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The Effects of Buffer Nature on Immobilized Lipase Stability Depend on Enzyme Support Loading

Pedro Abellanas-Perez [1], Diego Carballares [1], Javier Rocha-Martin [2,*] and Roberto Fernandez-Lafuente [1,*]

[1] Departamento de Biocatálisis, ICP-CSIC, Campus UAM-CSIC, 28049 Madrid, Spain; pabellanasperez@gmail.com (P.A.-P.); diego.carballares@csic.es (D.C.)
[2] Department of Biochemistry and Molecular Biology, Faculty of Biology, Complutense University of Madrid, José Antonio Novais 12, 28040 Madrid, Spain
* Correspondence: javrocha@ucm.es (J.R.-M.); rfl@icp.csic.es (R.F.-L.)

Abstract: The lipases from *Thermomyces lanuginosus* (TLL) and *Candida antarctica* (B) (CALB) were immobilized on octyl-agarose beads at 1 mg/g (a loading under the capacity of the support) and by overloading the support with the enzymes. These biocatalysts were compared in their stabilities in 10 mM of sodium phosphate, HEPES, and Tris-HCl at pH 7. Lowly loaded CALB was more stable than highly loaded CALB preparation, while with TLL this effect was smaller. Phosphate was very negative for the stability of the CALB biocatalyst and moderately negative using TLL at both loadings. The stability of the enzymes in HEPES and Tris-HCl presented a different response as a function of the enzyme loading (e.g., using lowly loaded CALB, the stabilities were similar in both buffers, but it was clearly smaller in HEPES using the highly loaded biocatalysts). Moreover, the specific activity of the immobilized enzymes versus *p*-nitrophenol butyrate, triacetin and *R*- or *S*-methyl mandelate depended on the buffer, enzyme loading, and interaction between them. In some cases, almost twice the expected activity could be obtained using highly loaded octyl-CALB, depending on the buffer. A co-interaction between the effects on enzyme activity and the specificity of support enzyme loading and buffer nature was detected.

Keywords: enzyme stability; enzyme activity; enzyme specificity; effects of buffers on enzyme properties; effects of enzyme support loading on enzyme properties

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1. Introduction

Lipases are among the most utilized enzymes in academic and applied research [1–4] due to their high stability under different media and conditions and their high versatility (catalyzing hydrolysis [5–8], esterifications [9–12], transesterifications [13–15], acidolysis [16–20], interesterifications [21,22], amidations [23–26], etc.). Lipases have a broad specificity, accepting substrates with very different structures, but in many instances exhibiting a high specificity or regio- or enantio-selectivity [27–31]. Lipases are among the enzymes able to catalyze promiscuous reactions [32–35].

The catalytic mechanism of lipases is peculiar and permits them to fulfill their biological function (the hydrolysis of oils, a substrate that is almost insoluble in water). They have the active center isolated from the medium by a polypeptide chain named lid or flat. In some instances, the lid is unable to fully block the active center of the lipase, like in the case of CALB [36]), while in other instances it is even more complex, with two polypeptide chains moving simultaneously [37–41]. This closed form is in equilibrium with a lipase conformation where the lid is shifted and makes the active center accessible to the medium [37–41]. The lipase open form presents a huge hydrophobic pocket and it is not stable in an aqueous homogeneous medium. This hydrophobic lipase pocket becomes adsorbed on the external surface of triglyceride droplets and, in that way, the lipases can interact with them. This mechanism is called interfacial activation [42–45].

Even with their positive catalytic features, the biological origin of lipases causes some of these properties to not fit some industrial requirements, such as ease of recovery and reuse and stability under operational conditions measured in weeks or even months. Moreover, the high activity, selectivity and specificity of enzymes are found using a physiological substrate; meanwhile, in some instances, the values of these features in the process of interest, sometimes involving substrates and conditions that are far-removed from the physiological ones, are not adequate [46]. Fortunately, there are many tools under intense development that can solve these limitations, such as new tools for the discovery of new enzymes (e.g., metagenomics) [47–50], protein engineering (e.g., directed evolution) [51–53], etc. Nowadays, enzyme molecules simultaneously bearing several active centers can be generated [54] (e.g., plurizymes [55–57] or fusion enzymes [58–70]). In this paper, we will devote our attention to enzyme immobilization [71–74], as it is a potent tool for the stabilization of enzymes (e.g., via multipoint covalent immobilization) [75–80] and for improving their activity, specificity, or selectivity [81–84]. An adequate immobilization protocol can permit an enzyme's coupling with enzyme purification [85–89].

The immobilization of enzymes usually has an optimal result in terms of stability when the support surface is inert after the enzyme immobilization and the enzyme is linked to the support via multipoint covalent attachment [75]. However, this did not happen with most lipases. The lipase immobilization on hydrophobic supports involves the adsorption on the hydrophobic surface of the open form of the enzyme via the large hydrophobic pocket formed by the internal face of the shifted lid and the surroundings of the active center (similar to interfacial activation versus substrate drops) [90]. This permits the one step purification, immobilization, hyperactivation, and stabilization of the lipases [90]. Hyperactivation is related to the stabilization of the open form of the lipases [91], while it has been shown that this adsorbed open form of the lipase is more stable than the lipase in the closing/opening equilibrium [92–94]. In fact, it is difficult to get an immobilized lipase using any other strategy with higher stability than when using this immobilization strategy, including lipase immobilization via multipoint covalent attachment [95]. The reversibility of this immobilization protocol can be considered an advantage, as it enables reuse of the support after enzyme inactivation, or after a problem. Although, in many cases, the operational stability of these biocatalysts is high, the lipase may be desorbed during utilization under certain conditions. In fact, even though it displays very strong adsorption, enzyme release from the support in the presence of organic cosolvents, at high temperatures, or in the presence of detergents (or substrates or products with detergent properties) may take place [96,97]. This can be prevented by using heterofunctional supports, which are those bearing—together with hydrophobic motifs—other groups able to establish ionic or covalent interactions with the enzyme [96].

The immobilization of lipases on hydrophobic supports is very fast. It is so fast that, under proper conditions (e.g., high enzyme concentration), the immobilization rate can be higher than the diffusion of the enzyme inside the pore [98]. This has made it possible to prepare immobilized enzyme preparations where the enzyme molecules are so near each other that they can interact with the immobilized molecules around them, and this has been shown to result in beneficial or negative effects on enzyme stability [99–101]. These effects on enzyme stability of enzyme loading were attributed to the existence of protein–protein interactions; these interactions are much less likely to occur in lowly loaded immobilized enzyme preparations where the distance between the immobilized enzyme molecules will be larger.

On the other hand, it has been found that these interfacially activated biocatalysts have some special "sensibilities" to the presence of some additives in the medium. For example, some cations have stabilizing effects mainly when the enzyme is immobilized on hydrophobic supports, while they are not so relevant in covalently immobilized preparations [102]. Similarly, phosphate anions have a strong destabilizing effect on this kind of biocatalyst, which is not so evident when using other lipase formulations [103]. The use of very different enzyme loadings has been shown to alter the possibilities of obtaining

intermolecular enzyme crosslinking, proving that when really high loadings are used, the distance between the enzyme molecules may be lower that when using low loadings [104]. Moreover, it has been shown that the effect of the chemical modification on immobilized enzyme stability depends on the buffer [105] and on enzyme loading [106].

In this paper, we studied if the effects of the nature of the buffer on the stability of the immobilized enzyme can depend on the immobilized enzyme–enzyme interactions; the intensity of these interactions should be controlled by the enzyme loading in the support. Hence, the objective is to analyze if there are interactions between the effects of support enzyme loading and the nature of the buffer on the features of the immobilized enzyme. No studies have been performed to date (in fact there are just some describing the individual effects of each of these variables) on this topic. As model support, we utilized octyl-agarose, as this can discard some unknown enzyme–support interactions [107]. The support's textural properties can be modified by organic solvents [108], and more so by agarose, which is mainly formed by water. For the enzymes, we selected two examples with very different structures, the one from *Thermomyces lanuginosus* (TLL) [109], bearing a large lid [110,111], and lipase B from *Candida antarctica* (CALB), [112,113] whose lid is so small that is unable to fully block the active center of the lipase [36,114]. Using octyl agarose with these enzymes, the high immobilization rate enables the formation of an enzyme crown on the external part of the pores of the agarose beads, if full loading is not intended [98]. The use of free enzymes in the case of lipases, as reference for any study, has some problems. These enzymes have a strong tendency to form lipase–lipase aggregates, where the open form of two lipase molecules are interacting and altering the functional properties of the enzymes, making studies on free lipases quite unreliable [115,116]. The immobilization in octyl-agarose guarantees the presence of lipase monomers in the open form, making much the comparison more reliable [90,91]. Moreover, lipase molecules may interact with any hydrophobic (even moderately hydrophobic) component of the crude and this can alter the lipase features [117–121], making it complex to also determine the intrinsic features of the free lipases. The use of washed immobilized enzymes prevents this situation.

2. Results

2.1. Immobilization of CALB and TLL on Octyl Agarose

Figures 1–4 show the immobilization courses of both enzymes at two different enzyme loads. Using low enzyme loading (1 mg/g), under the capacity of the support [88,89], TLL shows a clear hyperactivation upon immobilization, reaching an expressed activity 2.3-fold higher than that of the free enzyme. This result is described and explained by the stabilization of the open conformation of the enzyme [71]. Using CALB, the lowly loaded immobilized biocatalyst retains the activity of the free enzyme almost unaltered. This can be related to the lack of a lid large enough to isolate the active center of this enzyme [36,114].

