

Article

Tuning Almond Lipase Features by Using Different Immobilization Supports

Oumaima Cherni ^{1,2}, Diego Carballares ^{1,3} , El Hocine Siar ^{1,4} , Pedro Abellanas-Perez ¹ ,
Diandra de Andrade ^{1,5} , Javier Rocha-Martin ⁶ , Sellema Bahri ^{2,*} and Roberto Fernandez-Lafuente ^{1,*} 

- ¹ Department of Biocatalysis, Institute of Catalysis and Petrochemicals—Agencia Estatal Consejo Superior de Investigaciones Científicas Campus UAM-CSIC, 28049 Madrid, Spain; oumaimacherni96@gmail.com (O.C.); diego.carballares@csic.es (D.C.); hocines1@hotmail.fr (E.H.S.); pabellanaspez@csic.es (P.A.-P.); dcandrades@gmail.com (D.d.A.)
 - ² Department of Biology, Faculty of Science of Tunisia, University of Tunis-El-Manar, Tunis 2092, Tunisia
 - ³ Chemical and Materials Engineering Department, Faculty of Chemical Sciences, Complutense University of Madrid, Complutense Ave, 28040 Madrid, Spain
 - ⁴ Transformation and Food Product Elaboration Laboratory, Nutrition and Food Technology Institute (INATAA), University of Brothers Mentouri Constantine 1, Constantine 25017, Algeria
 - ⁵ Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Ribeirão Preto 14040-901, SP, Brazil
 - ⁶ Department of Biochemistry and Molecular Biology, Faculty of Biology, Complutense University of Madrid, José Antonio Novais 12, 28040 Madrid, Spain; javrocha@ucm.es
- * Correspondence: sellema.bahri@fst.utm.tn (S.B.); rfl@icp.csic.es (R.F.-L.)

Abstract: The lipase from *Prunus dulcis* almonds has been immobilized for the first time. For this purpose, two different supports, an octadecyl methacrylate particulate support, and aminated agarose (monoaminoethyl-N-aminoethyl) have been utilized. Both immobilized biocatalysts show improved enzyme stability, but great changes in enzyme specificity were detected. The enzyme immobilized via ion exchange maintained its activity intact versus *p*-nitrophenyl butyrate, while the enzyme immobilized on the hydrophobic support fully lost its activity versus this substrate, which was confirmed to be due to substrate adsorption on the support. However, this biocatalyst was much more active versus triacetin (more than 10-fold), *R*- or *S*-methyl mandelate at pH 7. At pH 9, a strong effect of using phosphate or bicarbonate as reaction buffers was detected. Using bicarbonate, the interfacially immobilized enzyme presented no activity versus *R*-isomer, but it was very active versus the *S*-isomer and triacetin. Using a phosphate buffer during the reaction, all compounds were recognized as substrates. The enzyme immobilized via ion exchange was significantly more active using phosphate; in fact, using bicarbonate, the enzyme was inactive versus both methyl mandelate isomers. This paper shows for the first time a great interaction between the effects of the immobilization protocol and buffer used during reaction on the enantiospecificity of lipases.

Keywords: lipase tuning by immobilization; lipase tuning by buffers; interfacially immobilized lipases; ionically exchanged lipase



Citation: Cherni, O.; Carballares, D.; Siar, E.H.; Abellanas-Perez, P.; de Andrade, D.; Rocha-Martin, J.; Bahri, S.; Fernandez-Lafuente, R. Tuning Almond Lipase Features by Using Different Immobilization Supports. *Catalysts* **2024**, *14*, 115. <https://doi.org/10.3390/catal14020115>

Academic Editors: Priscilla Amaral and Ivaldo Itabaiana Junior

Received: 18 December 2023

Revised: 22 January 2024

Accepted: 29 January 2024

Published: 31 January 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Lipases are among the most utilized enzymes in both academic and applied biocatalysis [1–6]. This is due to their robustness, lack of cofactors and wide specificity, and ability to accept a wide variety of substrates. Their high stability has enabled the use of lipases in a wide variety of reaction media (e.g., aqueous [7,8], organic solvents [2,9], supercritical fluids [10,11], ionic liquids [12–16], eutectic solvents [17,18], solvent-free systems [19]), and their variety of substrates permits to use them in a diversity of industrial areas [20] (wastewater treatment [21], food [22–24], energy [25,26], cosmetic [27], pharmaceutical [28–30], fine chemistry [31–36]). They can be used in hydrolysis [7,8], acidolysis [37,38], interesterifications [39,40], esterifications [19,41,42], transesterifications [25,43], amidations [44–46], etc.

Moreover, they are the most utilized enzymes in promiscuous reactions [47,48]. Due to this interest and diversity of applications, there are intense searches to find lipases that can fulfil the requirements of each specific application [49,50].

All lipases have a common mechanism of action: they are able to act in the interface of drops of oils, becoming “interfacial enzymes” [51,52]. This is due to their peculiar mechanism of action, called interfacial activation. They become adsorbed on hydrophobic surfaces via a huge hydrophobic pocket that surrounds the active site [53–58].

These enzymes would not be soluble in water, but they usually have the hydrophobic active site isolated from the medium, blocked by a mobile polypeptide chain whose external face is hydrophilic, while the internal one is hydrophobic, and can interact with the hydrophobic areas surrounding the active site (called lid or flap) [53–55]. In this situation, the substrate cannot reach the active site, and the enzyme is inactive. However, this lid is in conformational equilibrium with a form where the lid is shifted and the active site becomes exposed, the so-called open form, which is the active lipase form [53–55]. When a hydrophobic surface, like a drop of oil, is present, the open form of the lipase is adsorbed onto this hydrophobic surface. This trend of lipases to become adsorbed on hydrophobic surfaces can generate some problems for lipase characterization, as they can become adsorbed on other hydrophobic components of the medium such as other open lipases [59,60] or hydrophobic components in the crude mixture [61]. This aggregates formation changes the enzyme features, making understanding of the results using free enzymes quite hard. For this reason, we have not utilized the free enzyme in these studies. Some lipases have a very small lid that cannot fully isolate the active center, but they are still able to become adsorbed on hydrophobic surfaces, with the lipase B from *Candida antarctica* being one outstanding example [53,62]. However, this lipase still is considered to have interfacial activation mechanism of action [63–65].

One of the lipases sources that have been intensively explored are those contained in oleaginous plants seeds [66–74]. It can be assumed that the plant embryo should have the capability to degrade and consume the oils contained in the seed. Among these seeds, the *Prunus dulcis* almonds (also known as *Amygdalus communis*) may be of interest. However, we have been able to find just one paper devoted to this enzyme [75]. This paper describes this enzyme as a stable lipase (optimal temperature in soybean hydrolysis was 65 °C) and highlights its potential for a diverse range of applications.

Enzyme immobilization is a technique initially developed for the recovery and reuse of enzymes, which initially were very expensive biocatalysts. Moreover, it simplifies the control of the reactor and the downstream of the product [76–78]. Nowadays, with the improvements in the techniques for lipases production, the cost of the enzyme may no longer serve as a comprehensive justification for enzyme immobilization [26,79–81]. However, it should be considered that enzyme immobilization has shown to be able to improve many enzyme features [82–84]. Stability may be improved by multipoint [85–87] or multi-subunit immobilization [88], along with the other reasons recently reviewed [89]. The enzyme may be purified during the process of immobilization, if properly designed, and this can bypass any necessity for further enzyme purification, saving effort and expense [90]. Moreover, enzyme activity, selectivity, specificity, and inhibition may be tuned, and in some instances, convert an apparently inefficient enzyme into a very effective one [82–84]. The use of immobilization techniques to improve the enzyme specificity and selectivity has been reported in many instances [91–104].

