can take some time), while using octyl or octyl-VS perhaps could be explained by the reduction of the intensity of the enzyme adsorption to the octyl layer, perhaps decreasing the steric hindrances to the entry of the substrate generated by the support surface. The question may be if the activity reflects the enzyme stability, as a higher hyperactivation may reflect a higher mobility in the enzyme structure, really suggesting a lower rigidity of the enzyme.

3.3. Determination of the effect of the temperature on the activity and stability the different preparations of CALB

Fig. 4 shows the activity/temperature profile for the 3 CALB biocatalysts. While the optimal temperature is 50 °C for octyl-CALB, it is 55 °C for the other two preparations. The shape of the curve is very different for the biocatalysts. Octyl-CALB has an activity next to the maximum at 40 °C, with an increase smaller than 5% at 50 °C, almost doubling the activity at 25 °C. Amino-glutaraldehyde-CALB has more than double activity at 50 °C than at 25 °C, with a marginal increase at 55 °C. Octyl-VS-CALB presented an important increase in activity between 40 °C and 50° (around 40%), that further increased more than 15% when the activity is determined at 55 °C. At this temperature, this biocatalyst became the most active one versus this substrate (initially it was the least active). Moreover, the decrease of activity at 60 °C is much smaller using octyl-VS-CALB than using amino-glutaraldehyde-CALB, leaving this biocatalyst with a clearly higher activity at this temperature, while at 25 °C, the most active was the amino-glutaraldehyde-CALB.

Fig. 5 shows the inactivation of the different CALB biocatalyst using *p*-NPB and triacetin at 75 °C. Using *p*-NPB and 25 °C to determine the enzyme activity, the least stable biocatalyst is clearly amino-glutaraldehyde-CALB, almost fully inactive after 1 h. Octyl-CALB (more than 60% of retained activity after 4 h) is more stable than octyl-VS-CALB (less than 30% after 4 h). Using triacetin, the inactivation course is very similar to that using *p*-NPB when studying octyl-VS-CALB. However, the retained activity is clearly higher using amino-glutaraldehyde-CALB and this substrate, becoming similar to the inactivation course of octyl-VS-CALB. Octyl-CALB remains the most stable biocatalyst, although the decrease of activity using this substrate is slightly higher. When measuring the activity at 55 °C, the results were fairly different. The activity of octyl-CALB increased by more than a twofold factor after 15 min at 75 °C, and then remains unaltered for the next 3.5 h. Using amino-glutaraldehyde-CALB, the residual activity is even lower than if the activity is determined at 25 °C. Finally, using octyl-VS-CALB, after an initial increase of activity, it decreased to become less than half of the initial one after 4 h.

The immobilized CALB preparations were also inactivated at 70 °C (Fig. 6). Starting with the activity measurement at 25 °C, octyl-CALB activity decreased in a similar way using triacetin or *p*-NPB (to around 60% after 4 h, similar to the results at 75 °C). Octyl-VS-CALB is slightly less stable than octyl-CALB, maintaining 50-40% of the initial activity after 4 h with *p*-NPB or triacetin respectively. Results were very different using amino-glutaraldehyde-CALB after incubation at 70 or 75 °C. Now, the enzyme activity increased in a progressive way using triacetin as substrate (to reach a value of 175% after 4 h), while using *p*-NPB, the activity decreased more than when using the other two biocatalysts. That is, using triacetin this was the biocatalyst that retained the highest percentage of activity, while using *p*-NPB, it retained the lowest values. Next, we determined the activity of the samples versus triacetin but at 55 °C. Octyl-CALB increased its activity by almost fivefold after 15 min, and then slowly decreased this value (still maintaining threefold initial activity after 4 h). Octyl-VS-CALB increased twice its activity after 30 min, and then decreased, with a recovered activity just over 100% after 4 h. The amino-glutaraldehyde biocatalyst increased the activity under fourfold after 15 min and its activity decreased to maintain 174% activity after 4 h.

Again, the evolution of the enzyme activity for each biocatalyst depended on the substrate and temperature utilized to determine the residual activity.

3.4. Determination of the effect of the temperature on the activity and stability the different preparations of TLL

Using this enzyme, the activity employing the amino-glutaraldehyde support is too low for the experiments. That way, only octyl

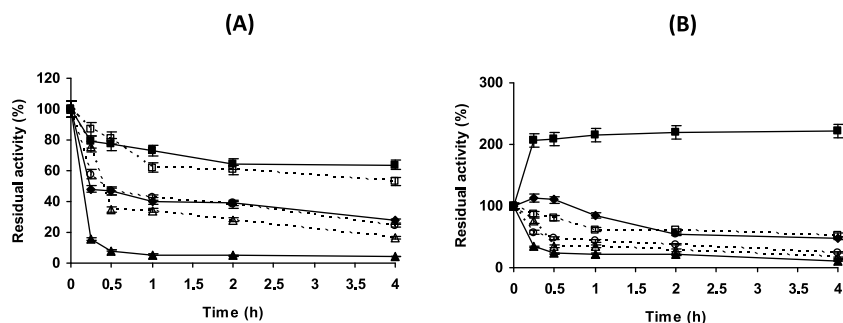


Fig. 5. Activity values of different CALB biocatalysts in the hydrolysis of 1 mM *p*-NPB or 50 mM triacetin. The samples were withdrawn during the inactivation courses performed at pH 7.0 and 75 °C. (A) Hydrolysis at 25 °C of *p*-NPB (pH 7.0) and triacetin (pH 5.0). Solid symbols: *p*-NPB and empty symbols: Triacetin. (B) Hydrolysis of triacetin at pH 5.0. Solid symbols: 55 °C and empty symbols: 25 °C. squares: Octyl biocatalysts; triangles: Glutaraldehyde biocatalysts; circles: Octyl-DVSF biocatalysts. Other specifications are described in Methods section.

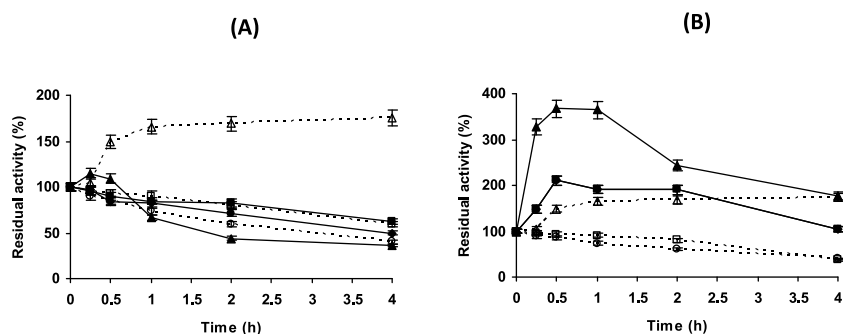


Fig. 6. Activity values of different CALB biocatalysts in the hydrolysis of 1 mM *p*-NPB or 50 mM triacetin. The samples were withdrawn during the inactivation courses performed at pH 7.0 and 70 °C. (A) Hydrolysis at 25 °C of *p*-NPB (pH 7.0) and triacetin (pH 5.0). Solid symbols: *p*-NPB and empty symbols: Triacetin. (B) Hydrolysis of triacetin at pH 5.0. Solid symbols: 55 °C and empty symbols: 25 °C. squares: Octyl biocatalysts; triangles: Glutaraldehyde biocatalysts; circles: Octyl-DVSF biocatalysts. Other specifications are described in Methods section.

and octyl-VS biocatalysts were compared. Fig. 7 shows the activity/temperature profiles for these biocatalysts using triacetin as substrate. Both biocatalysts had the same optimal temperature (40 °C), but while octyl-TLL multiplied its activity by 2 when going from 25 °C to 40 °C, octyl-VS-TLL increased by 3.5 folds its activity after this increase in temperature. That way, at 40 °C, the activity of both biocatalysts versus triacetin became similar. The decrease in activity at higher temperatures is different for each biocatalyst, while octyl-TLL maintained 95% of the maximum activity at 50 °C, octyl-VS-TLL decreased the activity to around 60%.