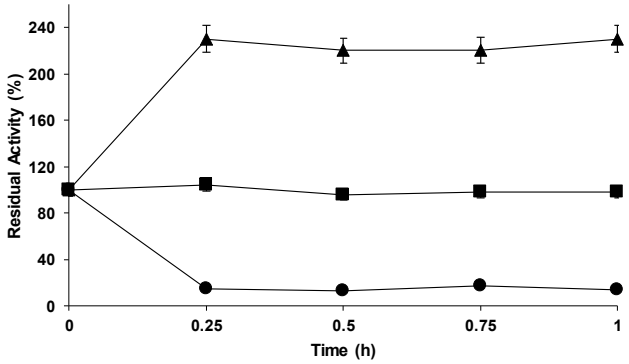

Figure 1. Immobilization course of TLL on octyl agarose beads offering 1 mg of enzyme per g of support. The immobilization was performed in 5 mM of phosphate buffer at pH 7.0 and 25 °C. Squares: reference suspension; triangles: immobilization suspension; and circles: supernatant of the immobilization suspension. Other specifications are described in Methods.

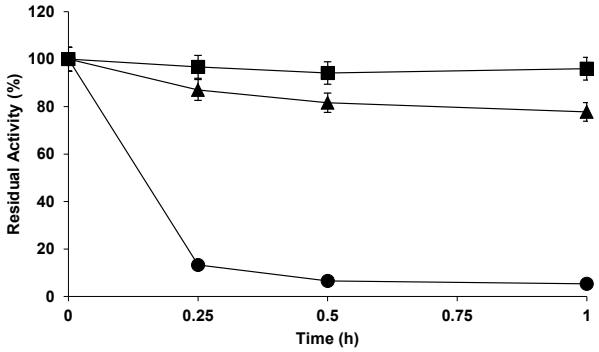

Figure 2. Immobilization course of CALB on octyl agarose beads offering 1 mg of enzyme per g of support. The immobilization was performed in 5 mM of phosphate buffer at pH 7.0 and 25 °C. Squares: reference suspension; triangles: immobilization suspension; and circles: supernatant of the immobilization suspension. Other specifications are described in Methods.

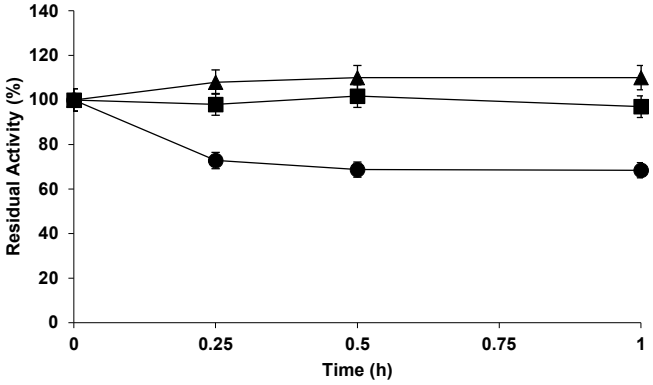

Figure 3. Immobilization course of TLL on octyl agarose beads offering 40 mg of enzyme per g of support. The immobilization was performed in 5 mM of phosphate buffer at pH 7.0 and 25 °C. Squares: reference suspension; triangles: immobilization suspension; and circles: supernatant of the immobilization suspension. Other specifications are described in Methods.

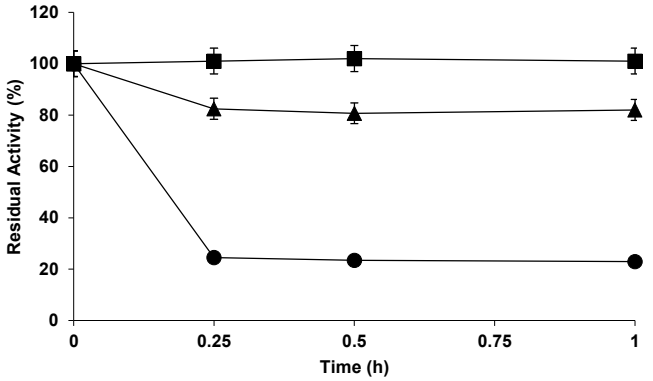

Figure 4. Immobilization course of CALB on octyl agarose beads offering 24 mg of enzyme per g of support. The immobilization was performed using 5 mM of phosphate buffer at pH 7.0 and 25 °C. Squares: reference; triangles: suspension; and circles: supernatant. Other specifications are described in Methods.

Next, we employed enzyme loadings that exceeded that of the support [88,89]: 40 mg/g for TLL and 24 mg/g for CALB [88,89]. In the case of TLL, only 30% of the enzyme was immobilized, and the suspension did not alter the activity, very likely because the activity decrease caused by the substrate diffusional limitations [59] compensated the hyperactivation of the enzyme molecules. Using CALB, 80% of the enzyme was immobilized and

the suspension activity slightly decreased, very likely also due to substrate diffusional limitations [59].

2.2. Effect of the Buffers on the Stability of the Different Biocatalysts

Using the lowly loaded TLL biocatalyst (Figure 5), it was possible to visualize a negative effect of the phosphate buffer, previously described for other octyl-lipase biocatalysts [92–94], giving half the activity after 2 h of inactivation compared to the other two buffers. HEPES permitted slightly lower enzyme stability than Tris-HCl, but the differences are very small. Figure 6 shows the inactivation of the highly and lowly loaded biocatalyst in the different buffers. In all cases, the highly loaded biocatalyst is more stable than the lowly loaded biocatalyst in the first moments of the inactivation, as may be expected from the artifacts that can be generated due to the substrate's diffusional limitations (that can decrease the observed activity); the inactivation of some of the enzyme molecules will decrease mass activity and may permit that the activity of enzymes located in the internal part of the particles can be expressed [59]. However, using HEPES and Tris-HCl, this advantage in the first moments of the inactivation disappeared when the inactivation was prolonged and the final residual activities were even lower than that of the lowly loaded biocatalyst: while using phosphate, a higher residual activity after 2 h was found for the highly loaded biocatalyst compared to the lowly loaded biocatalyst. Using Tris-HCl or HEPES, the situation is reversed, although the differences are not very relevant.

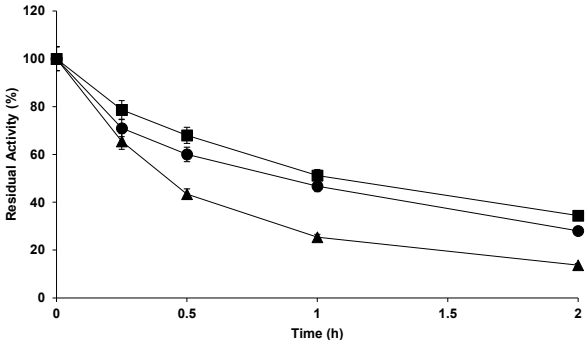

Figure 5. Effect of the buffer on the thermal inactivation courses of lowly loaded octyl-TLL (1 mg/g). The inactivation was performed using 10 mM of different buffers at pH 7.0 and 72 °C. Squares: Tris-HCl; triangles: Sodium phosphate; and circles: HEPES. Other specifications are described in Methods.

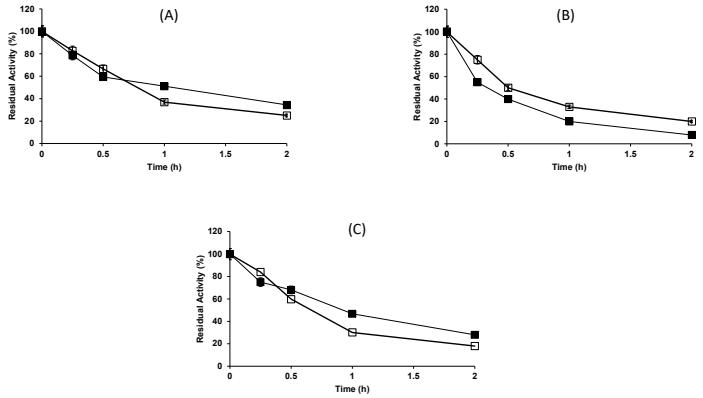

Figure 6. Effect of enzyme loading on the thermal inactivation courses of TLL immobilized on octyl-agarose beads in different buffers. Lowly (1 mg/g) (solid symbols) and highly (40 mg/g) (empty symbols) loaded TLL-octyl agarose biocatalysts (Figures 1 and 3) were used. The inactivation was performed using 10 mM of different buffers at pH 7.0 and 72 °C. Tris-HCl (**A**); sodium phosphate (**B**); and HEPES (**C**), at pH 7.0 and 72 °C. Other specifications are described in Methods.

In any case, it is maintained that phosphate is the buffer where lower octyl-TLL stability is detected also using the highly loaded biocatalyst (Figure S1), and again the stability in HEPES is slightly lower than in Tris-HCl.

When analyzing the lowly loaded biocatalyst from CALB (Figure 7), phosphate has a clear negative effect on enzyme stability, while the inactivation in HEPES and Tris-HCl yielded a higher and similar stability, respectively.

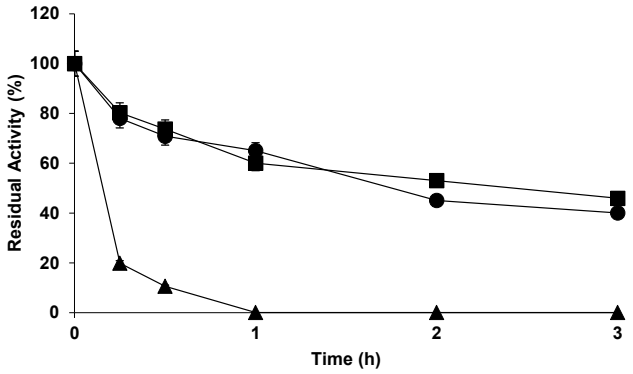

Figure 7. Effect of the buffer on the thermal inactivation courses of lowly loaded octyl-CALB (1 mg/g). The inactivation was performed using 10 mM of different buffers at pH 7.0 and 76 °C. Squares: Tris-HCl; triangles: Sodium phosphate; and circles: HEPES. Other specifications are described in Methods.