That way, even with the decrease in the enzyme price, enzyme immobilization can become relevant in the development of industrial biocatalysts [26,80], but it is far from being a mature discipline [105].

We have not found any published study regarding the immobilization of this lipase. In this paper, we have analyzed the immobilization of the enzyme on two different supports. On of the used methods utilizes aminated supports [106] for immobilizing the enzyme through multipoint ion exchange [107]. The second one is a hydrophobic support where the lipase becomes immobilized via interfacial activation [108]. This immobilization method

is among the most utilized ones in the literature, and it is expected to permit the one-step lipase immobilization, stabilization, purification, and hyperactivation [109]. The effects of enzyme immobilization on enzyme activity, stability, and specificity with different substrates at different pH values have also been analyzed. Moreover, the effect of the buffers on the lipase features have also been analyzed, as it has been shown that they can tune some enzyme features, either when they are present [110–112] or when used in the immobilization solution [113].

2. Results

2.1. Immobilization of Lipase from Almond on Different Supports

Figure 1 shows the lipase immobilization course on MANAE-agarose. Immobilization is quite fast: in 15 min, only around 10% of the initial activity remained in the supernatant. The *p*-NPB enzyme activity is maintained after enzyme immobilization, suggesting that the enzyme structure is fully maintained after the immobilization. In lipase immobilization via ion exchange, drastic changes in enzyme activity are not anticipated, and although a multipoint ionic exchange is required, each bond is relatively weak.

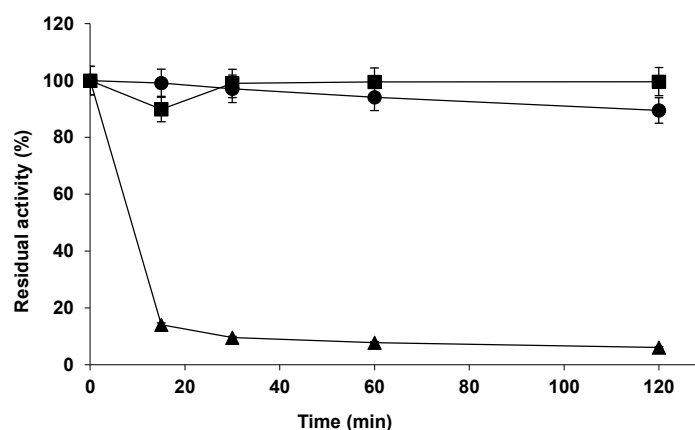


Figure 1. Almond lipase immobilization course on MANAE-agarose beads. The experiment was performed in 5 mM of glycine buffer at pH 7.0 and 4 °C. Other specifications can be found in the Materials and Methods sections. Solid triangles: supernatant; solid squares: suspension; and solid circles: reference.

Figure 2 shows the lipase immobilization course on hydrophobic Purolite. Immobilization is slower than using MANAE-agarose, which deviates from the typical behavior observed in other lipases immobilized on hydrophobic supports, where immobilization has traditionally been characterized by an extremely rapid rate [108]. However, the most relevant point is that the enzyme apparently lost almost all activity after immobilization on this support. This is unexpected, as it is expected that the enzyme can increase its activity because the open form of the lipase should be stabilized after this immobilization [108]. However, similar results have been previously reported using this support and other lipases [114]. To check if the problem was the adsorption of substrate or product on the support [84] (possible due to the very low concentration of the substrate and the small volumes used), the enzyme immobilized on MANAE-agarose was assayed after mixing the substrate with Purolite support. A very significant decrease on immobilized enzyme activity was found. To confirm this effect, other immobilized lipases were utilized, and in all cases, a drastic decrease on enzyme activity was detected when hydrophobic Purolite was added to the reaction, which was higher when the amount of Purolite particles was higher. This is a clear example where an artifact can lead to wrong conclusions, and using very low amounts of substrate the adsorption of the substrate to the support may become a problem to determinate the activity of an immobilized enzyme [84]. Apparently, the immobilization on this support fully inactivated the enzyme, and this method may be

discarded as immobilization protocol for this specific enzyme, but this may be a mistake if this “inactivation” is not fully confirmed.

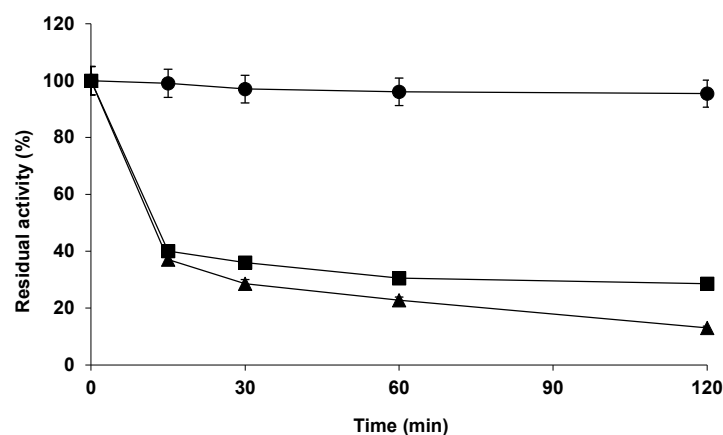


Figure 2. Almond lipase immobilization course on octadecyl Purolite beads. The experiment was performed using 5 mM of Gly buffer at pH 7.0 and 4 °C. Other specifications can be found in the Materials and Methods sections. Solid triangles: supernatant; solid squares: suspension; and solid circles: reference.

To assert if the enzyme immobilized on hydrophobic Purolite presented activity versus other substrates, that is, if the enzyme was not really inactivated, the activities of both biocatalysts were determined using triacetin at pH 7. The activity of hydrophobic Purolite biocatalyst was almost 11 U/g of support, while for the MANAE-agarose biocatalyst, the activity was under 1 U/g of support. Considering the similar amount of enzyme that was immobilized on both supports, this means that the activity versus triacetin was multiplied by more than one order of magnitude after immobilization on the hydrophobic support. That way, the enzyme immobilized on hydrophobic Purolite presented an activity versus triacetin much higher than the enzyme immobilized via ion exchange, as may be expected of a lipase with the open form stabilized versus the support.

Without considering the artifacts, the apparent enzyme specificity undergoes a substantial change upon immobilization. The enzyme immobilized on the hydrophobic support exhibits no activity towards *p*-NPB but displays high activity against triacetin. Considering the artifacts, this shows that the real effect is the decrease of available *p*-NPB, and that we can discard the use of *p*-NPB to follow the activity of lipases immobilized on these supports.

2.2. Stability of the Different Biocatalysts of Lipase from Almonds

Figure 3 shows the inactivation courses of the enzyme immobilized via ion exchange or interfacial activation in glycine at pH 7 and using triacetin as substrate. The inactivation was performed at 35 °C to achieve reliable inactivation courses. At 30 °C, the enzyme remained fully active for 24 h, while at 40 °C, inactivation was too fast. Both immobilized biocatalysts exhibited similar stabilities and were more stable than the free enzyme. That way, regarding the enzyme stability, the immobilization via interfacial activation on this support did not improve the enzyme stability more than the immobilization via ion exchange, although it has been reported that this interfacially activated lipase form should be more stable than the lipase in the conformational equilibrium [115–117].