Fig. 8 shows the thermal inactivations at 75 °C. Using *p*-NPB and 25 °C to determine the residual activity, the highest values are found using octyl-VS-TLL (44% after 4 h versus the 5% retained by octyl-TLL). Using triacetin and 25 °C to determine the residual activity, differences are shorter, as mainly octyl-VS-TLL lost much more activity than using *p*-NPB (maintained 15% after just 1 h) showing inactivation courses fairly similar to that of octyl-TLL. Results when determining the activity at 55 °C using triacetin were fairly similar.

The inactivation at 70 °C provides a somehow different picture (Fig. 9). Determining the activity at 25 °C, octyl-TLL shows a slightly higher loss of activity versus triacetin (27% residual activity after 4 h) than versus *p*-NPB (42% residual activity after 4 h). Octyl-VS-TLL retained less activity versus triacetin (23% residual activity after 4 h) than octyl-VS-TLL, but more activity was maintained using *p*-NPB (60%). If the activity was determined at 55 °C with triacetin, octyl-TLL showed an initial increase of activity (160% after 15 min) that slowly decreased along time (to 70% of the initial activity after 4 h). Octyl-VS-TLL presented a fully different inactivation course, decreasing its activity in a continue way from the beginning, maintaining under 20% of the initial activity after 4 h. Again, depending on the activity determination condition, the most stable preparation may be one or the other.

3.5. Determination of the effect of the temperature on the activity and stability the different preparations of CRL

Finally, the features of the different CRL preparations were analyzed. Fig. 10 shows the activity/temperature profiles of these biocatalysts. Octyl-CRL shows maximum activity at 60 °C. This is the highest optimal temperature found in this research, and this is surprising as in terms of stability, this is an enzyme considered less stable than the other 3 enzymes included in this study (Carballares et al., 2022). The other two biocatalysts presented the maximum activity at 50 °C, but while octyl-DVS activity presented almost a plateau from 50 to 60 °C, amino-glutaraldehyde-CRL lost 2/3 of its activity at 60 °C. Octyl-CRL increased its activity almost tenfold from 25 °C to 50 °C, while octyl-VS-CRL only increased the activity by a factor of 3.

The inactivation courses of these biocatalysts at 75 °C are shown in Fig. 11. Using *p*-NPB, the biocatalyst that retained the highest activity was amino-glutaraldehyde-CRL, being octyl CRL slightly more stable than octyl-VS-CRL. Using triacetin at 25 °C to determine

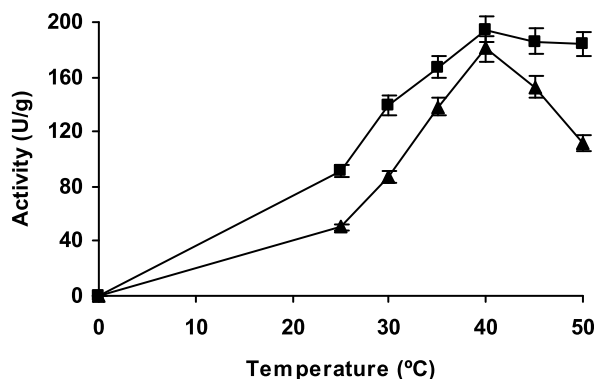


Fig. 7. Activities of the different immobilized TLL (12 mg/g) biocatalysts with 50 mM triacetin (pH 5.0) at different temperatures. Squares: Octyl-TLL and triangles: Octyl-VS-TLL. Experiments were performed as described in Methods.

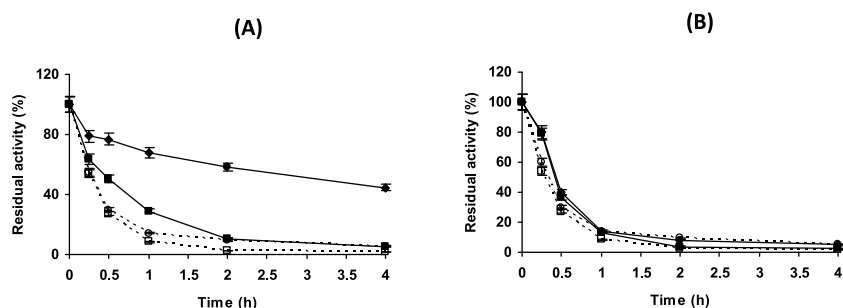


Fig. 8. Activity values of different TLL biocatalysts in the hydrolysis of 1 mM *p*-NPB or 50 mM triacetin. The samples were withdrawn during the inactivation courses performed at pH 7.0 and 75 °C. (A) Hydrolysis at 25 °C of *p*-NPB (pH 7.0) and triacetin (pH 5.0). Solid symbols: *p*-NPB and empty symbols: Triacetin. (B) Hydrolysis of triacetin at pH 5.0. Solid symbols: 55 °C and empty symbols: 25 °C. squares: Octyl biocatalysts; circles: Octyl-DVSF biocatalysts. Other specifications are described in Methods section.

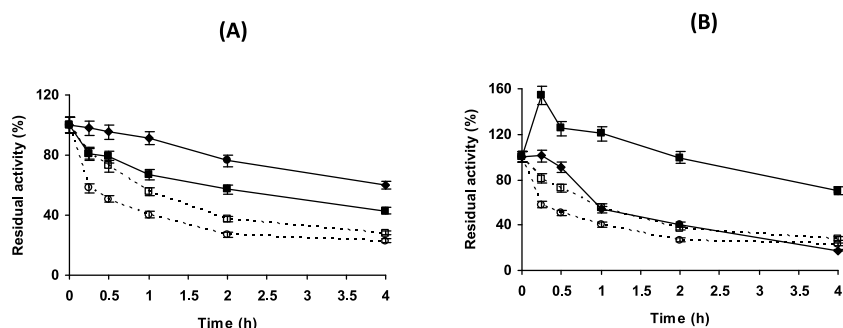


Fig. 9. Activity values of different TLL biocatalysts in the hydrolysis of 1 mM *p*-NPB or 50 mM triacetin. The samples were withdrawn during the inactivation courses performed at pH 7.0 and 70 °C. (A) Hydrolysis at 25 °C of *p*-NPB (pH 7.0) and triacetin (pH 5.0). Solid symbols: *p*-NPB and empty symbols: Triacetin. (B) Hydrolysis of triacetin at pH 5.0. Solid symbols: 55 °C and empty symbols: 25 °C. squares: Octyl biocatalysts; circles: Octyl-DVSF biocatalysts. Other specifications are described in Methods section.