Figure 8 shows the comparison of the inactivation of the highly and lowly loaded CALB biocatalysts in the different buffers. When the inactivation was performed in Tris-HCl, the lowly loaded biocatalyst was slightly less stable than the highly loaded biocatalyst. This small difference could be justified by the existence of diffusional problems in the highly loaded biocatalyst. Using HEPES as buffer, the lowly loaded biocatalyst was clearly more stable than the highly loaded biocatalyst, suggesting that, in the Tris-HCl buffer, the protein–protein interactions could generate some negative effects on enzyme stability. The comparison of the highly and lowly loaded biocatalysts in phosphate under the same temperatures in the other buffers gave too rapid an inactivation, making the comparison unreliable (Figure S2). For this reason, we repeated the inactivation at a lower temperature (Figure 8C). This inactivation showed that, in phosphate buffer too, the lowly loaded biocatalyst was more stable than the highly loaded biocatalyst; the difference being even larger than in HEPES. Therefore, the protein–protein interactions seemed to have no effect on octyl-CALB stability using Tris-HCl, but they were negative using HEPES or phosphate. Figure S3 shows that, when using the highly loaded biocatalyst too, the lowest stability was obtained in phosphate but, by using this highly loaded biocatalyst, the octyl-CALB stability in HEPES was clearly lower than that found using Tris-HCl. It should be borne in mind that these effects were detected using very low concentrations of buffers (10 mM).

2.3. Effect of Loading and Buffer on Enzyme Activity versus Different Substrates

If the buffers and enzyme loading can affect the enzyme stability to a great extent, as shown in the previous sections (mainly for CALB), it is possible that they can also alter enzyme activity, as the effects can be related to a certain alteration of enzyme–structure mobility. Therefore, we decided to analyze the activity of the different biocatalysts with triacetin and (*R*)- and (*S*)-methyl mandelate (Figure S4). We discarded *p*-NPB because the low concentration that must be used with this compound (due to its poor solubility) allowed us to observe substrate diffusional limitations just in the preliminary tests, and this is not the objective of the current research.

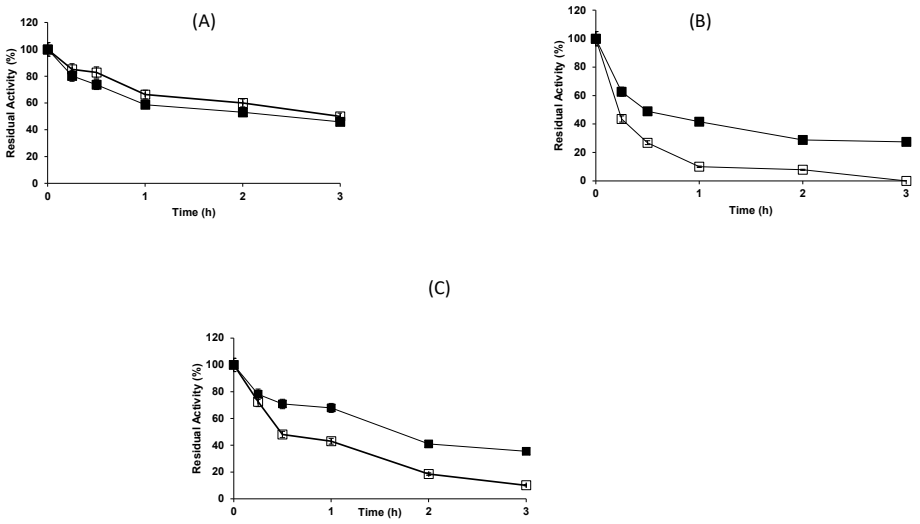

Figure 8. Effect of enzyme loading on the thermal inactivation courses of CALB immobilized on octyl-agarose beads in different buffers. Lowly (1 mg/g) (solid symbols) and highly (24 mg/g) (empty symbols) loaded CALB-octyl agarose biocatalysts (Figures 2 and 4) were used. The inactivation was performed using 10 mM of different buffers at pH 7.0 and 76 °C for (**A**,**C**), and 70 °C for (**B**). Tris-HCl (**A**); sodium phosphate (**B**); and HEPES (**C**). Other specifications are described in Methods.

TLL was much more active versus triacetin than versus both methyl mandelate isomers (Table 1). The lowly loaded TLL-biocatalyst expressed more activity versus triacetin in HEPES (25–40%) than in phosphate or Tris-HCl; these two buffers enable to the biocatalyst to express very similar activities (Table 1). Phosphate was the buffer that gave the lowest stability for this biocatalyst (see previous sections); hence, the effects of activity/stability seemed to be related not only to an increase in the enzyme structure mobility. Different structures of the enzyme when inactivated in different buffers have been previously reported [92]. Therefore, it is not possible to discard the possibility that these different enzyme conformations induced by the buffers can cause these different activities. The activity versus both mandelic esters was so low that it did not enable us to reach reliable conclusions. Moving to the highly loaded biocatalyst, the highest activity versus triacetin was obtained using Tris-HCl as the buffer, while HEPES and phosphate gave similar activities (in this case, the two buffers that gave the lowest stabilities produced lower activities, although, in these activity determination conditions, the enzyme activity remained unaltered for 24 h) (Table 1). With these highly loaded biocatalysts, it was possible to analyze the activity versus the mandelic esters. Using HEPES and Tris-HCl, the biocatalyst gave similar activities versus (*R*)-methyl mandelate, but lower than using phosphate (in this buffer, activity was 20–25% higher). However, using the (*S*)-isomer, in HEPES, 20% more activity was obtained than in Tris-HCl, and in Tris-HCl, a 20% increase in enzyme activity was observed when compared to the enzyme activity in phosphate. Therefore, in phosphate, the biocatalyst preferred to hydrolyze the (*R*)-isomer (the *R/S* activity ratio was around 1.3); in the other two buffers, the situation was different (with *R/S* activity ratios of 0.88 using Tris-HCl and 0.79 using HEPES as the buffer). Therefore, the biocatalyst enantiospecificity could be modulated and even inverted just by using a different buffer (Table 1). Similarly, the triacetin/(*R*)-methyl mandelate activity ratio was altered by the buffer; this value was 3600 in phosphate, almost 5200 in Tris-HCl, or over 4300 in HEPES (Table 1). Therefore, it seems that the different buffers produced different TLL-octyl conformations, bearing different stabilities and activities. And the effect of the buffers on enzyme activity was tuned by enzyme support loading.

Table 1. Effect of enzyme loading and buffer on the CALB and TLL biocatalysts in terms of the activity of the biocatalysts with different substrates under the defined conditions. The experiments were conducted as described in Methods. Activity is given per gram of biocatalyst.

Biocatalyst	Activity (U/g) with 50 mM Triacetin	Activity (U/g) with 10 mM (*R*)-Methyl Mandelate	Activity (U/g) with 10 mM (*S*)-Methyl Mandelate
Octyl-TLL (1 mg/g) Phosphate	18.16 ± 0.91	Under detection limit	Under detection limit
Octyl-TLL (1 mg/g) Tris-HCL	17.04 ± 0.68	Under detection limit	Under detection limit
Octyl-TLL (1 mg/g) Hepes	24.18 ± 1.21	Under detection limit	Under detection limit
Octyl-TLL (40 mg/g) Phosphate	**162.04 ± 6.43**	**0.045 ± 0.002**	**0.033 ± 0.001**
Octyl-TLL (40 mg/g) Tris-HCL	**187.10 ± 9.31**	**0.035 ± 0.001**	**0.040 ± 0.002**
Octyl-TLL (40 mg/g) Hepes	**165.67 ± 8.22**	**0.038 ± 0.001**	**0.047 ± 0.002**
Octyl-CALB (1 mg/g) Phosphate	2.61 ± 0.11	8.86 ± 0.35	0.523 ± 0.026
Octyl-CALB (1 mg/g) Tris-HCL	1.76 ± 0.08	6.85 ± 0.27	0.509 ± 0.025
Octyl-CALB (1 mg/g) Hepes	1.87 ± 0.07	6.77 ± 0.33	0.516 ± 0.020
Octyl-CALB (24 mg/g) Phosphate	**82.73 ± 3.30**	**148.69 ± 5.95**	**11.06 ± 0.44**
Octyl-CALB (24 mg/g) Tris-HCL	**62.89 ± 2.51**	**160.97 ± 8.13**	**15.22 ± 0.76**
Octyl-CALB (24 mg/g) Hepes	**51.03 ± 2.04**	**177.05 ± 8.81**	**12.75 ± 0.63**

In the case of CALB, the activity versus (*R*)-methyl mandelate (the preferred isomer) was higher than versus triacetin. Lowly loaded octyl-CALB presented more activity versus triacetin in phosphate than in HEPES or Tris-HCl (by around 45%), which gave similar activities. Utilizing the (*R*)-methyl mandelate, the situation was similar, with more activity in phosphate than in the other two buffers (by around 30%), while using the (*S*)-isomer the buffers had almost no influence on activity. Therefore, the *R*/*S* activity ratio was not very different when changing the buffer: 17 in phosphate and around 13 in Tris-HCl or HEPES. Considering the triacetin/*R*-methyl mandelate activity ratio, differences were small, ranging between 0.25 and 0.3. Again, it is not simple to correlate the effects on activity and stability just on the basis of higher enzyme structure mobility (although here the activity versus S-methyl mandelate is the only discrepancy).

Highly loaded octyl-CALB gave different activities using triacetin when changing the buffer. Again, the activity was the highest in phosphate, a 75% increase was obtained in Tris-HCl and around 60% in HEPES (as discussed above, HEPES produced a lower stability than Tris-HCl in this biocatalyst). The situation was completely different using methyl mandelate isomers. Using the (*R*)-isomer, the highest activity was obtained in HEPES, with 90% of this activity using Tris-HCl and less than 85% using phosphate. The activity in phosphate was the lowest using this substrate, while it was the highest using triacetin. Using the (*S*)-isomer, the lowest activity was obtained again using phosphate, a 15% higher activity was observed in HEPES and almost a 40% higher activity was found using Tris-HCl. This produced different *R*/*S* activity ratios: 13 using phosphate (slightly lower than using the lowly loaded biocatalyst), 10 using Tris-HCl, or 14 using HEPES. The

triacetin/*R*-methyl mandelate activity ratios were significantly altered by the buffer: 0.55 in phosphate, 0.4 in Tris-HCl, and 0.28 in HEPES. The values of these ratios were higher than using the lowly loaded biocatalysts.