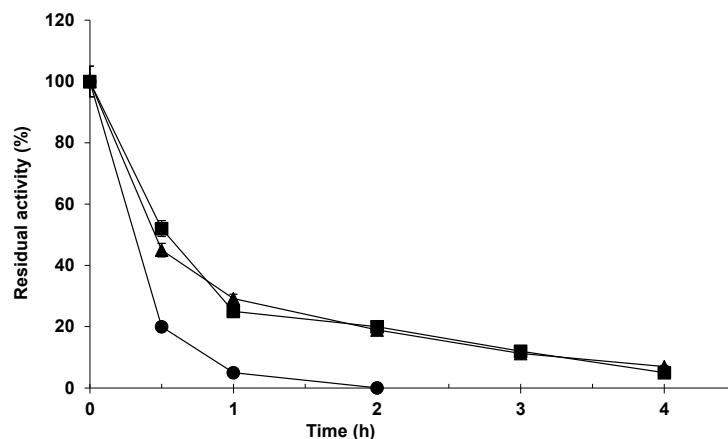


Figure 3. Inactivation course of different lipase preparations. Activity was determined using triacetin as indicated in the Methods section. The inactivation was performed using 50 mM of glycine buffer at pH 7.0 and 35 °C. Other specifications can be found in the Materials and Methods section. Solid circles: free enzyme; solid squares: MANAE-lipase; and solid triangles: Purolite-lipase.

2.3. Activity of the Different Almond Lipase Biocatalysts versus Different Substrates under Different Experimental Conditions

Next, with the aim to investigate possible changes on enzyme specificity caused by the enzyme immobilization, the activities of both biocatalysts versus both enantiomers of methyl mandelate and triacetin under different conditions were analyzed (Table 1). Free enzyme was not assayed due to the tendency of lipases to form aggregates that make the properties of the enzyme depend on the composition of the crude, the concentration of the lipase, etc. [59,60]. At pH 5, the enzyme stability even after immobilization is compromised, and only some activity could be detected using triacetin and the interfacially activated enzyme. Thus, we focused our studies at pH 7 and 9.

Table 1. Hydrolytic activity of the different immobilized lipase biocatalyst versus triacetin and *R*- or *S*-methyl mandelate in different conditions using lipase immobilized on different supports. Activity is given in U/g of biocatalyst. The experiments were performed as described in the Materials and Methods section.

Phosphate Buffer, pH 7			
Supports	Substrates		
	<i>R</i> -methyl mandelate	<i>S</i> -methyl mandelate	Triacetin
Purolite	4.75 ± 0.30	4.62 ± 0.34	10.81 ± 0.97
MANAE	0.16 ± 0.02	0.27 ± 0.02	0.95 ± 0.03
Bicarbonate buffer, pH 9			
Purolite	0	16.43 ± 0.89	2.09 ± 0.37
MANAE	0	0	6.33 ± 0.23
Phosphate buffer, pH 9			
Purolite	13.57 ± 0.93	11.7 ± 0.34	31.39 ± 1.29
MANAE	0.23 ± 0.02	0.24 ± 0.01	11.7 ± 0.74

At pH 7, results were very different depending on the biocatalyst. Lipase-hydrophobic Purolite more than doubled the activity versus triacetin when compared with the activity versus both isomers of methyl mandelate, which were hydrolyzed at similar rates. The activity of the enzyme immobilized on MANAE-agarose was much lower with the 3 substrates, and the difference in activities recorded using each substrate was clearly different. The enzyme preferred the *S*-isomer versus the *R*-isomer by a factor of almost 1.7, while the activity versus triacetin was 6-fold higher than versus *R*-methyl mandelate.

However, the most important result was obtained at pH 9. Using a bicarbonate buffer in the reaction, the lipase that was immobilized via ion exchange only exhibited activity

versus triacetin, completely losing the activity versus both isomers of methyl mandelate. However, the activity versus triacetin was more than 6-fold higher than at pH 7. In this way, the loss of activity versus the mandelic esters is not related to enzyme inactivation. The situation using the enzyme interfacially activated versus the hydrophobic Purolite was even more surprising. The activity versus triacetin was 5-fold lower than at pH 7. In fact, using triacetin, at pH 7 the lipase-hydrophobic Purolite was 11-fold more active than the lipase-MANAE-agarose, while at pH 9, it was around 3-fold less active. This biocatalyst was also fully inactive in the hydrolysis of *R*-methyl mandelate; however, it was almost 4-fold more active versus the *S*-isomer than at pH 7. As such, at pH 9, this biocatalyst seemed to be a very recommendable one for the resolution of racemic mixtures of *R/S* methyl mandelate, while at pH 7, it seemed completely inefficient. This suggests that the pH can exert drastic changes in enzyme conformations, and that these changes can be modulated by the immobilization support.

Considering previous reports that highlighted the importance of the buffer on the features of lipases immobilized via interfacial activation [51,52], the activity versus these 3 substrates at pH 9 was also determined using a phosphate buffer. Results changed drastically. Lipase-MANAE showed a significant activity versus both enantiomers of methyl mandelate, similar to the activity detected at pH 7, but without significant differences using both isomers. Using triacetin, now the activity doubled its value compared to the value observed using a bicarbonate buffer in the reaction (already much higher than at pH 7). Lipase-hydrophobic Purolite also increased its activity compared to the values observed in bicarbonate, but to a much higher extension; an improvement factor of almost 15 may be detected in its activity versus triacetin. Now, the biocatalyst presented similar activities with *R*- and *S*-methyl mandelate, even slightly higher using the *R*-isomer. These results showed the great effect of the buffers on the final properties of the immobilized enzyme. The medium composition seemed to alter the final conformation of the lipase, giving lipase forms with different activity and specificity, as has been shown on the immobilization of lipases via interfacial activation on hydrophobic supports using different medium composition present [110–112].

Phosphate at pH 9 seems to be able to produce enzyme forms with higher enzyme activities, but lower enzyme discrimination between the different substrates is another consequence of this conformational change caused by the buffer. From our knowledge, this is the more drastic effect of the buffer on enzyme specificity ever detected (from almost null to almost infinite). Moreover, this is also the first time a clear dependence on both the immobilization protocol and buffer conditions is shown in influencing the characteristics of the enzyme. This synergy can have very implications when designing biocatalysts for industrial purposes.

The activity values detected with mandelic esters are higher than those found using the lipase from *Thermomyces lanuginosus*, *Rhizomucor miehei*, or lipase A from *Candida antarctica* immobilized on octyl-agarose (even several magnitude order in some instances) [113]. The lipase B from *Candida antarctica* immobilized on octyl-agarose has a higher activity than that of the almond lipase versus mandelic esters [113]. Using triacetin, the activity is comparable to the activity of these enzymes, depending on the experimental conditions [113]. However, the drastic changes caused by the synergy between enzyme immobilization protocols and experimental conditions are among the highest reported in the literature, as we have found just one report totally nullifying the activity versus one of the isomers of methyl mandelate via immobilization and medium engineering [118].

3. Material and Methods

3.1. Materials

Triacetin and *p*-nitrophenyl butyrate (*p*-NPB) were obtained from GE Healthcare (Spain). *R*- and *S*-methyl mandelate were from Alfa Aesar (Fisher Scientific, Madrid, Spain). All reagents and solvents were of analytical grade. Agarose 4 BCL support was acquired from Agarose Bead Technologies (ABT), Madrid, Spain. Monoaminoethyl-N-aminoethyl

(MANAE)-support was produced using glyoxyl agarose beads [119,120] with a slight modification of the protocol previously described [121]. Purolite Lifetech™ ECR8804M (octadecyl methacrylate) was kindly gifted by Purolite Ltd. (Wales, UK). Purolite was treated as described by Tacias-Pascacio et al. [114,122] to fill the pores with water. A total of 10 g of Purolite beads was added to 100 mL of methanol and maintained under mild, continuous agitation for 1 h to eliminate the air inside the particles. Next, 100 mL of distilled water was added, and the suspension was stirred for 15 min. Afterward, the suspensions were vacuum filtered, washed with excess of distilled water, and stored at 4–6 °C.