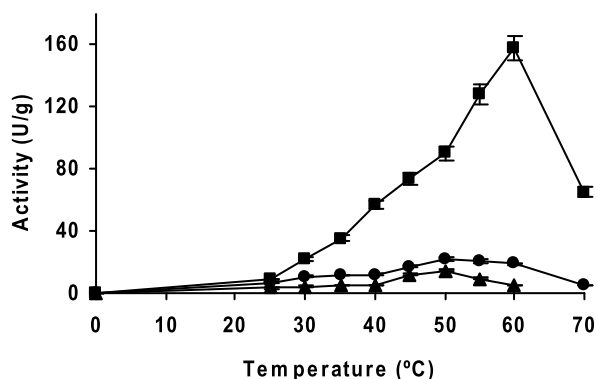


Fig. 10. Activities of the different immobilized CRL (4 mg/g) biocatalysts with 50 mM triacetin (pH 5.0) at different temperatures. Squares: Octyl-CRL; triangles: Glutaraldehyde-CRL and circles: Octyl-VS-CRL. Experiments were performed as described in Methods.

the enzyme activity, the octyl-CRL inactivation course is similar to that using *p*-NPB, but octyl-VS-CRL initially showed a slight increase in activity followed by a quick loss of activity. Amino-glutaraldehyde-CRL clearly increased its activity in the first inactivation times (to 80% after 30 min), but later the residual activity decreased, reaching values similar to those of octyl-VS-CRL after 4 h. The results are very different measuring the activities at 55 °C. Now, octyl-CRL retained a significant percentage of activity even after 4 h (15%), although it remained the one with the lowest recovered activity. Amino-glutaraldehyde-CRL presented a similar initial hyperactivation than when using 25 °C, but the activity decreased in a slower way, and octyl-VS-CRL had a similar behavior, but with a lower hyperactivation.

Fig. 12 shows the inactivation courses at 70 °C. Starting by the activity determination at 25 °C, octyl-CRL lost 2/3 of the initial triacetin activity after 4 h, while using *p*-NPB only maintained 5% after 2 h. Octyl-VS-CRL retained more activity after 4 h of

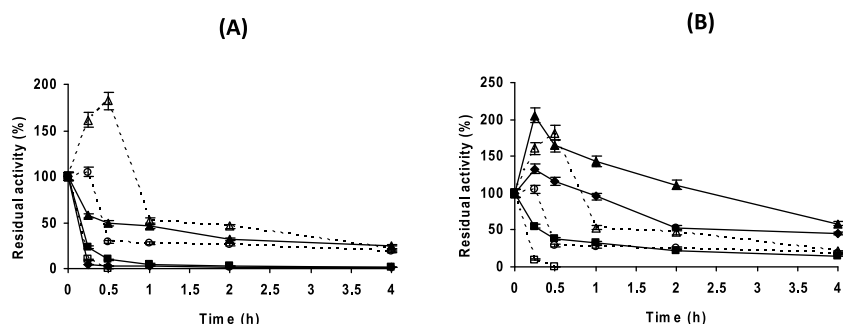


Fig. 11. Activity values of different CRL biocatalysts in the hydrolysis of 1 mM *p*-NPB or 50 mM triacetin. The samples were withdrawn during the inactivation courses performed at pH 7.0 and 75 °C. (A) Hydrolysis at 25 °C of *p*-NPB (pH 7.0) and triacetin (pH 5.0). Solid symbols: *p*-NPB and empty symbols: Triacetin. (B) Hydrolysis of triacetin at pH 5.0. Solid symbols: 55 °C and empty symbols: 25 °C. squares: Octyl biocatalysts; triangles: Glutaraldehyde biocatalysts; circles: Octyl-DVSF biocatalysts. Other specifications are described in Methods section.

inactivation, 76% versus triacetin (in fact the activity increased in the first inactivation times) and 10% versus *p*-NPB. Amino-glutaraldehyde-CRL maintained 15% of the initial activity versus triacetin, however, the activity versus *p*-NPB, after a drop to 40% in the first 2 h, was maintained after 4 h. Results were very different if determining the activity versus triacetin at 55 °C. Octyl-CRL showed a fourfold increase in the activity after 15 min, and then started to decrease; giving a 50% after 4 h. Octyl-VS-CRL did not show this increase in enzyme activity, but the residual activity was 33% after 4 h, much higher than when the activity was determined at 25 °C. Amino-glutaraldehyde-CRL showed the better activity retention, with an initial increment of more than threefold, and slow decrease to 95% after 4 h.

4. Discussion

The results showed in this paper confirm the complexity of immobilized enzyme inactivation and therefore, the complexity to even qualify the enzyme functional stability. It had been previous shown that the inactivation pathways of immobilized enzymes may be determined by the enzyme rigidification of specific areas or by enzyme-support interactions (Paiva Souza et al., 2021; Sanchez et al., 2016). Here, we show that the situation may be even more complex.

On the one hand, the optimal activity for the different immobilized enzymes included in this study is very short compared to the temperature where the enzymes activity starts to decrease when they are submitted to long term stress inactivation studies. Moreover, there is not a direct order in stability determined using stress inactivation and optimal temperature, neither to establish an order for the enzymes nor the different biocatalysts of each enzyme. In this sense, amino-glutaraldehyde-CRL could be an exception, it is the most stable CRL biocatalyst (at least using triacetin) and it is the one presenting the highest optimal temperature among the CRL preparations.

On the other hand, it is also evident that the “half” live of an enzyme in stress inactivation strongly depends on the activity determination conditions. It may be expected that the conformational changes caused by the enzyme inactivation can affect differently to the activity versus different substrates, and that way may be expected that an enzyme did not lose the same percentage of activity if the substrate used to determine the residual activity is different (Paiva Souza et al., 2021). In the examples utilized in this paper, only CALB biocatalyst shows relatively similar inactivation courses using triacetin and *p*-NPB at 25 °C. For TLL, the activity decreased quicker using triacetin, while for CALA and RML this substrate gave the highest residual activities (except for octyl-VS-CALB). In some instances, the activity decreased with one substrate (usually with *p*-NPB, the most used substrate to follow these processes using lipases) while with triacetin, the activity increased. This mainly occurred when the inactivation is performed at 70 °C (all enzymes have

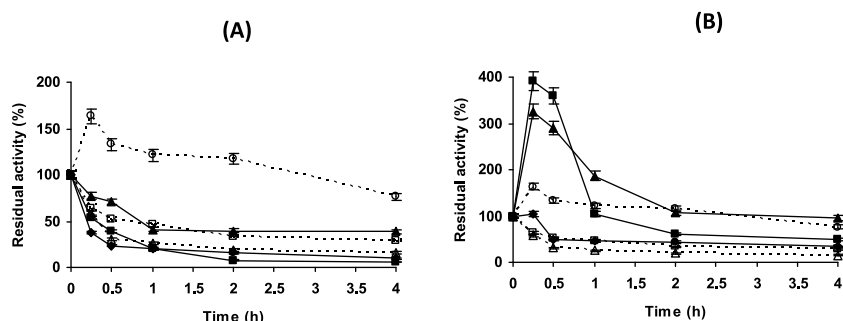


Fig. 12. Activity values of different CRL biocatalysts in the hydrolysis of 1 mM *p*-NPB or 50 mM triacetin. The samples were withdrawn during the inactivation courses performed at pH 7.0 and 70 °C. (A) Hydrolysis at 25 °C of *p*-NPB (pH 7.0) and triacetin (pH 5.0). Solid symbols: *p*-NPB and empty symbols: Triacetin. (B) Hydrolysis of triacetin at pH 5.0. Solid symbols: 55 °C and empty symbols: 25 °C. squares: Octyl biocatalysts; triangles: Glutaraldehyde biocatalysts; circles: Octyl-DVSF biocatalysts. Other specifications are described in Methods section.

some biocatalyst that increase the activity with some substrate), but also there are some examples when inactivating the enzyme at 75 °C (e.g., octyl or amino-glutaraldehyde-CALA) (see Fig. 2).