Next, we examined the effect of increasing CALB loading on biocatalyst activity versus the different substrates. The lowly loaded biocatalyst presented 1 mg/g, while the highly loaded biocatalyst presented less than 20 mg, as is shown in Figures 2 and 4. However, the activity of the highly loaded biocatalyst was 31-fold higher using triacetin in phosphate, 35-fold higher using Tris-HCl, and 27-fold higher using HEPES. This exceeds the increase in the amount of immobilized enzyme. The results suggest that the protein–protein interactions, that produced a negative effect on enzyme stability, also produced some alteration in terms of the enzyme structure that causes the enzyme to be more active versus triacetin. Using (*R*)-methyl mandelate, the increase in activity using the highly loaded CALB-octyl biocatalyst was around 17 in phosphate, 23 in Tris-HCl, and 26 in HEPES. Again, except in phosphate, the increase in activity was higher than expected, even ignoring the likely existence of substrate diffusional limitations that could decrease the activity of this biocatalyst exhibiting such high mass activity (largely exceeding the 100 U/g). Using the S-isomer, the increase in activity was 21 in phosphate, 30 in Tris-HCl, and 25 in HEPES. Therefore, it seems that these are not only effects of the buffer, but also of the protein–protein interactions that alter enzyme activity, as they also altered enzyme stability (see previous sections). These effects were not identical for the different buffers. Therefore, different *R/S* and triacetin/*R*-methyl mandelate activity ratios were obtained when comparing the biocatalysts at two different enzyme loadings.

3. Materials and Methods

3.1. Materials

The lipase B from *Candida antarctica* (CALB) (24.77 mg/mL) and the lipase from *Thermomyces lanuginosus* (TLL) (38.49 mg/mL), in liquid formulations, were obtained as kind donations from Novozymes (Madrid, Spain). The Octyl-Sepharose® 4BCL beads were acquired from GE Healthcare. The triacetin and *p*-nitrophenyl-butyrate (*p*-NPB) were purchased from Sigma-Aldrich (Madrid, Spain). The (*R*)- and (*S*)- methyl-mandelates were acquired from Thermo-Fisher (Alcobendas, Spain). The Bradford method was used for the quantification of protein concentrations [122]. All other compounds were of analytical grade.

3.2. Methods

All experiments were performed in triplicate and the values are given as average values and standard errors.

3.2.1. Determination of Enzyme Activity with *p*-NPB

A volume of 2.5 mL of 25 mM sodium phosphate (in some instances changed by Tris-HCl or HEPES) with a pH of 7.0 was added to a cuvette containing a magnetic stirrer along with 50 µL of *p*-NPB solution (50 mM dissolved in acetonitrile). The absorbance was read using a Jasco V-730 spectrophotometer (Jasco, Madrid, Spain) with magnetic stirring and controlled temperature (25 °C). To start the reaction, 50 µL of the free enzyme solution or immobilized enzyme suspension was added to the cuvette. The immobilized enzyme suspensions were prepared by adding 1 g of the different biocatalysts to 10 mL of the aqueous buffers. The increment of absorbance that occurs due to the released *p*-nitrophenol (*p*-NP), caused by the hydrolysis of *p*-NPB, was measured at 348 nm (isosbestic point of *p*-NP, ε is 5150 M^{-1} cm^{-1} under these conditions) [123] for 90 s. The activity was expressed in units (U) defined as the micromoles of the *p*-NP that are produced per minute.

3.2.2. Hydrolysis of Triacetin

The reaction was performed by adding 0.1–0.25 g of biocatalysts to 5–10 mL of 50 mM triacetin dissolved in 50 mM of sodium phosphate, HEPES, or Tris-HCl at a pH of 7.0 and

25 °C) (depending of the use of highly loaded or lowly loaded biocatalysts). The enzymes produced 1,2 diacetin, but this was subject to acyl migration, and a mixture with 1,3 diacetin was obtained [124]. The degree of conversion was calculated by HPLC (Kromasil C18 column of 15 cm × 0.46 cm). As the mobile phase, a solution of 15% acetonitrile-85% Milli-Q water was used. The flow rate was 1 mL/min. The UV–visible detector was settled at 230 nm and the retention times were 4 min for diacetins (these conditions promote the coelution of both diacetins) and 18 min for triacetin. To determine the initial rates, conversions between 15% and 20% were utilized.

3.2.3. Hydrolysis of (*R*) or (*S*)-Methyl Mandelate

The reaction was started by the addition of 0.02 or 0.5 g of biocatalysts to 3 or 5 mL of 10 mM of (*R*) or (*S*)-methyl mandelate dissolved in 50 mM of sodium phosphate, HEPES, or Tris-HCl buffers at pH 7.0 and 25 °C (depending on the use of highly loaded or lowly loaded biocatalysts). A HPLC (Kromasil C18 column of 15 cm × 0.46 cm) was utilized to follow the reaction. The mobile phase was a solution of 35% acetonitrile-65% Milli-Q water with 10 mM ammonium acetate at pH 2.8. The flow rate was 1 mL/min. The compounds were detected at 230 nm and the retention times were 2.5 min for the acid and 4.2 min for the ester. The initial rates were calculated utilizing conversions between 15% and 20%.

3.2.4. Immobilization of the Lipases on Octyl-Agarose Beads

Both enzymes (CALB and TLL) were immobilized on octyl-agarose beads using two enzyme loadings: one that ensures a full coating of the support surface with the enzyme going over the loading capability of the support, and the other one using a low load of enzyme. These loadings were selected following our previous results, where the effects of the enzyme interactions were detected [100,101]. Consequently, in the CALB case, 24 mg (high enzyme loading) and 1 mg (low enzyme loading) of protein were used per gram of support; while for TLL 40 mg (high enzyme loading) and 1 mg (low enzyme loading) per gram of support were used. The enzymes were immobilized in 5 mM of sodium phosphate at a pH of 7.0 at 25 °C in a 1/10 ratio (g/mL). The whole immobilization process was monitored by acquiring samples at different times of the suspension and supernatant—as well as via a reference suspension under identical conditions where agarose beads were used—and measuring their activity employing *p*-NPB as a substrate; the percentage of immobilization (immobilization yield) and the percentage of expressed activity of the immobilized enzyme (expressed activity) were supplied [125]. After one hour, the immobilized enzymes were washed with distilled water to dispose of the excess of lipases, vacuum filtered, and stored at 5–6 °C.

3.2.5. Enzyme Thermal Inactivation

The immobilized enzymes were resuspended in 10 mM of sodium phosphate, HEPES, or Tris-HCl at pH 7.0 in different ratios depending on the enzyme loading. For the highly loaded biocatalysts, a ratio of 1/30 (g support/mL) was employed, and for the low-concentration supports, a ratio of 1/10 (g support/mL) was used. Then, these suspensions were incubated in a thermostatized bath at temperatures depending on the enzyme: CALB was incubated at 76 °C or at 70 °C (using only sodium phosphate), and TLL at 72 °C in all cases. These conditions were selected to have fast but reliable inactivation courses. Samples were taken at the indicated times to quantify the residual activity retained by the different biocatalysts.

4. Conclusions

This paper has shown that the enzyme loading (very likely by favoring the enzyme molecules intermolecular interactions) and the nature of the buffer greatly affect not only enzyme stability but also other enzyme catalytic features, such as activity or specificity. The rapid immobilization of the enzymes in octyl-agarose may be a key point in explaining the existence of these enzyme–enzyme interactions. Furthermore, the effects of the buffers

differ depending on support loading and vice versa, suggesting a correlation between both phenomena. In the two enzymes used in this paper, octyl-CALB seems to be more sensitive to these variables, but TLL is also affected. Therefore, an integral analysis of the effects of the loading and the nature of the buffers on enzyme activity/stability/specificity should be performed and, very likely, compromise solutions must be reached in each specific process. The results obtained in an immobilized biocatalyst where there are no intermolecular interactions may be very different to those where these interactions exist. However, these enzyme–enzyme interactions only will be significant when the immobilization rate is very high and the loading of the support is very high.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal14020105/s1, Figure S1. Effect of the buffer in the thermal inactivation courses of highly loaded octyl-TLL (40 mg/g). The inactivation was performed using 10 mM of different buffers at pH 7.0 and 72 °C. Squares: Tris-HCl; Triangles: Sodium phosphate and Circles: HEPES. Other specifications were described in Methods. Figure S2. Effect of the enzyme loading on the thermal inactivation courses of CALB immobilized on octyl-agarose beads in phosphate. Lowly (1 mg/g) (solid symbols) and highly (24 mg/g) (empty symbols) CALB-octyl agarose biocatalysts (Figures 2 and 4) were used, The inactivation was performed using 10 mM of sodium phosphate at pH 7.0 and 76 °C. Other specifications were described in Methods. Figure S3. Effect of the buffer in the thermal inactivation courses of highly loaded octyl-CALB (24 mg/g). The inactivation was performed using 10 mM of different buffers at pH 7.0 and 76 °C. Squares: Tris-HCl; Triangles: Sodium phosphate and Circles: HEPES. Other specifications were described in Methods. Figure S4. Schematic representation of the enzymatic hydrolysis of (A) Triacetin and (B) (*R*)-(*S*) methyl mandelate.