3.2. Methods

All experiments have been performed by triplicate (at least), and results are given as mean values together with their respective standard deviations.

3.2.1. Preparation of Lipase Extract

Almonds were collected in Sfax in the South of Tunisia in July 2023 and used in September/October. After 3 days of germination, the radicles were discarded, and the cotyledons were collected. Extraction was performed using the method described by Bahri [123], with slight modifications. The extraction was carried out at 4 °C (significant activity losses were observed at room temperature probably due to the action of the various proteolytic enzymes released during extraction). A volume of 30 mL of 50 mM glycine buffer at pH 7 containing 1 mM benzamidine and 1 mM dithiothreitol was added to 10 g of the material previously collected, which were crushed in a porcelain mortar at 4 °C. The suspension was vacuum filtered using a sintered filter 3, and the solid was discarded. The obtained supernatant was centrifuged at $10,000\times g$ for 20 min at 4 °C. The insoluble material was discarded, and the supernatant (25 mg protein/mL) was retained, stored at 4–6 °C, and utilized further.

The protein concentration was quantified by the method reported by Bradford [124]. BSA was used as the standard protein.

3.2.2. Determination of the Enzyme Activity Using Different Substrates

Hydrolysis of *p*-NPB

This assay was employed to spectrophotometrically quantify the activity of the enzyme by determining the augmentation in absorbance at 348 nm (isobestic point of pNP) [125] produced by the pNP that is released after the hydrolysis of 0.4 mM *p*-NPB in 25 mM sodium phosphate at pH 7.0 and 25 °C. The reaction was started by the addition of 200 µL of lipase suspension or solution to 2.3 mL of substrate solution. One international unit of activity (U) was defined as the amount of enzyme that hydrolyses 1 µmol of *p*-NPB per minute under the above conditions.

Hydrolysis of Triacetin

A mass of 0.2 g of wet biocatalysts (lipase immobilized on MANAE or Purolite) was added to 2 mL of 50 mM of triacetin in 50 mM sodium phosphate solution at 25 °C and pH 7.0, under incessant stirring. The produced 1,2 diacetin suffers acyl migration under these conditions, producing a mixture with 1,3 diacetin [126]. The determination of the reaction progress was determined using a HPLC Kromasil C18 column of (15 cm \times 0.46 cm) with a UV detector at 230 nm. The mobile phase was a solution of 15% acetonitrile–85% Milli-Q water and the flow rate was 1 mL/min. At conversion degrees between 15 and 20%, the production of 1,2 diacetin and 1,3 diacetin (both products co-eluted) was determined. The retention times of the compounds were 18 min for the triacetin and 4 min for the diacetins.

R- or *S*-Methyl Mandelate Hydrolysis

To 2–3 mL of substrate solution at 25 °C, 0.2–0.3 g of wet biocatalyst was added. The suspension was subjected to continuous stirring. The reaction was performed using 10 mM of *R*- or *S*-methyl mandelate in 50 mM of sodium phosphate at pH 7.0. The product was

identified by using a HPLC using a Kromasil C18 column (15 cm × 0.46 cm). The mobile phase was a solution of 35% acetonitrile-65% 10 mM ammonium acetate in Milli-Q water at pH 2.8 with a flow of 1 mL/min. A UV/VIS detector at 230 nm was utilized, with the retention times being 2.4 min for mandelic acid and 4.2 min for methyl mandelate. The initial reaction rates were calculated using conversions between 15 and 20%.

3.2.3. Immobilization of Lipase

The immobilizations were followed by determining the activities of supernatant, suspension, and a reference of the enzyme incubated under identical conditions but in absence of support [84]. Lipase immobilizations were carried out using 25 mg of enzyme/g of wet support, diluting the enzyme solution with distilled water. Immobilizations were performed at 4 °C. The stock enzyme solution was prepared in 50 mM Gly.

The support/enzyme solution had a relation of 1 g/10 mL of the enzyme solution. The activity was determined employing *p*-NPB. After immobilization, the biocatalysts were washed with excess of distilled water, vacuum dried, and stored at 4 °C.

3.2.4. Thermal Inactivations of the Biocatalysts

Biocatalysts stabilities were determined using triacetin or *S*-mandelate as substrates. A total of 0.2 g of immobilized enzymes was incubated in 2 mL of 50 mM Gly buffer at pH 7.0 and 35 °C. At the indicated times, samples were taken, and their activities were determined using triacetin and *S*-mandelate as substrates, as described above. Residual activities were calculated as a percentage of the initial activity and half-lives were calculated using the inactivation curves.

4. Conclusions

The lipase from *Prunus dulcis* almonds is a poorly studied enzyme that had never been immobilized before this paper; however, it may be an interesting one. For example, it is a very tunable enzyme via immobilization. It also shows one of the strongest modulations of enzyme specificity via immobilization. Its immobilization via physical adsorption (interfacial activation or ion exchange) improves enzyme stability, but the most interesting effects are the changes on enzyme specificity. The immobilization on hydrophobic Purolite greatly decreases the activity of the enzyme versus *p*-NPB, whereas with MANAE, the activity is fairly preserved. This effect was shown to be caused by an artifact where the substrate becomes adsorbed on the hydrophobic support.

The interfacially activated enzyme is much more active than the enzyme ionically exchanged using triacetin or both isomers of methyl mandelate as substrates at pH 7. At pH 9, an intense effect from the buffer was found. While using bicarbonate, the interfacially activated enzyme was less active using triacetin than the ionically exchanged enzyme, both biocatalysts were fully inactive using *R*-methyl mandelate, and the ionically exchanged enzyme was not active neither with *S*-methyl mandelate. However, the interfacially activated enzyme was active versus the *S*-isomer, in fact, far more active than at pH 7 using this substrate. Using phosphate at pH 9, both biocatalysts were active versus all substrates. This shows for first time the combined effects of enzyme immobilization protocols, pH values, and buffers in the specificity of immobilized lipases. This means that in the biocatalysts and process design and optimization, these synergic effects must be considered. We have not found similar effects in the literature, and the ones found in this paper are very drastic, but if this is a general case, then an enzyme or medium may be discarded for a specific reaction using a kind of immobilized enzyme and become a very suitable one using another immobilization technique. Considering the outcomes with other biocatalysts of a specific enzyme, it is not advisable to assume any granted influence of the medium on the properties of the immobilized enzyme.

The easy change of the enzyme catalytic features makes this enzyme very interesting for possible applications, while the lack of specific research makes it difficult to predict the

potential of this enzyme for industrial applications (not the subject of this paper). For these industrial applications, economic balances, patent issues, etc., should be considered.

For industrial applications, the moderate stability of the enzyme even after immobilization makes it necessary to apply stabilization strategies to this enzyme (e.g., immobilization protocols that can stabilize the enzyme, further chemical modifications, etc.). The use of the enzyme biocatalysts in other reaction media (solvent free, organic solvents, ionic liquids, etc.) and applications (esterifications, transesterifications, etc.) described in the introduction should be also analyzed.