However, the most surprising results have been observed when determining the enzyme activity at 55 °C. This temperature is over the optimal temperature when using the immobilized biocatalyst from CALA, TLL and CRL (except the glutaraldehyde preparation) and is the optimal temperature for some CALB preparations (except octyl that has the maximum activity at 50 °C). As explained in the introduction, the optimal temperature is expected to be a consequence of the enzyme structure distortion (Glowacki et al., 2012) together to altered temperature sensitivity of substrate-enzyme, enzyme-product transitions stages, heat capacity of enzyme-bound intermediates, ionic status at the enzyme active center (DeLong et al., 2017; Glowacki et al., 2012; Gürdaş et al., 2012; Kekene-Huskey et al., 2016; Yadav and Prakash, 2009). When measuring the enzyme activity well below this optimal temperature (e.g., at 25 °C), it is expected that the enzyme submitted to much harsher conditions can partially reactivate at this milder temperature even during the activity determination time, and that way the determined activity may be higher than that will maintain the enzyme under the inactivation conditions, and this effect may be more significant when the incubations under the milder conditions are longer (Rodrigues et al., 2009a, 2009b, 2009c; Romero et al., 2009; Rueda et al., 2015; Soler et al., 1997; Suescun et al., 2015).

However, using temperatures above this temperature, this reactivation should be reduced, as it is supposed to be a temperature that affects the enzyme structure (Glowacki et al., 2012). However, our results point that in a general way, the immobilized enzymes used in this study shows a significant increase of the activity at 55 °C when they are previously incubated at 70 or 75 °C, with exception of TLL. Octyl-TLL only increased its activity by 60% and in a very transient way, when incubated at 70 °C (Fig. 9). For the other preparations, this increased activity reached values as high as 15 fold for octyl-CALA incubated at 75 °C or almost 25 fold for the glutaraldehyde when incubated at 70 °C, and the values remained over 100% for all the studied times (Figs. 2 and 3). This suggests that if the enzyme is incubated at 25 °C and then exposed to 55 °C, the conformation that reaches is different to the one that it presents if incubated previously at 70 or 75 °C. And that, for some reason, the previous high Temperature incubation is positive for the activity versus triacetin. It may be related to the opening of the enzyme (that may be easier at high temperatures but that can need some time and it is not observed when directly measuring at 55 °C samples stored at 25 °C) or an accommodation of the enzyme in their interactions with the support, that somehow permits a higher activity (perhaps reducing steric hindrances to the entry of the substrate, perhaps direct conformational changes in the enzyme). This increased activity is not necessarily an advantage, as it can suggest an enzyme flexibility that can alter all enzyme features (e.g., if the enzyme is utilized in resolution of racemic mixtures). It is also a problem if using coimmobilized enzymes, as if one enzyme decreases or maintains its activity while the other enzyme increases it, the careful design of the combi-biocatalyst, which involve the selection of the activity ratios of the enzymes, will be lost (Hwang and Lee, 2019; Ren et al., 2019; Rocha-Martín et al., 2012; Zhang, 2011). This may be critical if the enzyme that increases the activity is the first enzyme in the chain and the formed product is unstable and requires the rapid enzyme modification to be converted to the target product (Araña-Peña et al., 2021). That way, one open question is if this increment on activity, that suggests some mobility of the enzyme structure, actually means a higher or a lower stability than a biocatalysts whose activity remains unaltered, considering that stability means to keep the initial enzyme features.

This also raises the question on the results achieved in the determination of optimal temperature. In most of the examples in this paper, the activity at 55 °C had significantly increased if the enzyme was previously incubated at high temperature.

From a practical point of view, it seems evident that to select a biocatalyst for a specific process, even using stress inactivation conditions, the residual activity should be determined under the exact conditions where the enzyme is going to be utilized (temperature, substrate, and other conditions).

5. Conclusion

The results showed in this paper confirm that there is not a direct relation between optimal temperature value and enzyme stability in all cases, and that both data should be used to make a better picture of the enzyme stability. In fact, operational stability may be the optimal form to establish the enzyme stability of different biocatalyst, perhaps even under stress conditions to make the experiment time reasonable.

The conditions of residual enzyme activity also determined the value and even the order of enzyme biocatalysts stability, being both temperature and substrate able to fully alter the inactivation pattern.

Author statement

Thays N. da Rocha, Diego Carballares, José R. Guimarãe and Diego Carballares performed the experiments, Javier Rocha-Martin, Paulo W. Tardioli, Luciana R.B Gonçalves and Roberto Fernandez-Lafuente designed the experiments. Roberto Fernandez-Lafuente and Javier Rocha-Martin supervised the experiments. All authors contributed to the writing and final editing of the paper.

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.

Data availability

Data will be made available on request.

Acknowledgments

We gratefully recognize the financial support from Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación/10.13039/501100011033/(PID2021-122398OB-I00). DC thanks to Ministerio de Ciencia e Innovación-Spanish Government by a FPI (PRE2018-084809). The help and suggestions from Dr. Ángel Berenguer (Departamento de Química Inorgánica, Universidad de Alicante) are gratefully recognized. Thays Nogueira da Rocha thanks CNPq for the predoctoral fellowship (CNPq—Brazil).