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References

1. Rabbani, G.; Ahmad, E.; Ahmad, A.; Khan, R.H. Structural Features, Temperature Adaptation and Industrial Applications of Microbial Lipases from Psychrophilic, Mesophilic and Thermophilic Origins. *Int. J. Biol. Macromol.* **2023**, *225*, 822–839. [CrossRef]
2. Mahfoudhi, A.; Benmabrouk, S.; Fendri, A.; Sayari, A. Fungal Lipases as Biocatalysts: A Promising Platform in Several Industrial Biotechnology Applications. *Biotechnol. Bioeng.* **2022**, *119*, 3370–3392. [CrossRef]
3. Remonatto, D.; Miotti, R.H.; Monti, R.; Bassan, J.C.; de Paula, A.V. Applications of Immobilized Lipases in Enzymatic Reactors: A Review. *Process Biochem.* **2022**, *114*, 1–20. [CrossRef]
4. Salgado, C.A.; dos Santos, C.I.A.; Vanetti, M.C.D. Microbial Lipases: Propitious Biocatalysts for the Food Industry. *Food Biosci.* **2022**, *45*, 101509. [CrossRef]
5. He, H.; Han, H.; Shi, H.; Tian, Y.; Sun, F.; Song, Y.; Li, Q.; Zhu, G. Construction of Thermophilic Lipase-Embedded Metal-Organic Frameworks via Biomimetic Mineralization: A Biocatalyst for Ester Hydrolysis and Kinetic Resolution. *ACS Appl. Mater. Interfaces* **2016**, *8*, 24517–24524. [CrossRef]
6. Goswami, D.; Basu, J.K.; De, S. Lipase Applications in Oil Hydrolysis with a Case Study on Castor Oil: A Review. *Crit. Rev. Biotechnol.* **2013**, *33*, 81–96. [CrossRef]
7. Sun, B.; Chen, J.; Sun, C.; Jiang, B.; Zhang, P.; Ma, Y.; Zheng, S.; Tang, K. Enhanced MOF-Immobilized Lipase CALA with Polyethylene Glycol for Efficient Stereoselective Hydrolysis of Aromatic Acid Esters. *Biochem. Eng. J.* **2022**, *189*, 108707. [CrossRef]
8. Carvalho, A.C.L.d.M.; de Oliveira, B.R.; Lima, G.V.; Negreiro, J.M.; Oliveira, M.C.F.; de Lemos, T.L.G.; da Silva, M.R.; Fonseca, T.d.S.; Bezerra, R.M.; dos Santos, J.C.S.; et al. Resolution of Racemic Aryloxy-Propan-2-Yl Acetates via Lipase-Catalyzed Hydrolysis: Preparation of Enantiomerically Pure/Enantioenriched Mexiletine Intermediates and Analogs. *Catalysts* **2022**, *12*, 1566. [CrossRef]

9. Stergiou, P.Y.; Foukis, A.; Filippou, M.; Koukouritaki, M.; Parapouli, M.; Theodorou, L.G.; Hatziloukas, E.; Afendra, A.; Pandey, A.; Papamichael, E.M. Advances in Lipase-Catalyzed Esterification Reactions. *Biotechnol. Adv.* **2013**, *31*, 1846–1859. [CrossRef] [PubMed]
10. Dhake, K.P.; Thakare, D.D.; Bhanage, B.M. Lipase: A Potential Biocatalyst for the Synthesis of Valuable Flavour and Fragrance Ester Compounds. *Flavour. Fragr. J.* **2013**, *28*, 71–83. [CrossRef]
11. Sousa, R.R.; Silva, A.S.A.; Fernandez-Lafuente, R.; Ferreira-Leitão, V.S. Solvent-Free Esterifications Mediated by Immobilized Lipases: A Review from Thermodynamic and Kinetic Perspectives. *Catal. Sci. Technol.* **2021**, *11*, 5696–5711. [CrossRef]
12. Pereira, A.d.S.; de Souza, A.H.; Fraga, J.L.; Villeneuve, P.; Torres, A.G.; Amaral, P.F.F. Lipases as Effective Green Biocatalysts for Phytosterol Esters’ Production: A Review. *Catalysts* **2022**, *12*, 88. [CrossRef]
13. Moussavou Mounguengui, R.W.; Brunschwig, C.; Baréa, B.; Villeneuve, P.; Blin, J. Are Plant Lipases a Promising Alternative to Catalyze Transesterification for Biodiesel Production? *Prog. Energy Combust. Sci.* **2013**, *39*, 441–456. [CrossRef]
14. Narwal, S.K.; Gupta, R. Biodiesel Production by Transesterification Using Immobilized Lipase. *Biotechnol. Lett.* **2013**, *35*, 479–490. [CrossRef]
15. Bajaj, A.; Lohan, P.; Jha, P.N.; Mehrotra, R. Biodiesel Production through Lipase Catalyzed Transesterification: An Overview. *J. Mol. Catal. B Enzym.* **2010**, *62*, 9–14. [CrossRef]
16. Sahin, N.; Akoh, C.C.; Karaali, A. Lipase-Catalyzed Acidolysis of Tripalmitin with Hazelnut Oil Fatty Acids and Stearic Acid to Produce Human Milk Fat Substitutes. *J. Agric. Food Chem.* **2005**, *53*, 5779–5783. [CrossRef]
17. Akil, E.; Pereira, A.d.S.; El-Bacha, T.; Amaral, P.F.F.; Torres, A.G. Efficient Production of Bioactive Structured Lipids by Fast Acidolysis Catalyzed by *Yarrowia lipolytica* Lipase, Free and Immobilized in Chitosan-Alginate Beads, in Solvent-Free Medium. *Int. J. Biol. Macromol.* **2020**, *163*, 910–918. [CrossRef]
18. Shimada, Y.; Maruyama, K.; Sugihara, A.; Moriyama, S.; Tominaga, Y. Purification of Docosahexaenoic Acid from Tuna Oil by a Two-Step Enzymatic Method: Hydrolysis and Selective Esterification. *J. Am. Oil Chem. Soc.* **1997**, *74*, 1441–1446. [CrossRef]
19. Zou, X.; Nadege, K.; Ninette, I.; Wen, Y.; Wu, S.; Jiang, X.; Zhang, H.; Jin, Q.; Wang, X. Preparation of Docosahexaenoic Acid-Rich Diacylglycerol-Rich Oil by Lipase-Catalyzed Glycerolysis of Microbial Oil from *Schizochytrium* Sp. in a Solvent-Free System. *JAOCS J. Am. Oil Chem. Soc.* **2020**, *97*, 263–270. [CrossRef]
20. Okulus, M.; Rychlicka, M.; Gliszczyńska, A. Enzymatic Production of Biologically Active 3-Methoxycinnamoylated Lysophosphatidylcholine via Regioselctive Lipase-Catalyzed Acidolysis. *Foods* **2022**, *11*, 7. [CrossRef] [PubMed]
21. Soumanou, M.M.; Pérignon, M.; Villeneuve, P. Lipase-Catalyzed Interesterification Reactions for Human Milk Fat Substitutes Production: A Review. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 270–285. [CrossRef]
22. Utama, Q.D.; Sitanggang, A.B.; Adawiyah, D.R.; Hariyadi, P. Lipase-Catalyzed Interesterification for the Synthesis of Medium-Long-Medium (MLM) Structured Lipids. *Food Technol. Biotechnol.* **2019**, *57*, 305–318. [CrossRef] [PubMed]
23. Sun, M.; Nie, K.; Wang, F.; Deng, L. Optimization of the Lipase-Catalyzed Selective Amidation of Phenylglycinol. *Front. Bioeng. Biotechnol.* **2020**, *7*, 486. [CrossRef] [PubMed]
24. Manova, D.; Gallier, F.; Tak-Tak, L.; Yotava, L.; Lubin-Germain, N. Lipase-Catalyzed Amidation of Carboxylic Acid and Amines. *Tetrahedron Lett.* **2018**, *59*, 2086–2090. [CrossRef]
25. Zhang, L.; Li, F.; Wang, C.; Zheng, L.; Wang, Z.; Zhao, R.; Wang, L. Lipase-Mediated Amidation of Anilines with 1,3-Diketones via C–C Bond Cleavage. *Catalysts* **2017**, *7*, 115. [CrossRef]
26. Vongvilai, P.; Ramström, O. Dynamic Asymmetric Multicomponent Resolution: Lipase-Mediated Amidation of a Double Dynamic Covalent System. *J. Am. Chem. Soc.* **2009**, *131*, 14419–14425. [CrossRef] [PubMed]
27. Park, J.Y.; Park, K.M. Lipase and Its Unique Selectivity: A Mini-Review. *J. Chem.* **2022**, *2022*, 7609019. [CrossRef]
28. Chen, H.; Meng, X.; Xu, X.; Liu, W.; Li, S. The Molecular Basis for Lipase Stereoselectivity. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 3487–3495. [CrossRef]
29. Tsai, S.W. Enantiopreference of *Candida antarctica* Lipase B toward Carboxylic Acids: Substrate Models and Enantioselectivity Thereof. *J. Mol. Catal. B Enzym.* **2016**, *127*, 98–116. [CrossRef]
30. Lee, J.; Kim, N.H.; Choi, Y.; Yang, E.; Yu, H.; Kwon, C.W.; Chang, P.S. Divergent Substrate Specificities and Regioselectivities of Three Lipase Isoforms from Cordyceps Militaris: Combinatorial Advantages for Entomopathogenicity and Prospects as Biocatalysts. *Enzym. Microb. Technol.* **2022**, *161*, 110117. [CrossRef]
31. Dong, Z.; Olofsson, K.; Linares-Pastén, J.A.; Karlsson, E.N. Investigation of Structural Features of Two Related Lipases and the Impact on Fatty Acid Specificity in Vegetable Fats. *Int. J. Mol. Sci.* **2022**, *23*, 7072. [CrossRef] [PubMed]
32. Patti, A.; Sanfilippo, C. Stereoselective Promiscuous Reactions Catalyzed by Lipases. *Int. J. Mol. Sci.* **2022**, *23*, 2675. [CrossRef]
33. Samsonowicz-Górski, J.; Koszelewski, D.; Kowalczyk, P.; Śmigielski, P.; Hrunyk, A.; Kramkowski, K.; Wypych, A.; Szymczak, M.; Lizut, R.; Ostaszewski, R. Promiscuous Lipase-Catalyzed Knoevenagel–Phospha–Michael Reaction for the Synthesis of Antimicrobial β-Phosphono Malonates. *Int. J. Mol. Sci.* **2022**, *23*, 8819. [CrossRef]
34. Kowalczyk, P.; Koszelewski, D.; Gawdzik, B.; Samsonowicz-Górski, J.; Kramkowski, K.; Wypych, A.; Lizut, R.; Ostaszewski, R. Promiscuous Lipase-Catalyzed Markovnikov Addition of H-Phosphites to Vinyl Esters for the Synthesis of Cytotoxic α-Acyloxy Phosphonate Derivatives. *Materials* **2022**, *15*, 1975. [CrossRef] [PubMed]
35. Guezane-Lakoud, S.; Toffano, M.; Aribi-Zouioueche, L. Promiscuous Lipase Catalyzed a New P–C Bond Formation: Green and Efficient Protocol for One-Pot Synthesis of α-Aminophosphonates. *Heteroat. Chem.* **2017**, *28*, e21408. [CrossRef]