Author Contributions: O.C., D.C., E.H.S. and P.A.-P., formal analysis, investigation, writing review and editing. D.d.A., S.B. and J.R.-M. data curation, writing—original draft preparation; R.F.-L. Conceptualization, data curation supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: Ministerio de Ciencia e Innovación and Agencia Estatal de Investigación (Spanish Government) (PID2022-136535OB-I00).

Data Availability Statement: Data are contained within the article.

Acknowledgments: The authors gratefully acknowledge FAPESP (São Paulo Research Foundation) by research scholarship to DA (Grant No: 2023/01338-7). Oumaima Cherni thanks the Tunisian Ministry of Higher Education and Scientific Research for her fellowship. The help and suggestions from Ángel Berenguer (Departamento de Química Inorgánica, Universidad de Alicante) are gratefully recognized.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Kumar, A.; Verma, V.; Dubey, V.K.; Srivastava, A.; Garg, S.K.; Singh, V.P.; Arora, P.K. Industrial applications of fungal lipases: A review. *Front. Microbiol.* **2023**, *14*, 1142536. [\[CrossRef\]](#)
2. Ismail, A.R.; Kashtoh, H.; Baek, K.H. Temperature-resistant and solvent-tolerant lipases as industrial biocatalysts: Biotechnological approaches and applications. *Int. J. Biol. Macromol.* **2021**, *187*, 127–142. [\[CrossRef\]](#)
3. Ali, S.; Khan, S.A.; Hamayun, M.; Lee, I.J. The Recent Advances in the Utility of Microbial Lipases: A Review. *Microorganisms* **2023**, *11*, 510. [\[CrossRef\]](#)
4. dos Santos, L.N.; Perna, R.F.; Vieira, A.C.; de Almeida, A.F.; Ferreira, N.R. Trends in the Use of Lipases: A Systematic Review and Bibliometric Analysis. *Foods* **2023**, *12*, 3058. [\[CrossRef\]](#)
5. Akram, F.; Mir, A.S.; ul Haq, I.; Roohi, A. An Appraisal on Prominent Industrial and Biotechnological Applications of Bacterial Lipases. *Mol. Biotechnol.* **2023**, *65*, 521–543. [\[CrossRef\]](#)
6. 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. [\[CrossRef\]](#)
7. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Murty, V.R.; Bhat, J.; Muniswaran, P.K.A. Hydrolysis of oils by using immobilized lipase enzyme: A review. *Biotechnol. Bioprocess Eng.* **2002**, *7*, 57–66. [\[CrossRef\]](#)
9. Kumar, A.; Dhar, K.; Kanwar, S.S.; Arora, P.K. Lipase catalysis in organic solvents: Advantages and applications. *Biol. Proced. Online* **2016**, *18*, 2. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Fan, Y.; Qian, J. Lipase catalysis in ionic liquids/supercritical carbon dioxide and its applications. *J. Mol. Catal. B Enzym.* **2010**, *66*, 1–7. [\[CrossRef\]](#)
11. Dias, A.L.B.; dos Santos, P.; Martínez, J. Supercritical CO₂ technology applied to the production of flavor ester compounds through lipase-catalyzed reaction: A review. *J. CO₂ Util.* **2018**, *23*, 159–178. [\[CrossRef\]](#)
12. Šibalić, D.; Šalić, A.; Zelić, B.; Tran, N.N.; Hessel, V.; Nigam, K.D.P.; Tišma, M. Synergism of ionic liquids and lipases for lignocellulosic biomass valorization. *Chem. Eng. J.* **2023**, *461*, 142011. [\[CrossRef\]](#)
13. Alvarez, E.; Rodriguez, J.; Villa, R.; Gomez, C.; Nieto, S.; Donaire, A.; Lozano, P. Clean enzymatic production of flavor esters in spongelike ionic liquids. *ACS Sustain. Chem. Eng.* **2019**, *7*, 13307–13314. [\[CrossRef\]](#)
14. Lozano, P.; Villa, R.; Nieto, S.; Donaire, A.; García-Verdugo, E. Clean biocatalysis in sponge-like ionic liquids. In *Biocatalysis in Green Solvents*; Academic Press: Cambridge, MA, USA, 2022; pp. 155–182. [\[CrossRef\]](#)
15. Nieto, S.; Villa, R.; Donaire, A.; Lozano, P. Ultrasound-assisted enzymatic synthesis of xylitol fatty acid esters in solvent-free conditions. *Ultrason. Sonochem.* **2021**, *75*, 105606. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Elgharbawy, A.A.; Riyadi, F.A.; Alam, M.Z.; Moniruzzaman, M. Ionic liquids as a potential solvent for lipase-catalysed reactions: A review. *J. Mol. Liq.* **2018**, *251*, 150–166. [\[CrossRef\]](#)