References

- Ahammad, S.Z., Gomes, J., Sreekrishnan, T.R., 2008. Improvement of enantioselectivity and stability of *Klebsiella oxytoca* hydrolase immobilized on Eupergit C 250L. J. Chem. Technol. Biotechnol. 83, 1163–1169. <https://doi.org/10.1002/jctb>.
- Albuquerque, T.L.D., Rueda, N., Dos Santos, J.C.S., Barbosa, O., Ortiz, C., Binay, B., Özdemir, E., Gonçalves, L.R.B., Fernandez-Lafuente, R., 2016. Easy stabilization of interfacially activated lipases using heterofunctional divinyl sulfone activated-octyl agarose beads. Modulation of the immobilized enzymes by altering their nanoenvironment. Process Biochem. 51, 865–874. <https://doi.org/10.1016/j.procbio.2016.04.002>.
- Anderson, E.M., Larsson, K.M., Kirk, O., 1998. One biocatalyst - many applications: the use of *Candida antarctica* B-lipase in organic synthesis. Biocatal. Biotransform. 16, 181–204. <https://doi.org/10.3109/10242429809003198>.
- Arana-Peña, S., Carballares, D., Morellon-Sterling, R., Rocha-Martin, J., Fernandez-Lafuente, R., 2022. The combination of covalent and ionic exchange immobilizations enables the coimmobilization on vinyl sulfone activated supports and the reuse of the most stable immobilized enzyme. Int. J. Biol. Macromol. 199, 51–60. <https://doi.org/10.1016/j.ijbiomac.2021.12.148>.
- Arana-Peña, S., Carballares, D., Morellon-Sterling, R., Berenguer-Murcia, Á., Alcántara, A.R., Rodrigues, R.C., Fernandez-Lafuente, R., 2021. Enzyme co-immobilization: always the biocatalyst designers' choice...or not? Biotechnol. Adv. 51, 107584. <https://doi.org/10.1016/j.biotechadv.2020.107584>.
- Arana-Peña, S., Rios, N.S., Carballares, D., Mendez-Sanchez, C., Lokha, Y., Gonçalves, L.R.B., Fernandez-Lafuente, R., 2020. Effects of enzyme loading and immobilization conditions on the catalytic features of lipase from *Pseudomonas fluorescens* immobilized on octyl-agarose beads. Front. Bioeng. Biotechnol. 8, 36. <https://doi.org/10.3389/fbioe.2020.00036>.
- Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R.C., Fernandez-Lafuente, R., 2014. Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization. RSC Adv. 4, 1583–1600. <https://doi.org/10.1039/c3ra45991h>.
- Benítez-Mateos, A.L., Huber, C., Nidetzky, B., Bolívar, J.M., López-Gallego, F., 2020. Design of the enzyme-carrier interface to overcome the O₂ and NADH mass transfer limitations of an immobilized flavin oxidase. ACS Appl. Mater. Interfaces 12, 56027–56038. <https://doi.org/10.1021/acsami.0c17568>.
- Benjamin, S., Pandey, A., 1998. *Candida rugosa* lipases: molecular biology and versatility in biotechnology. Yeast 14, 1069–1087. [https://doi.org/10.1002/\(SICI\)1097-0061\(19980915\)14:12<1069::AID-YEA303>3.0.CO;2-K](https://doi.org/10.1002/(SICI)1097-0061(19980915)14:12<1069::AID-YEA303>3.0.CO;2-K).
- Betancor, L., López-Gallego, F., Hidalgo, A., Alonso-Morales, N., Mateo, G.D.-O.C., Fernández-Lafuente, R., Guisán, J.M., 2006. Different mechanisms of protein immobilization on glutaraldehyde activated supports: effect of support activation and immobilization conditions. Enzym. Microb. Technol. 39, 877–882. <https://doi.org/10.1016/j.enzmictec.2006.01.014>.
- Błaszczak, J., Kiebasinski, P., 2020. Quarter of a century after: a glimpse at the conformation and mechanism of *Candida antarctica* lipase b. Crystals 10. <https://doi.org/10.3390/cryst10050404>.
- Bolívar, J.M., Eisl, I., Nidetzky, B., 2016. Advanced characterization of immobilized enzymes as heterogeneous biocatalysts. Catal. Today 259, 66–80. <https://doi.org/10.1016/j.cattod.2015.05.004>.
- Bolívar, J.M., Woodley, J.M., Fernandez-Lafuente, R., 2022. Is enzyme immobilization a mature discipline? Some critical considerations to capitalize on the benefits of immobilization. Chem. Soc. Rev. <https://doi.org/10.1039/D2CS00083K> (in press).
- Bommarius, A.S., Paye, M.F., 2013. Stabilizing biocatalysts. Chem. Soc. Rev. 42, 6534–6565. <https://doi.org/10.1039/c3cs60137d>.
- Bortone, N., Fidalgo, M., Moresi, M., 2014. Internal and external mass transfer limitations on the activity of immobilised acid urease derivatives differing in enzyme loading. Biochem. Eng. J. 82, 22–33. <https://doi.org/10.1016/j.bej.2013.10.017>.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
- Braham, S.A., Siar, E.H., Arana-Peña, S., Carballares, D., Morellon-Sterling, R., Bavandi, H., de Andrade, D., Kornecki, J.F., Fernandez-Lafuente, R., 2021a. Effect of concentrated salts solutions on the stability of immobilized enzymes: influence of inactivation conditions and immobilization protocol. Molecules 26, 968. <https://doi.org/10.3390/molecules26040968>.
- Braham, S.A., Siar, E.H., Arana-Peña, S., Bavandi, H., Carballares, D., Morellon-Sterling, R., de Andrade, D., Kornecki, J.F., Fernandez-Lafuente, R., 2021b. Positive effect of glycerol on the stability of immobilized enzymes: is it a universal fact? Process Biochem. 102, 108–121. <https://doi.org/10.1016/j.procbio.2020.12.015>.
- Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Høge-Jensen, B., Patkar, S.A., Thim, L., 1991. A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. Nature 351, 491–494. <https://doi.org/10.1038/351491a0>.
- Carballares, D., Rocha-Martin, J., Fernandez-Lafuente, R., 2022. Coimmobilization of lipases exhibiting three very different stability ranges. Reuse of the active enzymes and selective discarding of the inactivated ones. Int. J. Biol. Macromol. 206, 580–590. <https://doi.org/10.1016/j.ijbiomac.2022.02.084>.
- Chaubey, A., Parshad, R., Koul, S., Taneja, S.C., Qazi, G.N., 2006. Enantioselectivity modulation through immobilization of *Arthrobacter* sp. lipase: kinetic resolution of fluoxetine intermediate. J. Mol. Catal. B Enzym. 42, 39–44. <https://doi.org/10.1016/j.molcatb.2006.06.011>.
- Daniel, R.M., Danson, M.J., 2010. A new understanding of how temperature affects the catalytic activity of enzymes. Trends Biochem. Sci. 35, 584–591. <https://doi.org/10.1016/j.tibs.2010.05.001>.
- DeLong, J.P., Gibert, J.P., Luhring, T.M., Bachman, G., Reed, B., Neyer, A., Montooth, K.L., 2017. The combined effects of reactant kinetics and enzyme stability explain the temperature dependence of metabolic rates. Ecol. Evol. 7, 3940–3950. <https://doi.org/10.1002/ece3.2955>.
- DiCosimo, R., McAuliffe, J., Poulou, A.J., Bohlmann, G., 2013. Industrial use of immobilized enzymes. Chem. Soc. Rev. 42, 6437. <https://doi.org/10.1039/c3cs35506c>.
- Dill, K.A., Ghosh, K., Schmit, J.D., 2011. Physical limits of cells and proteomes. Proc. Natl. Acad. Sci. U.S.A. 108, 17876–17882. <https://doi.org/10.1073/pnas.1114477108>.
- Domínguez De María, P., Carboni-Oerlemans, C., Tuin, B., Bargeman, G., Van Der Meer, A., Van Gemert, R., 2005. Biotechnological applications of *Candida antarctica* lipase A: state-of-the-art. J. Mol. Catal. B Enzym. 37, 36–46. <https://doi.org/10.1016/j.molcatb.2005.09.001>.
- Domínguez De María, P., Sánchez-Montero, J.M., Sinisterra, J.V., Alcántara, A.R., 2006. Understanding *Candida rugosa* lipases: an overview. Biotechnol. Adv. 24, 180–196. <https://doi.org/10.1016/j.biotechadv.2005.09.003>.
- dos Santos, J.C.S., Rueda, N., Gonçalves, L.R.B., Fernandez-Lafuente, R., 2015. Tuning the catalytic properties of lipases immobilized on divinylsulfone activated agarose by altering its nanoenvironment. Enzym. Microb. Technol. 77, 1–7. <https://doi.org/10.1016/j.enzmictec.2015.05.001>.
- Dos Santos, J.C.S., Rueda, N., Sanchez, A., Villalonga, R., Gonçalves, L.R.B., Fernandez-Lafuente, R., 2015a. Versatility of divinylsulfone supports permits the tuning of CALB properties during its immobilization. RSC Adv. 5, 35801–35810. <https://doi.org/10.1039/c5ra03798k>.
- Dos Santos, J.C.S., Rueda, N., Torres, P., Barbosa, O., Gonçalves, L.R.B., Fernandez-Lafuente, R., 2015b. Evaluation of divinylsulfone activated agarose to immobilize lipases and to tune their catalytic properties. Process Biochem. 50, 918–927. <https://doi.org/10.1016/j.procbio.2015.03.018>.
- Du, C., Zhao, B., Li, C., Wang, P., Wang, Z., Tang, J., Wang, L., 2009. Improvement of the enantioselectivity and activity of lipase from *Pseudomonas* sp. via adsorption on a hydrophobic support: kinetic resolution of 2-octanol. Biocatal. Biotransform. 27, 340–347. <https://doi.org/10.3109/10242420903225230>.
- Fernandez-Lafuente, R., 2010. Lipase from *Thermomyces lanuginosus*: uses and prospects as an industrial biocatalyst. J. Mol. Catal. B Enzym. 62, 197–212. <https://doi.org/10.1016/j.molcatb.2009.11.010>.