36. Uppenberg, J.; Hansen, M.T.; Patkar, S.; Jones, T.A. The Sequence, Crystal Structure Determination and Refinement of Two Crystal Forms of Lipase B from *Candida antarctica*. *Structure* **1994**, *2*, 293–308. [CrossRef]
37. Brzozowski, A.M.; Derewenda, U.; Derewenda, Z.S.; Dodson, G.G.; Lawson, D.M.; Turkenburg, J.P.; Bjorkling, F.; Huge-Jensen, B.; Patkar, S.A.; Thim, L. A Model for Interfacial Activation in Lipases from the Structure of a Fungal Lipase-Inhibitor Complex. *Nature* **1991**, *351*, 491–494. [CrossRef] [PubMed]
38. Verger, R. 'Interfacial Activation' of Lipases: Facts and Artifacts. *Trends Biotechnol.* **1997**, *15*, 32–38. [CrossRef]
39. van Tilbeurgh, H.; Egloff, M.-P.; Martinez, C.; Rugani, N.; Verger, R.; Cambillau, C. Interfacial Activation of the Lipase–Procolipase Complex by Mixed Micelles Revealed by X-ray Crystallography. *Nature* **1993**, *362*, 814–820. [CrossRef]
40. Grochulski, P.; Li, Y.; Schrag, J.D.; Bouthillier, F.; Smith, P.; Harrison, D.; Rubin, B.; Cygler, M. Insights into Interfacial Activation from an Open Structure of *Candida rugosa* Lipase. *J. Biol. Chem.* **1993**, *268*, 12843–12847. [CrossRef]
41. Derewenda, U.; Brzozowski, A.M.; Lawson, D.M.; Derewenda, Z.S. Catalysis at the Interface: The Anatomy of a Conformational Change in a Triglyceride Lipase. *Biochemistry* **1992**, *31*, 1532–1541. [CrossRef]
42. Reis, P.; Holmberg, K.; Watzke, H.; Leser, M.E.; Miller, R. Lipases at Interfaces: A Review. *Adv. Colloid. Interface Sci.* **2009**, *147–148*, 237–250. [CrossRef]
43. Aloulou, A.; Rodriguez, J.A.; Fernandez, S.; van Oosterhout, D.; Puccinelli, D.; Carrière, F. Exploring the Specific Features of Interfacial Enzymology Based on Lipase Studies. *Biochim. Et Biophys. Acta (BBA)-Mol. Cell Biol. Lipids* **2006**, *1761*, 995–1013. [CrossRef]
44. Dragoi, B.; Dumitriu, E. Are Interfacial Biocatalysts Important Tools For Nonpolluting Technologies? 1. Production, Structural Aspects And Mechanism of Lipases. *Environ. Eng. Manag. J.* **2005**, *4*, 1–24. [CrossRef]
45. Schmid, R.D.; Verger, R. Lipases: Interfacial Enzymes with Attractive Applications. *Angew. Chem.* **1998**, *37*, 1608–1633. [CrossRef]
46. Schoemaker, H.E.; Mink, D.L.; WubboLts, M.G. Dispelling the Myths—Biocatalysis in Industrial Synthesis. *Science* **2003**, *299*, 1694–1697. [CrossRef]
47. Ferrer, M.; Martínez-Martínez, M.; Bargiela, R.; Streit, W.R.; Golyshina, O.V.; Golyshin, P.N. Estimating the Success of Enzyme Bioprospecting through Metagenomics: Current Status and Future Trends. *Microb. Biotechnol.* **2016**, *9*, 22–34. [CrossRef] [PubMed]
48. Vieites, J.M.; Guazzaroni, M.-E.; Beloqui, A.; Golyshin, P.N.; Ferrer, M. Metagenomics Approaches in Systems Microbiology. *FEMS Microbiol. Rev.* **2009**, *33*, 236–255. [CrossRef]
49. Martínez-Martínez, M.; Alcaide, M.; Tchigvintsev, A.; Reva, O.; Polaina, J.; Bargiela, R.; Guazzaroni, M.-E.; Chicote, Á.; Canet, A.; Valero, F.; et al. Biochemical Diversity of Carboxyl Esterases and Lipases from Lake Arreo (Spain): A Metagenomic Approach. *Appl. Environ. Microbiol.* **2013**, *79*, 3553–3562. [CrossRef] [PubMed]
50. Peña-García, C.; Martínez-Martínez, M.; Reyes-Duarte, D.; Ferrer, M. High Throughput Screening of Esterases, Lipases and Phospholipases in Mutant and Metagenomic Libraries: A Review. *Comb. Chem. High. Throughput Screen.* **2016**, *19*, 605–615. [CrossRef]
51. Romero, P.A.; Arnold, F.H. Exploring Protein Fitness Landscapes by Directed Evolution. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 866–876. [CrossRef] [PubMed]
52. Packer, M.S.; Liu, D.R. Methods for the Directed Evolution of Proteins. *Nat. Rev. Genet.* **2015**, *16*, 379–394. [CrossRef] [PubMed]
53. Renata, H.; Wang, Z.J.; Arnold, F.H. Expanding the Enzyme Universe: Accessing Non-Natural Reactions by Mechanism-Guided Directed Evolution. *Angew. Chem. Int. Ed.* **2015**, *54*, 3351–3367. [CrossRef] [PubMed]
54. Carballares, D.; Morellon-Sterling, R.; Fernandez-Lafuente, R. Design of Artificial Enzymes Bearing Several Active Centers: New Trends, Opportunities and Problems. *Int. J. Mol. Sci.* **2022**, *23*, 5304. [CrossRef] [PubMed]
55. Alonso, S.; Santiago, G.; Cea-Rama, I.; Fernandez-Lopez, L.; Coscolín, C.; Modregger, J.; Ressmann, A.K.; Martínez-Martínez, M.; Marrero, H.; Bargiela, R.; et al. Genetically Engineered Proteins with Two Active Sites for Enhanced Biocatalysis and Synergistic Chemo- and Biocatalysis. *Nat. Catal.* **2020**, *3*, 319–328. [CrossRef]
56. Fernandez-Lopez, L.; Roda, S.; Gonzalez-Alfonso, J.L.; Plou, F.J.; Guallar, V.; Ferrer, M. Design and Characterization of In-One Protease-Esterase PluriZyme. *Int. J. Mol. Sci.* **2022**, *23*, 13337. [CrossRef] [PubMed]
57. Santiago, G.; Martínez-Martínez, M.; Alonso, S.; Bargiela, R.; Coscolín, C.; Golyshin, P.N.; Guallar, V.; Ferrer, M. Rational Engineering of Multiple Active Sites in an Ester Hydrolase. *Biochemistry* **2018**, *57*, 2245–2255. [CrossRef]
58. Kan, S.; Aoyagi-Scharber, M.; Le, S.Q.; Vincelette, J.; Ohmi, K.; Bullens, S.; Wendt, D.J.; Christianson, T.M.; Tiger, P.M.N.; Brown, J.R.; et al. Delivery of an Enzyme-IGFII Fusion Protein to the Mouse Brain Is Therapeutic for Mucopolysaccharidosis Type IIIB. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 14870–14875. [CrossRef]
59. Lee, D.; Lloyd, N.D.R.; Pretorius, I.S.; Borneman, A.R. Heterologous Production of Raspberry Ketone in the Wine Yeast *Saccharomyces cerevisiae* via Pathway Engineering and Synthetic Enzyme Fusion. *Microb. Cell Fact.* **2016**, *15*, 49. [CrossRef]
60. Liu, H.-H.; Wang, C.; Lu, X.-Y.; Huang, H.; Tian, Y.; Ji, X.-J. Improved Production of Arachidonic Acid by Combined Pathway Engineering and Synthetic Enzyme Fusion in *Yarrowia lipolytica*. *J. Agric. Food Chem.* **2019**, *67*, 9851–9857. [CrossRef]
61. Rabeharindranto, H.; Castaño-Cerezo, S.; Lautier, T.; Garcia-Alles, L.F.; Treitz, C.; Tholey, A.; Truan, G. Enzyme-Fusion Strategies for Redirecting and Improving Carotenoid Synthesis in *S. cerevisiae*. *Metab. Eng. Commun.* **2019**, *8*, e00086. [CrossRef]
62. Camagna, M.; Grundmann, A.; Bär, C.; Koschmieder, J.; Beyer, P.; Welsch, R. Enzyme Fusion Removes Competition for Geranylgeranyl Diphosphate in Carotenogenesis. *Plant Physiol.* **2019**, *179*, 1013–1027. [CrossRef]