17. Tan, J.-N.; Dou, Y. Deep eutectic solvents for biocatalytic transformations: Focused lipase-catalyzed organic reactions. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 1481–1496. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Durand, E.; Lecomte, J.; Villeneuve, P. Deep eutectic solvents: Synthesis, application, and focus on lipase-catalyzed reactions. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 379–385. [\[CrossRef\]](#)
19. 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. [\[CrossRef\]](#)
20. Liu, Y.Q.; WeiZhuo, X.; Wei, X. A review on lipase-catalyzed synthesis of geranyl esters as flavor additives for food, pharmaceutical and cosmetic applications. *Food Chem. Adv.* **2022**, *1*, 100052. [\[CrossRef\]](#)
21. Nimkande, V.D.; Bafana, A. A review on the utility of microbial lipases in wastewater treatment. *J. Water Process Eng.* **2022**, *46*, 102591. [\[CrossRef\]](#)
22. 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. [\[CrossRef\]](#)
23. Cheng, W.; Nian, B. Computer-Aided Lipase Engineering for Improving Their Stability and Activity in the Food Industry: State of the Art. *Molecules* **2023**, *28*, 5848. [\[CrossRef\]](#) [\[PubMed\]](#)
24. 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. [\[CrossRef\]](#)
25. Moya Joëlle Carole, A.; Konan Edmond, K.; Abollé, A.; Esaie Kouadio Appiah, K.; Kouassi Benjamin, Y. Transesterification of vegetable oils into biodiesel by an immobilized lipase: A review. *Biofuels* **2023**, *14*, 1087–1101. [\[CrossRef\]](#)
26. Monteiro, R.R.C.; Arana-Peña, S.; da Rocha, T.N.; Miranda, L.P.; Berenguer-Murcia, Á.; Tardioli, P.W.; dos Santos, J.C.S.; Fernandez-Lafuente, R. Liquid lipase preparations designed for industrial production of biodiesel. Is it really an optimal solution? *Renew. Energy* **2021**, *164*, 1566–1587. [\[CrossRef\]](#)
27. Alvarez, E.; Villa, R.; Nieto, S.; Donaire, A.; García-Verdugo, E.; Luis, S.V.; Lozano, P. The Suitability of Lipases for the Synthesis of Bioactive Compounds with Cosmeceutical Applications. *Mini-Rev. Org. Chem.* **2020**, *18*, 515–528. [\[CrossRef\]](#)
28. Wang, H.; Zhang, Q.; Yu, X.; Liang, J.; Zhang, Y.; Jiang, Y.; Su, W. Application of Lipase B from *Candida antarctica* in the Pharmaceutical Industry. *Ind. Eng. Chem. Res.* **2023**, *62*, 15733–15751. [\[CrossRef\]](#)
29. Pinto, G.B.; Mendes, F.M.L.; Antunes, A.M.d.S. Technological Profile of Lipases in the Pharmaceutical Industry. *Mini-Rev. Org. Chem.* **2019**, *17*, 701–716. [\[CrossRef\]](#)
30. Verma, S.; Choudhary, R.N.; Kanadje, A.P.; Banerjee, U.C. Diversifying arena of drug synthesis: In the realm of lipase mediated waves of biocatalysis. *Catalysts* **2021**, *11*, 1328. [\[CrossRef\]](#)
31. Patti, A.; Sanfilippo, C. Stereoselective Promiscuous Reactions Catalyzed by Lipases. *Int. J. Mol. Sci.* **2022**, *23*, 2675. [\[CrossRef\]](#)
32. Budhiraja, M.; Ali, A.; Tyagi, V. Recent Advances in Lipase Catalyzed Multicomponent Reactions to Synthesize N-Heterocycles. *Asian J. Org. Chem.* **2023**, *12*, e202300427. [\[CrossRef\]](#)
33. Monteiro, R.R.C.; Berenguer-Murcia, Á.; Rocha-Martin, J.; Vieira, R.S.; Fernandez-Lafuente, R. Biocatalytic production of biolubricants: Strategies, problems and future trends. *Biotechnol. Adv.* **2023**, *68*, 108215. [\[CrossRef\]](#)
34. Bolina, I.C.A.; Gomes, R.A.B.; Mendes, A.A. Biolubricant Production from Several Oleaginous Feedstocks Using Lipases as Catalysts: Current Scenario and Future Perspectives. *Bioenergy Res.* **2021**, *14*, 1039–1057. [\[CrossRef\]](#)
35. Vilas Bôas, R.N.; Castro, H.F. A review of synthesis of esters with aromatic, emulsifying, and lubricant properties by biotransformation using lipases. *Biotechnol. Bioeng.* **2022**, *119*, 725–742. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Serra, S.; De Simeis, D. Stereoselective Synthesis of Terpenoids through Lipase-Mediated Resolution Approaches. *Catalysts* **2020**, *10*, 504. [\[CrossRef\]](#)
37. Zou, X.; Su, H.; Zhang, F.; Zhang, H.; Yeerbolati, Y.; Xu, X.; Chao, Z.; Zheng, L.; Jiang, B. Bioimprinted lipase-catalyzed synthesis of medium- and long-chain structured lipids rich in docosahexaenoic acid for infant formula. *Food Chem.* **2023**, *424*, 136450. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Yoshioka, T.; Kuroiwa, T.; Wakahara, K.; Ozono, S. Accelerated acidolysis for triglyceride modification using commercial lipases activated by a hydration-aggregation pretreatment. *Process Biochem.* **2023**, *132*, 130–139. [\[CrossRef\]](#)
39. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
40. 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. [\[CrossRef\]](#)
41. 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. [\[CrossRef\]](#)
42. Cha, H.J.; Park, J.B.; Park, S. Esterification of secondary alcohols and multi-hydroxyl compounds by *Candida antarctica* lipase B and subtilisin. *Biotechnol. Bioprocess Eng.* **2019**, *24*, 41–47. [\[CrossRef\]](#)
43. Toldrá-Reig, F.; Mora, L.; Toldrá, F. Developments in the use of lipase transesterification for biodiesel production from animal fat waste. *Appl. Sci.* **2020**, *10*, 5085. [\[CrossRef\]](#)
44. Gotor, V. Non-conventional hydrolase chemistry: Amide and carbamate bond formation catalyzed by lipases. *Bioorg. Med. Chem.* **1999**, *7*, 2189–2197. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Sun, M.; Nie, K.; Wang, F.; Deng, L. Optimization of the Lipase-Catalyzed Selective Amidation of Phenylglycinol. *Front. Bioeng. Biotechnol.* **2020**, *7*, 486. [\[CrossRef\]](#) [\[PubMed\]](#)

46. Nian, B.; Liao, G.; Song, Y.; Su, Y.; Cao, C.; Liu, Y. Ionic hydrogen-bonding interaction controlled electrophilicity and nucleophilicity: Mechanistic insights into the synergistic catalytic effect of lipase and natural deep eutectic solvents in amidation reaction. *J. Catal.* **2020**, *384*, 159–168. [\[CrossRef\]](#)
47. Kapoor, M.; Gupta, M.N. Lipase promiscuity and its biochemical applications. *Process Biochem.* **2012**, *47*, 555–569. [\[CrossRef\]](#)
48. Dwivedee, B.P.; Soni, S.; Sharma, M.; Bhaumik, J.; Laha, J.K.; Banerjee, U.C. Promiscuity of lipase-catalyzed reactions for organic synthesis: A recent update. *ChemistrySelect* **2018**, *3*, 2441–2466. [\[CrossRef\]](#)
49. Vivek, K.; Sandhia, G.S.; Subramaniyan, S. Extremophilic lipases for industrial applications: A general review. *Biotechnol. Adv.* **2022**, *60*, 108002. [\[CrossRef\]](#)
50. Mhetras, N.; Mapare, V.; Gokhale, D. Cold active lipases: Biocatalytic tools for greener technology. *Appl. Biochem. Biotechnol.* **2021**, *193*, 2245–2266. [\[CrossRef\]](#)
51. Schmid, R.D.; Verger, R. Lipases: Interfacial enzymes with attractive applications. *Angew. Chem.* **1998**, *37*, 1608–1633. [\[CrossRef\]](#)
52. Verger, R. ‘Interfacial activation’ of lipases: Facts and artifacts. *Trends Biotechnol.* **1997**, *15*, 32–38. [\[CrossRef\]](#)
53. Martinelle, M.; Holmquist, M.; Hult, K. On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochim. Biophys. Acta Lipids Lipid Metab.* **1995**, *1258*, 272–276. [\[CrossRef\]](#) [\[PubMed\]](#)
54. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
55. 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. A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* **1991**, *351*, 491–494. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Cheng, C.; Jiang, T.; Wu, Y.; Cui, L.; Qin, S.; He, B. Elucidation of lid open and orientation of lipase activated in interfacial activation by amphiphilic environment. *Int. J. Biol. Macromol.* **2018**, *119*, 1211–1217. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Angkawidjaja, C.; Matsumura, H.; Koga, Y.; Takano, K.; Kanaya, S. X-ray crystallographic and MD simulation studies on the mechanism of interfacial activation of a family I.3 lipase with two lids. *J. Mol. Biol.* **2010**, *400*, 82–95. [\[CrossRef\]](#)
58. Wang, S.; Xu, Y.; Yu, X.W. A phenylalanine dynamic switch controls the interfacial activation of *Rhizopus chinensis* lipase. *Int. J. Biol. Macromol.* **2021**, *173*, 1–12. [\[CrossRef\]](#)
59. Palomo, J.M.; Fuentes, M.; Fernández-Lorente, G.; Mateo, C.; Guisan, J.M.; Fernández-Lafuente, R. General trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the enzyme functionality. *Biomacromolecules* **2003**, *4*, 1–6. [\[CrossRef\]](#)
60. 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. [\[CrossRef\]](#)
61. 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. [\[CrossRef\]](#)
62. 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. [\[CrossRef\]](#)
63. Stauch, B.; Fisher, S.J.; Cianci, M. Open and closed states of *Candida antarctica* lipase B: Protonation and the mechanism of interfacial activation. *J. Lipid Res.* **2015**, *56*, 2348–2358. [\[CrossRef\]](#)
64. Zisis, T.; Freddolino, P.L.; Turunen, P.; Van Teeseling, M.C.F.; Rowan, A.E.; Blank, K.G. Interfacial Activation of *Candida antarctica* Lipase B: Combined Evidence from Experiment and Simulation. *Biochemistry* **2015**, *54*, 5969–5979. [\[CrossRef\]](#) [\[PubMed\]](#)
65. Foresti, M.L.; Ferreira, M.L. Computational Approach to Solvent-Free Synthesis of Ethyl Oleate Using *Candida rugosa* *Candida antarctica* B lipases. I. Interfacial Activation and Substrate (Ethanol, Oleic Acid) Adsorption. *Biomacromolecules* **2004**, *5*, 2366–2375. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Kublicki, M.; Koszelewski, D.; Brodzka, A.; Ostaszewski, R. Wheat germ lipase: Isolation, purification and applications. *Crit. Rev. Biotechnol.* **2022**, *42*, 184–200. [\[CrossRef\]](#)
67. Kumar, R.R.; Bhargava, D.V.; Pandit, K.; Goswami, S.; Mukesh Shankar, S.; Singh, S.P.; Rai, G.K.; Satyavathi, C.T.; Praveen, S. Lipase—The fascinating dynamics of enzyme in seed storage and germination—A real challenge to pearl millet. *Food Chem.* **2021**, *361*, 130031. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Barros, M.; Fleuri, L.F.; MacEdo, G.A. Seed lipases: Sources, applications and properties—A review. *Braz. J. Chem. Eng.* **2010**, *27*, 15–29. [\[CrossRef\]](#)
69. Jung, H.; Moon, S.J. Purification, distribution, and characterization activity of lipase from oat seeds (*Avena sativa* L.). *J. Korean Soc. Appl. Biol. Chem.* **2013**, *56*, 639–645. [\[CrossRef\]](#)
70. Polizelli, P.P.; Facchini, F.D.A.; Cabral, H.; Bonilla-Rodriguez, G.O. A new lipase isolated from oleaginous seeds from *Pachira aquatica* (Bombacaceae). *Appl. Biochem. Biotechnol.* **2008**, *150*, 233–242. [\[CrossRef\]](#) [\[PubMed\]](#)
71. Nanssou Kouteu, P.A.; Baréa, B.; Barouh, N.; Blin, J.; Villeneuve, P. Lipase Activity of Tropical Oilseed Plants for Ethyl Biodiesel Synthesis and Their Typo- and Regioselectivity. *J. Agric. Food Chem.* **2016**, *64*, 8838–8847. [\[CrossRef\]](#)
72. Moussavou, M.R.W.; Brunschwig, C.; Baréa, B.; Villeneuve, P.; Blin, J. Assessing the Enzyme Activity of Different Plant Extracts of Biomasses from Sub-Saharan Africa for Ethyl Biodiesel Production. *Energy Fuels* **2016**, *30*, 2356–2364. [\[CrossRef\]](#)
73. Seth, S.; Chakravorty, D.; Dubey, V.K.; Patra, S. An insight into plant lipase research—Challenges encountered. *Protein Expr. Purif.* **2014**, *95*, 13–21. [\[CrossRef\]](#) [\[PubMed\]](#)