- Fernandez-Lafuente, R., 2009. Stabilization of multimeric enzymes: strategies to prevent subunit dissociation. *Enzym. Microb. Technol.* 45, 405–418. <https://doi.org/10.1016/j.enzmictec.2009.08.009>.
- Fernandez-Lafuente, R., Rosell, C.M., Rodriguez, V., Santana, C., Soler, G., Bastida, A., Guisán, J.M., 1993. Preparation of activated supports containing low pK amino groups. A new tool for protein immobilization via the carboxyl coupling method. *Enzym. Microb. Technol.* 15, 546–550. [https://doi.org/10.1016/0141-0229\(93\)90016-U](https://doi.org/10.1016/0141-0229(93)90016-U).
- Fernandez-Lopez, L., Bartolome-Cabrero, R., Rodriguez, M.D., Dos Santos, C.S., Rueda, N., Fernandez-Lafuente, R., 2015. Stabilizing effects of cations on lipases depend on the immobilization protocol. *RSC Adv.* 5, 83868–83875. <https://doi.org/10.1039/c5ra18344h>.
- Fernandez-Lopez, L., Pedrero, S.G., Lopez-Carrolles, N., Gorines, B.C., Virgen-Ortiz, J.J., Fernandez-Lafuente, R., 2017. Effect of protein load on stability of immobilized enzymes. *Enzym. Microb. Technol.* 98, 18–25. <https://doi.org/10.1016/j.enzmictec.2016.12.002>.
- Fernandez-Lopez, L., Rueda, N., Bartolome-Cabrero, R., Rodriguez, M.D., Albuquerque, T.L., Dos Santos, J.C.S., Barbosa, O., Fernandez-Lafuente, R., 2016. Improved immobilization and stabilization of lipase from *Rhizomucor miehei* on octyl-glyoxyl agarose beads by using CaCl₂. *Process Biochem.* 51, 48–52. <https://doi.org/10.1016/j.procbio.2015.11.015>.
- Fernández-Lorente, G., Palomo, J.M., Fuentes, M., Mateo, C., Guisán, J.M., Fernández-Lafuente, R., 2003. Self-assembly of *Pseudomonas fluorescens* lipase into bimolecular aggregates dramatically affects functional properties. *Biotechnol. Bioeng.* 82, 232–237. <https://doi.org/10.1002/bit.10560>.
- Fernandez-Lafuente, R., Guisán, J.M., Ali, S., Cowan, D., 2000. Immobilization of functionally unstable catechol-2,3-dioxygenase greatly improves operational stability. *Enzym. Microb. Technol.* 26, 568–573. [https://doi.org/10.1016/S0141-0229\(00\)00144-7](https://doi.org/10.1016/S0141-0229(00)00144-7).
- García-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., Rodríguez, R.C., 2011. Potential of different enzyme immobilization strategies to improve enzyme performance. *Adv. Synth. Catal.* 353, 2885–2904. <https://doi.org/10.1002/adsc.201100534>.
- Gillooly, J.F., Brown, J.H., West, G.B., Savage, V.M., Charnov, E.L., 2001. Effects of size and temperature on metabolic rate. *Science* (80- 293), 2248–2251. <https://doi.org/10.1126/science.1061967>.
- Glowacki, D.R., Harvey, J.N., Mulholland, A.J., 2012. Taking Ockham's razor to enzyme dynamics and catalysis. *Nat. Chem.* 4, 169–176. <https://doi.org/10.1038/nchem.1244>.
- Gotor-Fernández, V., Busto, E., Gotor, V., 2006. *Candida antarctica* lipase B: an ideal biocatalyst for the preparation of nitrogenated organic compounds. *Adv. Synth. Catal.* 348, 797–812. <https://doi.org/10.1002/adsc.200606057>.
- Grochulski, P., Li, Y., Schrag, J.D., Bouthillier, F., Smith, P., Harrison, D., Rubin, B., Cygler, M., 1993. Insights into interfacial activation from an open structure of *Candida rugosa* lipase. *J. Biol. Chem.* 268, 12843–12847. <https://doi.org/10.2210/pdb1crl/pdb>.
- Grubecki, I., 2021. Analytical determination of the optimal feed temperature for hydrogen peroxide decomposition process occurring in bioreactor with a fixed-bed of commercial catalase. *Catalysts* 11, 1–18. <https://doi.org/10.3390/catal11010035>.
- Grubecki, I., 2018a. Optimal feed temperature for hydrogen peroxide decomposition process occurring in a bioreactor with fixed-bed of commercial catalase: a case study on thermal deactivation of the enzyme. *Chem. Process Eng. - Inz. Chem. i Proces.* 39, 491–501. <https://doi.org/10.24425/cpe.2018.124974>.
- Grubecki, I., 2018b. Optimal feed temperature for an immobilized enzyme fixed-bed reactor: a case study on hydrogen peroxide decomposition by commercial catalase. *Chem. Process Eng. - Inz. Chem. i Proces.* 39, 39–57. <https://doi.org/10.24425/119098>.
- Grubecki, I., 2016. How to run biotransformations - at the optimal temperature control or isothermally? Mathematical assessment. *J. Process Control* 44, 79–91. <https://doi.org/10.1016/j.jprocont.2016.05.005>.
- Guimarães, J.R., Miranda, L.P., Fernandez-lafuente, R., Tardioli, P.W., 2021. Immobilization of eversa® transform via CLEA technology using waste cooking oil. *Molecules* 23, 193.
- Gürdaş, S., Güleç, H.A., Mutlu, M., 2012. Immobilization of *Aspergillus oryzae* β -galactosidase onto duolite A568 resin via simple adsorption mechanism. *Food Bioprocess Technol.* 5, 904–911. <https://doi.org/10.1007/s11947-010-0384-7>.
- Hernandez, K., Berenguer-Murcia, A., Rodriguez, R.C., Fernandez-Lafuente, R., 2012. Hydrogen peroxide in biocatalysis. A dangerous liaison. *Curr. Org. Chem.* 16, 2652–2672. <https://doi.org/10.2174/138527212804004526>.