63. Nogueira, M.; Enfissi, E.M.A.; Welsch, R.; Beyer, P.; Zurbriggen, M.D.; Fraser, P.D. Construction of a Fusion Enzyme for Astaxanthin Formation and Its Characterisation in Microbial and Plant Hosts: A New Tool for Engineering Ketocarotenoids. *Metab. Eng.* **2019**, *52*, 243–252. [CrossRef]
64. Du, L.; Cui, X.; Li, H.; Wang, Y.; Fan, L.; He, R.; Jiang, F.; Yu, A.; Xiao, D.; Ma, L. Enhancing the Enzymatic Hydrolysis Efficiency of Lignocellulose Assisted by Artificial Fusion Enzyme of Swollenin-Xylanase. *Ind. Crop. Prod.* **2021**, *173*, 114106. [CrossRef]
65. Xia, Y.; Wu, Z.; He, R.; Gao, Y.; Qiu, Y.; Cheng, Q.; Ma, X.; Wang, Z. Simultaneous Degradation of Two Mycotoxins Enabled by a Fusion Enzyme in Food-Grade Recombinant *Kluyveromyces lactis*. *Bioresour. Bioprocess.* **2021**, *8*, 62. [CrossRef]
66. Liao, L.; Zhang, Y.; Wang, Y.; Fu, Y.; Zhang, A.; Qiu, R.; Yang, S.; Fang, B. Construction and Characterization of a Novel Glucose Dehydrogenase-Leucine Dehydrogenase Fusion Enzyme for the Biosynthesis of l-Tert-Leucine. *Microb. Cell Fact.* **2021**, *20*, 3. [CrossRef]
67. Fabara, A.N.; Fraaije, M.W. Production of Indigo through the Use of a Dual-Function Substrate and a Bifunctional Fusion Enzyme. *Enzym. Microb. Technol.* **2020**, *142*, 109692. [CrossRef] [PubMed]
68. Mourelle-Insua, Á.; Aalbers, F.S.; Lavandera, I.; Gotor-Fernández, V.; Fraaije, M.W. What to Sacrifice? Fusions of Cofactor Regenerating Enzymes with Baeyer-Villiger Monooxygenases and Alcohol Dehydrogenases for Self-Sufficient Redox Biocatalysis. *Tetrahedron* **2019**, *75*, 1832–1839. [CrossRef]
69. Baklouti, Z.; Delattre, C.; Pierre, G.; Gardarin, C.; Abdelkafi, S.; Michaud, P.; Dubessay, P. Biochemical Characterization of a Bifunctional Enzyme Constructed by the Fusion of a Glucuronan Lyase and a Chitinase from *Trichoderma* sp. *Life* **2020**, *10*, 234. [CrossRef] [PubMed]
70. Ying, X.; Wang, C.; Shao, S.; Wang, Q.; Zhou, X.; Bai, Y.; Chen, L.; Lu, C.; Zhao, M.; Wang, Z. Efficient Oxidation of Methyl Glycolate to Methyl Glyoxylate Using a Fusion Enzyme of Glycolate Oxidase, Catalase and Hemoglobin. *Catalysts* **2020**, *10*, 943. [CrossRef]
71. Sheldon, R.A.; van Pelt, S. Enzyme Immobilisation in Biocatalysis: Why, What and How. *Chem. Soc. Rev.* **2013**, *42*, 6223–6235. [CrossRef]
72. Bolivar, J.M.; Woodley, J.M.; Fernandez-Lafuente, R. Is Enzyme Immobilization a Mature Discipline? Some Critical Considerations to Capitalize on the Benefits of Immobilization. *Chem. Soc. Rev.* **2022**, *51*, 6251–6290. [CrossRef]
73. Di Cosimo, R.; Mc Auliffe, J.; Poulose, A.J.; Bohlmann, G. Industrial Use of Immobilized Enzymes. *Chem. Soc. Rev.* **2013**, *42*, 6437–6474. [CrossRef]
74. Liese, A.; Hilterhaus, L. Evaluation of Immobilized Enzymes for Industrial Applications. *Chem. Soc. Rev.* **2013**, *42*, 6236–6249. [CrossRef]
75. Rodrigues, R.C.; Berenguer-Murcia, Á.; Carballares, D.; Morellon-Sterling, R.; Fernandez-Lafuente, R. Stabilization of Enzymes via Immobilization: Multipoint Covalent Attachment and Other Stabilization Strategies. *Biotechnol. Adv.* **2021**, *52*, 107821. [CrossRef] [PubMed]
76. Hwang, E.T.; Gu, M.B. Enzyme Stabilization by Nano/Microsized Hybrid Materials. *Eng. Life Sci.* **2013**, *13*, 49–61. [CrossRef]
77. Kim, J.; Grate, J.W.; Wang, P. Nanostructures for Enzyme Stabilization. *Chem. Eng. Sci.* **2006**, *61*, 1017–1026. [CrossRef]
78. Stepankova, V.; Bidmanova, S.; Koudelakova, T.; Prokop, Z.; Chaloupkova, R.; Damborsky, J. Strategies for Stabilization of Enzymes in Organic Solvents. *ACS Catal.* **2013**, *3*, 2823–2836. [CrossRef]
79. Klibanov, A.M. Stabilization of Enzymes against Thermal Inactivation. *Adv. Appl. Microbiol.* **1983**, *29*, 1–28. [CrossRef] [PubMed]
80. Iyer, P.V.; Ananthanarayan, L. Enzyme Stability and Stabilization—Aqueous and Non-Aqueous Environment. *Process Biochem.* **2008**, *43*, 1019–1032. [CrossRef]
81. Garcia-Galan, C.; Berenguer-Murcia, Á.; Fernandez-Lafuente, R.; Rodrigues, R.C. Potential of Different Enzyme Immobilization Strategies to Improve Enzyme Performance. *Adv. Synth. Catal.* **2011**, *353*, 2885–2904. [CrossRef]
82. Bilal, M.; Asgher, M.; Cheng, H.; Yan, Y.; Iqbal, H.M.N. Multi-Point Enzyme Immobilization, Surface Chemistry, and Novel Platforms: A Paradigm Shift in Biocatalyst Design. *Crit. Rev. Biotechnol.* **2019**, *39*, 202–219. [CrossRef] [PubMed]
83. Wahab, R.A.; Elias, N.; Abdullah, F.; Ghoshal, S.K. On the Taught New Tricks of Enzymes Immobilization: An All-Inclusive Overview. *React. Funct. Polym.* **2020**, *152*, 104613. [CrossRef]
84. Bilal, M.; Zhao, Y.; Noreen, S.; Shah, S.Z.H.; Bharagava, R.N.; Iqbal, H.M.N. Modifying Bio-Catalytic Properties of Enzymes for Efficient Biocatalysis: A Review from Immobilization Strategies Viewpoint. *Biocatal. Biotransformation* **2019**, *37*, 159–182. [CrossRef]
85. El-Aziz, S.M.A.; Faraag, A.H.I.; Ibrahim, A.M.; Albrakati, A.; Bakkar, M.R. Tyrosinase Enzyme Purification and Immobilization from *Pseudomonas* sp. EG22 Using Cellulose Coated Magnetic Nanoparticles: Characterization and Application in Melanin Production. *World J. Microbiol. Biotechnol.* **2023**, *40*, 10. [CrossRef] [PubMed]
86. Lau, E.C.H.T.; Dodds, K.C.; McKenna, C.; Cowan, R.M.; Ganin, A.Y.; Campopiano, D.J.; Yiu, H.H.P. Direct Purification and Immobilization of His-Tagged Enzymes Using Unmodified Nickel Ferrite NiFe2O4 Magnetic Nanoparticles. *Sci. Rep.* **2023**, *13*, 21549. [CrossRef]
87. Zhou, L.; Ouyang, Y.; Kong, W.; Ma, T.; Zhao, H.; Jiang, Y.; Gao, J.; Ma, L. One Pot Purification and Co-Immobilization of His-Tagged Old Yellow Enzyme and Glucose Dehydrogenase for Asymmetric Hydrogenation. *Enzym. Microb. Technol.* **2022**, *156*, 110001. [CrossRef]