74. Jorge, N.; Luzia, D.M.M. Characterization of seed oil *Pachira aquatica* Aublet for food utilization. *Acta Amaz.* **2012**, *42*, 149–156. [\[CrossRef\]](#)
75. Yeşiloğlu, Y.; Başkurt, L. Partial Purification and Characterization of Almond Seed Lipase. *Prep. Biochem. Biotechnol.* **2008**, *38*, 397–410. [\[CrossRef\]](#)
76. Sheldon, R.A.; van Pelt, S. Enzyme immobilisation in biocatalysis: Why, what and how. *Chem. Soc. Rev.* **2013**, *42*, 6223–6235. [\[CrossRef\]](#)
77. Di Cosimo, R.; Mc Auliffe, J.; Poulouse, A.J.; Bohlmann, G. Industrial use of immobilized enzymes. *Chem. Soc. Rev.* **2013**, *42*, 6437–6474. [\[CrossRef\]](#)
78. Liese, A.; Hilterhaus, L. Evaluation of immobilized enzymes for industrial applications. *Chem. Soc. Rev.* **2013**, *42*, 6236–6249. [\[CrossRef\]](#) [\[PubMed\]](#)
79. Wancura, J.H.C.; Tres, M.V.; Jahn, S.L.; Oliveira, J.V. Lipases in liquid formulation for biodiesel production: Current status and challenges. *Biotechnol. Appl. Biochem.* **2019**, *67*, 648–667. [\[CrossRef\]](#)
80. Kim, B.H.; Hwang, J.; Akoh, C.C. Liquid microbial lipase—Recent applications and expanded use through immobilization. *Curr. Opin. Food Sci.* **2023**, *50*, 100987. [\[CrossRef\]](#)
81. Bhatt, C.; Nielsen, P.M.; Rancke-Madsen, A.; Woodley, J.M. Combining technology with liquid-formulated lipases for in-spec biodiesel production. *Biotechnol. Appl. Biochem.* **2022**, *69*, 7–19. [\[CrossRef\]](#)
82. 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. [\[CrossRef\]](#)
83. Guzik, U.; Hupert-Kocurek, K.; Wojcieszynska, D. Immobilization as a strategy for improving enzyme properties—Application to oxidoreductases. *Molecules* **2014**, *19*, 8995–9018. [\[CrossRef\]](#)
84. Boudrant, J.; Woodley, J.M.; Fernandez-Lafuente, R. Parameters necessary to define an immobilized enzyme preparation. *Process Biochem.* **2020**, *90*, 66–80. [\[CrossRef\]](#)
85. Klibanov, A.M. Enzyme stabilization by immobilization. *Anal. Biochem.* **1979**, *93*, 1–25. [\[CrossRef\]](#)
86. Iyer, P.V.; Ananthanarayan, L. Enzyme stability and stabilization—Aqueous and non-aqueous environment. *Process Biochem.* **2008**, *43*, 1019–1032. [\[CrossRef\]](#)
87. Hwang, E.T.; Gu, M.B. Enzyme stabilization by nano/microsized hybrid materials. *Eng. Life Sci.* **2013**, *13*, 49–61. [\[CrossRef\]](#)
88. Fernandez-Lafuente, R. Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. *Enzym. Microb. Technol.* **2009**, *45*, 405–418. [\[CrossRef\]](#)
89. 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. [\[CrossRef\]](#)
90. Barbosa, O.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Rodrigues, R.C.; Fernandez-Lafuente, R. Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts. *Biotechnol. Adv.* **2015**, *33*, 435–456. [\[CrossRef\]](#)
91. Chen, N.; Zhang, C.; Liu, Y.; Dong, X.; Sun, Y. Cysteine-modified poly(glycidyl methacrylate) grafted onto silica nanoparticles: New supports for significantly enhanced performance of immobilized lipase. *Biochem. Eng. J.* **2019**, *145*, 137–144. [\[CrossRef\]](#)
92. de Moraes Júnior, W.G.; Moura Maia, A.; Alves Martins, P.; Fernández-Lorente, G.; Guisán, J.M.; Pessela, B.C. Influence of different immobilization techniques to improve the enantioselectivity of lipase from *Geotrichum candidum* applied on the resolution of mandelic acid. *Mol. Catal.* **2018**, *458*, 89–96. [\[CrossRef\]](#)
93. Moure, V.R.; Fabrício, C.; Frensch, G.; Marques, F.A.; Mitchell, D.A.; Krieger, N. Enhancing the enantioselectivity of the lipase from *Burkholderia cepacia* LTEB11 towards the resolution of secondary allylic alcohols. *Biocatal. Agric. Biotechnol.* **2014**, *3*, 146–153. [\[CrossRef\]](#)
94. Ghattas, N.; Filice, M.; Abidi, F.; Guisan, J.M.; Ben Salah, A. Purification and improvement of the functional properties of *Rhizopus oryzae* lipase using immobilization techniques. *J. Mol. Catal. B Enzym.* **2014**, *110*, 111–116. [\[CrossRef\]](#)
95. Naya, M.; Imai, M. Regulation of the hydrolysis reactivity of immobilized *Candida rugosa* lipase with the aid of a hydrophobic porous carrier. *Asia-Pac. J. Chem. Eng.* **2012**, *7* (Suppl. S1), S157–S165. [\[CrossRef\]](#)
96. Uyanik, A.; Sen, N.; Yilmaz, M. Improvement of catalytic activity of lipase from *Candida rugosa* via sol-gel encapsulation in the presence of calix(aza)crown. *Bioresour. Technol.* **2011**, *102*, 4313–4318. [\[CrossRef\]](#) [\[PubMed\]](#)
97. Du, C.; Zhao, B.; Li, C.; Wang, P.; Wang, Z.; Tang, J.; Wang, L. Improvement of the enantioselectivity and activity of lipase from *Pseudomonas* sp. via adsorption on a hydrophobic support: Kinetic resolution of 2-octanol. *Biocatal. Biotransform.* **2009**, *27*, 340–347. [\[CrossRef\]](#)
98. Volpato, G.; Filice, M.; Rodrigues, R.C.; Heck, J.X.; Guisan, J.M.; Mateo, C.; Ayub, M.A.Z. Modulation of a lipase from *Staphylococcus warneri* EX17 using immobilization techniques. *J. Mol. Catal. B Enzym.* **2009**, *60*, 125–132. [\[CrossRef\]](#)
99. Yang, G.; Wu, J.; Xu, G.; Yang, L. Enhancement of the activity and enantioselectivity of lipase in organic systems by immobilization onto low-cost support. *J. Mol. Catal. B Enzym.* **2009**, *57*, 96–103. [\[CrossRef\]](#)
100. Wang, P.Y.; Tsai, S.W.; Chen, T.L. Improvement of enantioselectivity and stability of *Klebsiella oxytoca* hydrolase immobilized on Eupergit C 250L. *J. Chem. Technol. Biotechnol.* **2008**, *83*, 1518–1525. [\[CrossRef\]](#)
101. Takaç, S.; Bakkal, M. Impressive effect of immobilization conditions on the catalytic activity and enantioselectivity of *Candida rugosa* lipase toward S-Naproxen production. *Process Biochem.* **2007**, *42*, 1021–1027. [\[CrossRef\]](#)