- Hernandez, K., García-Verdugo, E., Porcar, R., Fernandez-Lafuente, R., 2011. Hydrolysis of triacetin catalyzed by immobilized lipases: effect of the immobilization protocol and experimental conditions on diacetin yield. *Enzym. Microb. Technol.* 48, 510–517. <https://doi.org/10.1016/j.enzmictec.2011.02.005>.
- Hwang, E.T., Lee, S., 2019. Multienzymatic cascade reactions via enzyme complex by immobilization. *ACS Catal.* 9, 4402–4425. <https://doi.org/10.1021/acscatal.8b04921>.
- Illanes, A., 1999. Stability of biocatalysts. *Electron. J. Biotechnol.* 2, 1–9. <https://doi.org/10.2225/vol2-issue1-fulltext-2>.
- Illanes, A., Wilson, L., 2003. Enzyme reactor design under thermal inactivation. *Crit. Rev. Biotechnol.* 23, 61–93. <https://doi.org/10.1080/1713609298>.
- Illanes, A., Wilson, L., Altamirano, C., Aillapán, A., 1998. Reactor performance under thermal inactivation and temperature optimization with chitin-immobilized lactase. *Prog. Biotechnol.* 15, 27–34. [https://doi.org/10.1016/S0921-0423\(98\)80005-1](https://doi.org/10.1016/S0921-0423(98)80005-1).
- Iyer, P.V., Ananthanarayan, L., 2008. Enzyme stability and stabilization—aqueous and non-aqueous environment. *Process Biochem.* 43, 1019–1032. <https://doi.org/10.1016/j.procbio.2008.06.004>.
- Kadisich, M., Willrodt, C., Hillen, M., Bühler, B., Schmid, A., 2017. Maximizing the stability of metabolic engineering-derived whole-cell biocatalysts. *Biotechnol. J.* 12. <https://doi.org/10.1002/biot.201600170>.
- Kekenes-Huskey, P.M., Scott, C.E., Atalay, S., 2016. Quantifying the influence of the crowded cytoplasm on small molecule diffusion. *J. Phys. Chem. B* 120, 8696–8706. <https://doi.org/10.1021/acs.jpcc.6b03887>.
- Klibanov, A.M., 1979. Enzyme stabilization by immobilization. *Anal. Biochem.* 93, 1–25. [https://doi.org/10.1016/S0003-2697\(79\)80110-4](https://doi.org/10.1016/S0003-2697(79)80110-4).
- Liese, A., Hilterhaus, L., 2013. Evaluation of immobilized enzymes for industrial applications. *Chem. Soc. Rev.* 42, 6236–6249. <https://doi.org/10.1039/c3cs35511j>.
- Lokha, Y., Arana-Peña, S., Rios, N.S., Mendez-Sanchez, C., Gonçalves, L.R.B., Lopez-Gallego, F., Fernandez-Lafuente, R., 2020. Modulating the properties of the lipase from *Thermomyces lanuginosus* immobilized on octyl agarose beads by altering the immobilization conditions. *Enzym. Microb. Technol.* 133, 109461. <https://doi.org/10.1016/j.enzmictec.2019.109461>.
- Lombardo, D., Guy, O., 1981. Effect of alcohols on the hydrolysis catalyzed by human pancreatic carboxylic-ester hydrolase. *Biochim. Biophys. Acta Enzymol.* 657, 425–437. [https://doi.org/10.1016/0005-2744\(81\)90328-4](https://doi.org/10.1016/0005-2744(81)90328-4).
- Manoel, E.A., dos Santos, J.C.S., Freire, D.M.G., Rueda, N., Fernandez-Lafuente, R., 2015. Immobilization of lipases on hydrophobic supports involves the open form of the enzyme. *Enzym. Microb. Technol.* 71, 53–57. <https://doi.org/10.1016/j.enzmictec.2015.02.001>.
- Martinelle, M., Holmquist, M., Hult, K., 1995. On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochim. Biophys. Acta Lipids Lipid. Metabol.* 1258, 272–276. [https://doi.org/10.1016/0005-2760\(95\)00131-U](https://doi.org/10.1016/0005-2760(95)00131-U).
- Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisán, J.M., Fernandez-Lafuente, R., 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzym. Microb. Technol.* 40, 1451–1463. <https://doi.org/10.1016/j.enzmictec.2007.01.018>.
- Meissner, M.P., Woodley, J.M., 2022. Mass-based biocatalyst metrics to guide protein engineering and bioprocess development. *Nat. Catal.* 5, 2–4. <https://doi.org/10.1038/s41929-021-00728-5>.
- Monsan, P., 1978. Optimization of glutaraldehyde activation of a support for enzyme immobilization. *J. Mol. Catal.* 3, 371–384. [https://doi.org/10.1016/0304-5102\(78\)80026-1](https://doi.org/10.1016/0304-5102(78)80026-1).
- Monteiro, R.R.C., Virgen-Ortiz, J.J., Berenguer-Murcia, Á., da Rocha, T.N., dos Santos, J.C.S., Alcántara, A.R., Fernandez-Lafuente, R., 2021. Biotechnological relevance of the lipase A from *Candida antarctica*. *Catal. Today* 362, 141–154. <https://doi.org/10.1016/j.cattod.2020.03.026>.
- Paiva Souza, P.M., Carballeares, D., Lopez-Carrolles, N., Gonçalves, L.R.B., Lopez-Gallego, F., Rodriguez, S., Fernandez-Lafuente, R., 2021. Enzyme-support interactions and inactivation conditions determine *Thermomyces lanuginosus* lipase inactivation pathways: functional and fluorescence studies. *Int. J. Biol. Macromol.* 191, 79–91. <https://doi.org/10.1016/j.ijbiomac.2021.09.061>.
- Palomo, J.M., Fuentes, M., Fernández-Lorente, G., Mateo, C., Guisán, J.M., Fernández-Lafuente, R., 2003. General trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the enzyme functionality. *Biomacromolecules* 4, 1–6. <https://doi.org/10.1021/bm025729+>.