88. Zhou, J.; Chen, J.; Zhuang, N.; Zhang, A.; Chen, K.; Xu, N.; Xin, F.; Zhang, W.; Dong, W.; Jiang, M. Immobilization and Purification of Enzymes With the Novel Affinity Tag ChBD-AB From *Chitinolyticbacter meiyuanensis* SYBC-H1. *Front. Bioeng. Biotechnol.* **2020**, *8*, 544642. [CrossRef]
89. Ahmad, A.; Patta, A.M.; Natsir, H. Purification and Immobilization of L-Asparaginase Enzyme from the Thermophilic Bacteria *Bacillus licheniformis* Strain HSA3-1a. *Int. J. Pharma Bio Sci.* **2013**, *4*, B274–B280.
90. Rodrigues, R.C.; Virgen-Ortíz, J.J.; dos Santos, J.C.S.; Berenguer-Murcia, Á.; Alcantara, A.R.; Barbosa, O.; Ortiz, C.; Fernandez-Lafuente, R. Immobilization of Lipases on Hydrophobic Supports: Immobilization Mechanism, Advantages, Problems, and Solutions. *Biotechnol. Adv.* **2019**, *37*, 746–770. [CrossRef] [PubMed]
91. Manoel, E.A.; dos Santos, J.C.S.; Freire, D.M.G.; Rueda, N.; Fernandez-Lafuente, R. Immobilization of Lipases on Hydrophobic Supports Involves the Open Form of the Enzyme. *Enzym. Microb. Technol.* **2015**, *71*, 53–57. [CrossRef]
92. Kim, K.K.; Song, H.K.; Shin, D.H.; Hwang, K.Y.; Suh, S.W. The Crystal Structure of a Triacylglycerol Lipase from *Pseudomonas cepacia* Reveals a Highly Open Conformation in the Absence of a Bound Inhibitor. *Structure* **1997**, *5*, 173–185. [CrossRef] [PubMed]
93. Cygler, M.; Schrag, J.D. Structure and Conformational Flexibility of *Candida rugosa* Lipase. *Biochim. Et Biophys. Acta (BBA)-Mol. Cell Biol. Lipids* **1999**, *1441*, 205–214. [CrossRef]
94. Jaeger, K.-E.; Ransac, S.; Koch, H.B.; Ferrato, F.; Dijkstra, B.W. Topological Characterization and Modeling of the 3D Structure of Lipase from *Pseudomonas aeruginosa*. *FEBS Lett.* **1993**, *332*, 143–149. [CrossRef]
95. dos Santos, J.C.S.; Rueda, N.; Gonçalves, L.R.B.; Fernandez-Lafuente, R. Tuning the Catalytic Properties of Lipases Immobilized on Divinylsulfone Activated Agarose by Altering Its Nanoenvironment. *Enzym. Microb. Technol.* **2015**, *77*, 1–7. [CrossRef] [PubMed]
96. Rueda, N.; dos Santos, J.C.S.; Torres, R.; Ortiz, C.; Barbosa, O.; Fernandez-Lafuente, R. Improved Performance of Lipases Immobilized on Heterofunctional Octyl-Glyoxyl Agarose Beads. *RSC Adv.* **2015**, *5*, 11212–11222. [CrossRef]
97. Virgen-Ortíz, J.J.; Tacias-Pascacio, V.G.; Hirata, D.B.; Torrestiana-Sanchez, B.; Rosales-Quintero, A.; Fernandez-Lafuente, R. Relevance of Substrates and Products on the Desorption of Lipases Physically Adsorbed on Hydrophobic Supports. *Enzym. Microb. Technol.* **2017**, *96*, 30–35. [CrossRef] [PubMed]
98. Diamanti, E.; Arana-Peña, S.; Ramos-Cabrer, P.; Comino, N.; Carballares, D.; Fernandez-Lafuente, R.; López-Gallego, F. Intraparticle Macromolecular Migration Alters the Structure and Function of Proteins Reversibly Immobilized on Porous Microbeads. *Adv. Mater. Interfaces* **2022**, *9*, 2200263. [CrossRef]
99. Arana-Peña, S.; Rios, N.S.; Carballares, D.; Mendez-Sanchez, C.; Lokha, Y.; Gonçalves, L.R.B.; Fernandez-Lafuente, R. Effects of Enzyme Loading and Immobilization Conditions on the Catalytic Features of Lipase from *Pseudomonas fluorescens* Immobilized on Octyl-Agarose Beads. *Front. Bioeng. Biotechnol.* **2020**, *8*, 36. [CrossRef]
100. Zaak, H.; Siar, E.-H.; Kornecki, J.F.; Fernandez-Lopez, L.; Pedrero, S.G.; Virgen-Ortíz, J.J.; Fernandez-Lafuente, R. 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.* **2017**, *56*, 117–123. [CrossRef]
101. Fernandez-Lopez, L.; Pedrero, S.G.; Lopez-Carrobles, N.; Gorines, B.C.; Virgen-Ortíz, J.J.; Fernandez-Lafuente, R. Effect of Protein Load on Stability of Immobilized Enzymes. *Enzym. Microb. Technol.* **2017**, *98*, 18–25. [CrossRef] [PubMed]
102. Fernandez-Lopez, L.; Bartolome-Cabrero, R.; Rodriguez, M.D.; Dos Santos, C.S.; Rueda, N.; Fernandez-Lafuente, R. Stabilizing Effects of Cations on Lipases Depend on the Immobilization Protocol. *RSC Adv.* **2015**, *5*, 83868–83875. [CrossRef]
103. Kornecki, J.F.; Carballares, D.; Morellon-Sterling, R.; Siar, E.H.; Kashefi, S.; Chafiaa, M.; Arana-Peña, S.; Rios, N.S.; Gonçalves, L.R.B.B.; Fernandez-Lafuente, R. Influence of Phosphate Anions on the Stability of Immobilized Enzymes. Effect of Enzyme Nature, Immobilization Protocol and Inactivation Conditions. *Process Biochem.* **2020**, *95*, 288–296. [CrossRef]
104. Abellanas-Perez, P.; Carballares, D.; Fernandez-Lafuente, R.; Rocha-Martin, J. Glutaraldehyde Modification of Lipases Immobilized on Octyl Agarose Beads: Roles of the Support Enzyme Loading and Chemical Amination of the Enzyme on the Final Enzyme Features. *Int. J. Biol. Macromol.* **2023**, *248*, 125853. [CrossRef] [PubMed]
105. Abellanas-Perez, P.; Carballares, D.; Rocha-Martin, J.; Fernandez-Lafuente, R. The Nature of the Buffer Alters the Effects of the Chemical Modification on the Stability of Immobilized Lipases. *Process Biochem.* **2023**, *133*, 20–27. [CrossRef]
106. Abellanas-Perez, P.; Carballares, D.; Rocha-Martin, J.; Fernandez-Lafuente, R. The Effects of the Chemical Modification on Immobilized Lipase Features Are Affected by the Enzyme Crowding in the Support. *Biotechnol. Prog.* **2023**, *12*, e3394. [CrossRef] [PubMed]
107. Zucca, P.; Fernandez-Lafuente, R.; Sanjust, E. Agarose and Its Derivatives as Supports for Enzyme Immobilization. *Molecules* **2016**, *21*, 1577. [CrossRef] [PubMed]
108. Mangiagalli, M.; Ami, D.; Divitiis, M.; Brocca, S.; Catelani, T.; Natalello, A.; Lotti, M. Short-chain Alcohols Inactivate an Immobilized Industrial Lipase through Two Different Mechanisms. *Biotechnol. J.* **2022**, *17*, 2100712. [CrossRef]
109. Fernandez-Lafuente, R. Lipase from *Thermomyces lanuginosus*: Uses and Prospects as an Industrial Biocatalyst. *J. Mol. Catal. B Enzym.* **2010**, *62*, 197–212. [CrossRef]
110. Brzozowski, A.M.; Savage, H.; Verma, C.S.; Turkenburg, J.P.; Lawson, D.M.; Svendsen, A.; Patkar, S. Structural Origins of the Interfacial Activation in *Thermomyces (Humicola) lanuginosa* Lipase. *Biochemistry* **2000**, *39*, 15071–15082. [CrossRef]
111. Cajal, Y.; Svendsen, A.; Girona, V.; Patkar, S.A.; Alsina, M.A. Interfacial Control of Lid Opening in Thermomyces Lanuginosa Lipase. *Biochemistry* **2000**, *39*, 413–423. [CrossRef] [PubMed]

112. Anderson, E.M.; Larsson, K.M.; Kirk, O. One Biocatalyst—Many Applications: The Use of *Candida antarctica* B-Lipase in Organic Synthesis. *Biocatal. Biotransform.* **1998**, *16*, 181–204. [CrossRef]
113. Kirk, O.; Christensen, M.W. Lipases from *Candida antarctica*: Unique Biocatalysts from a Unique Origin. *Org. Process Res. Dev.* **2002**, *6*, 446–451. [CrossRef]
114. Martinelle, M.; Holmquist, M.; Hult, K. On the Interfacial Activation of *Candida antarctica* Lipase A and B as Compared with *Humicola lanuginosa* Lipase. *Biochim. Et Biophys. Acta-Lipids Lipid Metab.* **1995**, *1258*, 272–276. [CrossRef] [PubMed]
115. Volpato, G.; Filice, M.; Ayub, M.A.Z.; Guisan, J.M.; Palomo, J.M. Single-Step Purification of Different Lipases from Staphylococcus Warneri. *J. Chromatogr. A* **2010**, *1217*, 473–478. [CrossRef] [PubMed]
116. Fernández-Lorente, G.; Palomo, J.M.; Fuentes, M.; Mateo, C.; Guisán, J.M.; Fernández-Lafuente, R. Self-Assembly of *Pseudomonas fluorescens* Lipase into Bimolecular Aggregates Dramatically Affects Functional Properties. *Biotechnol. Bioeng.* **2003**, *82*, 232–237. [CrossRef] [PubMed]
117. Palomo, J.M.; Peñas, M.M.; Fernández-Lorente, G.; Mateo, C.; Pisabarro, A.G.; Fernández-Lafuente, R.; Ramírez, L.; Guisán, J.M. Solid-Phase Handling of Hydrophobins: Immobilized Hydrophobins as a New Tool to Study Lipases. *Biomacromolecules* **2003**, *4*, 204–210. [CrossRef] [PubMed]
118. Wang, P.; He, J.; Sun, Y.; Reynolds, M.; Zhang, L.; Han, S.; Liang, S.; Sui, H.; Lin, Y. Display of Fungal Hydrophobin on the *Pichia pastoris* Cell Surface and Its Influence on *Candida antarctica* Lipase B. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 5883–5895. [CrossRef]
119. Zhang, K.; Jin, Z.; Wang, P.; Zheng, S.P.; Han, S.Y.; Lin, Y. Improving the Catalytic Characteristics of Lipase-Displaying Yeast Cells by Hydrophobic Modification. *Bioprocess. Biosyst. Eng.* **2017**, *40*, 1689–1699. [CrossRef]
120. Ribitsch, D.; Guebitz, G.M. Tuning of Adsorption of Enzymes to Polymer. In *Methods in Enzymology*; Academic Press: Cambridge, MA, USA, 2021; pp. 293–315.
121. Rocha-Pino, Z.; Ramos-López, J.I.; Gimeno, M.; Barragán-Aroche, F.; Durán-Valencia, C.; López-Ramírez, S.; Shirai, K. Enhanced Oil Recovery by Hydrophobins from *Lecanicillium lecanii*. *Fuel* **2018**, *224*, 10–16. [CrossRef]
122. Bradford, M.M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
123. Lombardo, D.; Guy, O. Effect of Alcohols on the Hydrolysis Catalyzed by Human Pancreatic Carboxylic-Ester Hydrolase. *Biochim. Et Biophys. Acta (BBA)-Enzymol.* **1981**, *657*, 425–437. [CrossRef]
124. Hernandez, K.; Garcia-Verdugo, E.; Porcar, R.; Fernandez-Lafuente, R. Hydrolysis of Triacetin Catalyzed by Immobilized Lipases: Effect of the Immobilization Protocol and Experimental Conditions on Diacetin Yield. *Enzym. Microb. Technol.* **2011**, *48*, 510–517. [CrossRef] [PubMed]
125. Boudrant, J.; Woodley, J.M.; Fernandez-Lafuente, R. Parameters Necessary to Define an Immobilized Enzyme Preparation. *Process Biochem.* **2020**, *90*, 66–80. [CrossRef]