- Palomo, J.M., Ortiz, C., Fernández-Lorente, G., Fuentes, M., Guisán, J.M., Fernández-Lafuente, R., 2005. Lipase-lipase interactions as a new tool to immobilize and modulate the lipase properties. *Enzym. Microb. Technol.* 36, 447–454. <https://doi.org/10.1016/j.enzmictec.2004.09.013>.
- Palomo, J.M., Ortiz, C., Fuentes, M., Fernández-Lorente, G., Guisán, J.M., Fernández-Lafuente, R., 2004. Use of immobilized lipases for lipase purification via specific lipase-lipase interactions. *J. Chromatogr. A* 1038, 267–273. <https://doi.org/10.1016/j.chroma.2004.03.058>.
- Polizzi, K.M., Bommaris, A.S., Broering, J.M., Chaparro-Riggers, J.F., 2007. Stability of biocatalysts. *Curr. Opin. Chem. Biol.* 11, 220–225. <https://doi.org/10.1016/j.cbpa.2007.01.685>.
- Ren, S., Li, C., Jiao, X., Jia, S., Jiang, Y., Bilal, M., Cui, J., 2019. Recent progress in multienzymes co-immobilization and multienzyme system applications. *Chem. Eng. J.* 373, 1254–1278. <https://doi.org/10.1016/j.cej.2019.05.141>.
- Ritchie, M.E., 2018. Reaction and diffusion thermodynamics explain optimal temperatures of biochemical reactions. *Sci. Rep.* 8, 1–10. <https://doi.org/10.1038/s41598-018-28833-9>.
- Rocha-Martín, J., Rivas, B. de las, Muñoz, R., Guisán, J.M., López-Gallego, F., 2012. Rational co-immobilization of bi-enzyme cascades on porous supports and their applications in bio-redox reactions with in situ recycling of soluble cofactors. *ChemCatChem* 4, 1279–1288. <https://doi.org/10.1002/cctc.201200146>.
- Rodriguez, R.C., Berenguer-Murcia, Á., Carballares, D., Morellon-Sterling, R., Fernandez-Lafuente, R., 2021. Stabilization of enzymes via immobilization: multipoint covalent attachment and other stabilization strategies. *Biotechnol. Adv.* 52, 107821. <https://doi.org/10.1016/j.biotechadv.2021.107821>.
- Rodriguez, R.C., Bolívar, J.M., Palau-Ors, A., Volpato, G., Ayub, M.A.Z., Fernandez-Lafuente, R., Guisán, J.M., 2009a. Positive effects of the multipoint covalent immobilization in the reactivation of partially inactivated derivatives of lipase from *Thermomyces lanuginosus*. *Enzym. Microb. Technol.* 44, 386–393. <https://doi.org/10.1016/j.enzmictec.2009.02.009>.
- Rodriguez, R.C., Bolívar, J.M., Volpato, G., Filice, M., Godoy, C., Fernandez-Lafuente, R., Guisán, J.M., 2009b. Improved reactivation of immobilized-stabilized lipase from *Thermomyces lanuginosus* by its coating with highly hydrophilic polymers. *J. Biotechnol.* 144, 113–119. <https://doi.org/10.1016/j.jbiotec.2009.09.002>.
- Rodriguez, R.C., Godoy, C.A., Filice, M., Bolívar, J.M., Palau-Ors, A., Garcia-Vargas, J.M., Romero, O., Wilson, L., Ayub, M.A.Z., Fernandez-Lafuente, R., Guisán, J.M., 2009c. Reactivation of covalently immobilized lipase from *Thermomyces lanuginosus*. *Process Biochem.* 44, 641–646. <https://doi.org/10.1016/j.procbio.2009.02.001>.
- Rodriguez, R.C., Virgen-Ortiz, J.J., dos Santos, J.C.S., Berenguer-Murcia, Á., Alcantara, A.R., Barbosa, O., Ortiz, C., Fernandez-Lafuente, R., 2019. Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions. *Biotechnol. Adv.* 37, 746–770. <https://doi.org/10.1016/j.biotechadv.2019.04.003>.
- Romero, O., Vergara, J., Fernández-Lafuente, R., Guisán, J.M., Illanes, A., Wilson, L., 2009. Simple strategy of reactivation of a partially inactivated penicillin G acylase biocatalyst in organic solvent and its impact on the synthesis of β -lactam antibiotics. *Biotechnol. Bioeng.* 103, 472–479. <https://doi.org/10.1002/bit.22264>.
- Rueda, N., Dos Santos, J.C.S., Torres, R., Barbosa, O., Ortiz, C., Fernandez-Lafuente, R., 2015. Reactivation of lipases by the unfolding and refolding of covalently immobilized biocatalysts. *RSC Adv.* 5, 55588–55594. <https://doi.org/10.1039/c5ra07379k>.
- Sabbani, S., Hedenström, E., Nordin, O., 2006. The enantioselectivity of *Candida rugosa* lipase is influenced by the particle size of the immobilising support material. *Accred. J. Mol. Catal. B Enzym.* 42, 1–9. <https://doi.org/10.1016/j.molcatb.2006.05.003>.
- Sanchez, A., Cruz, J., Rueda, N., dos Santos, J.C.S., Torres, R., Ortiz, C., Villalonga, R., Fernandez-Lafuente, R., 2016. Inactivation of immobilized trypsin under dissimilar conditions produces trypsin molecules with different structures. *RSC Adv.* 6, 27329–27334. <https://doi.org/10.1039/C6RA03627A>.
- Schmig, B., Nguyen, M., Franzreb, M., 2020. Simulative minimization of mass transfer limitations within hydrogel-based 3D-printed enzyme carriers. *Front. Bioeng. Biotechnol.* 8, 1–13. <https://doi.org/10.3389/fbioe.2020.00365>.
- Schnell, S., Turner, T.E., 2004. Reaction kinetics in intracellular environments with macromolecular crowding: simulations and rate laws. *Prog. Biophys. Mol. Biol.* 85, 235–260. <https://doi.org/10.1016/j.pbiomolbio.2004.01.012>.
- Sheldon, R.A., van Pelt, S., 2013. Enzyme immobilisation in biocatalysis: why, what and how. *Chem. Soc. Rev.* 42, 6223–6235. <https://doi.org/10.1039/C3CS60075K>.
- Siar, E.-H., Arana-Peña, S., Barbosa, O., Zidoune, M., Fernandez-Lafuente, R., 2018a. Immobilization/stabilization of ficin extract on glutaraldehyde-activated agarose beads. Variables that control the final stability and activity in protein hydrolyses. *Catalysts* 8, 149. <https://doi.org/10.3390/catal8040149>.
- Siar, E.-H., Arana-Peña, S., Barbosa, O., Zidoune, M.N., Fernandez-Lafuente, R., 2018b. Solid phase chemical modification of agarose glyoxyl-ficin: improving activity and stability properties by amination and modification with glutaraldehyde. *Process Biochem.* 73, 109–116. <https://doi.org/10.1016/j.procbio.2018.07.013>.
- Siar, E.-H.H., Morellon-Sterling, R., Zidoune, M.N., Fernandez-Lafuente, R., 2019. Amination of ficin extract to improve its immobilization on glyoxyl-agarose: improved stability and activity versus casein. *Int. J. Biol. Macromol.* 133, 412–419. <https://doi.org/10.1016/j.jbiomac.2019.04.123>.
- Soler, G., Bastida, A., Blanco, R.M., Fernández-Lafuente, R., Guisán, J.M., 1997. Reactivation strategies by unfolding/refolding of chymotrypsin derivatives after inactivation by organic solvents. *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* 1339, 167–175. [https://doi.org/10.1016/S0167-4838\(96\)00223-3](https://doi.org/10.1016/S0167-4838(96)00223-3).
- Suescun, A., Rueda, N., dos Santos, J.C.S., Castillo, J.J., Ortiz, C., Torres, R., Barbosa, O., Fernandez-Lafuente, R., 2015. Immobilization of lipases on glyoxyl-octyl supports: improved stability and reactivation strategies. *Process Biochem.* 50, 1211–1217. <https://doi.org/10.1016/j.procbio.2015.05.010>.
- Takaç, S., Bakka, M., 2007. Impressive effect of immobilization conditions on the catalytic activity and enantioselectivity of *Candida rugosa* lipase toward S-Naproxen production. *Process Biochem.* 42, 1021–1027. <https://doi.org/10.1016/j.procbio.2007.03.013>.
- Van Daele, T., Fernandes del Pozo, D., Van Hauwermeiren, D., Gernaey, K.V., Wohlgemuth, R., Nopens, I., 2016. A generic model-based methodology for quantification of mass transfer limitations in microreactors. *Chem. Eng. J.* 300, 193–208. <https://doi.org/10.1016/j.cej.2016.04.117>.
- Verger, R., 1997. 'Interfacial activation' of lipases: facts and artifacts. *Trends Biotechnol.* 15, 32–38. [https://doi.org/10.1016/S0167-7799\(96\)10064-0](https://doi.org/10.1016/S0167-7799(96)10064-0).
- Wilson, L., Illanes, A., 2020. Parameters for the Evaluation of Immobilized Enzymes under Process Conditions 2100, pp. 65–81.
- Wilson, L., Palomo, J.M., Fernández-Lorente, G., Illanes, A., Guisán, J.M., Fernández-Lafuente, R., 2006. Effect of lipase-lipase interactions in the activity, stability and specificity of a lipase from *Alcaligenes* sp. *Enzym. Microb. Technol.* 39, 259–264. <https://doi.org/10.1016/j.enzmictec.2005.10.015>.
- Xiu, G.H., Jiang, L., Li, P., 2001. Mass-transfer limitations for immobilized enzyme-catalyzed kinetic resolution of racemate in a fixed-bed reactor. *Biotechnol. Bioeng.* 74, 29–39. <https://doi.org/10.1002/bit.1092>.
- Yadav, J.K., Prakash, V., 2009. Thermal stability of α -amylase in aqueous cosolvent systems. *J. Biosci.* 34, 377–387. <https://doi.org/10.1007/s12038-009-0044-0>.
- Zaak, H., Fernandez-Lopez, L., Velasco-Lozano, S., Alcaraz-Fructuoso, M.T., Sassi, M., Lopez-Gallego, F., Fernandez-Lafuente, R., 2017a. Effect of high salt concentrations on the stability of immobilized lipases: dramatic deleterious effects of phosphate anions. *Process Biochem.* 62, 128–134. <https://doi.org/10.1016/j.procbio.2017.07.018>.
- Zaak, H., Siar, E.-H., Kornecki, J.F., Fernandez-Lopez, L., Pedrero, S.G., Virgen-Ortiz, J.J., Fernandez-Lafuente, R., 2017b. Effect of immobilization rate and enzyme crowding on enzyme stability under different conditions. The case of lipase from *Thermomyces lanuginosus* immobilized on octyl agarose beads. *Process Biochem.* 56, 117–123. <https://doi.org/10.1016/j.procbio.2017.02.024>.
- Zhang, Y.H.P., 2011. Substrate channeling and enzyme complexes for biotechnological applications. *Biotechnol. Adv.* 29, 715–725. <https://doi.org/10.1016/j.biotechadv.2011.05.020>.
- Zucca, P., Fernandez-Lafuente, R., Sanjust, E., 2016. Agarose and its derivatives as supports for enzyme immobilization. *Molecules* 21, 1577. <https://doi.org/10.3390/molecules21111577>.