102. Chaubey, A.; Parshad, R.; Koul, S.; Taneja, S.C.; Qazi, G.N. Enantioselectivity modulation through immobilization of *Arthrobacter* sp. lipase: Kinetic resolution of fluoxetine intermediate. *J. Mol. Catal. B Enzym.* **2006**, *42*, 39–44. [\[CrossRef\]](#)
103. Sabbani, S.; Hedenström, E.; Nordin, O. The enantioselectivity of *Candida rugosa* lipase is influenced by the particle size of the immobilising support material Accurel. *J. Mol. Catal. B Enzym.* **2006**, *42*, 1–9. [\[CrossRef\]](#)
104. Yu, H.; Wu, J.; Chi, B.C. Enhanced activity and enantioselectivity of *Candida rugosa* lipase immobilized on macroporous adsorptive resins for ibuprofen resolution. *Biotechnol. Lett.* **2004**, *26*, 629–633. [\[CrossRef\]](#) [\[PubMed\]](#)
105. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
106. Jesionowski, T.; Zdarta, J.; Krajewska, B. Enzyme immobilization by adsorption: A review. *Adsorption* **2014**, *20*, 801–821. [\[CrossRef\]](#)
107. Pessela, B.C.C.; Munilla, R.; Betancor, L.; Fuentes, M.; Carrascosa, A.V.; Vian, A.; Fernández-Lafuente, R.; Guisán, J.M. Ion exchange using poorly activated supports, an easy way for purification of large proteins. *J. Chromatogr. A* **2004**, *1034*, 155–159. [\[CrossRef\]](#) [\[PubMed\]](#)
108. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
109. Rodrigues, R.C.; Virgen-Ortiz, 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. [\[CrossRef\]](#)
110. 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.; 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. [\[CrossRef\]](#)
111. 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. [\[CrossRef\]](#)
112. 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. [\[CrossRef\]](#)
113. Arana-Peña, S.; Rios, N.S.; Carballares, D.; Gonçalves, L.R.B.; Fernandez-Lafuente, R. Immobilization of lipases via interfacial activation on hydrophobic supports: Production of biocatalysts libraries by altering the immobilization conditions. *Catal. Today* **2021**, *362*, 130–140. [\[CrossRef\]](#)
114. Tacias-Pascacio, V.G.; Peirce, S.; Torrestiana-Sanchez, B.; Yates, M.; Rosales-Quintero, A.; Virgen-Ortiz, J.J.; Fernandez-Lafuente, R. Evaluation of different commercial hydrophobic supports for the immobilization of lipases: Tuning their stability, activity and specificity. *RSC Adv.* **2016**, *6*, 100281–100294. [\[CrossRef\]](#)
115. Cygler, M.; Schrag, J.D. Structure and conformational flexibility of *Candida rugosa* lipase. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **1999**, *1441*, 205–214. [\[CrossRef\]](#) [\[PubMed\]](#)
116. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
117. 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. [\[CrossRef\]](#)
118. Palomo, J.M.; Fernandez-Lorente, G.; Mateo, C.; Ortiz, C.; Fernandez-Lafuente, R.; Guisan, J.M. Modulation of the enantioselectivity of lipases via controlled immobilization and medium engineering: Hydrolytic resolution of mandelic acid esters. *Enzym. Microb. Technol.* **2002**, *31*, 775–783. [\[CrossRef\]](#)
119. Guisán, J.M. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. *Enzym. Microb. Technol.* **1988**, *10*, 375–382. [\[CrossRef\]](#)
120. Mateo, C.; Palomo, J.M.; Fuentes, M.; Betancor, L.; Grazu, V.; López-Gallego, F.; Pessela, B.C.C.; Hidalgo, A.; Fernández-Lorente, G.; Fernández-Lafuente, R.; et al. Glyoxyl agarose: A fully inert and hydrophilic support for immobilization and high stabilization of proteins. *Enzym. Microb. Technol.* **2006**, *39*, 274–280. [\[CrossRef\]](#)
121. Fernandez-Lafuente, R.; Rosell, C.M.; Rodriguez, V.; Santana, C.; Soler, G.; Bastida, A.; Guisán, J.M. Preparation of activated supports containing low pK amino groups. A new tool for protein immobilization via the carboxyl coupling method. *Enzym. Microb. Technol.* **1993**, *15*, 546–550. [\[CrossRef\]](#)
122. Tacias-Pascacio, V.G.; Virgen-Ortiz, J.J.; Jiménez-Pérez, M.; Yates, M.; Torrestiana-Sanchez, B.; Rosales-Quintero, A.; Fernandez-Lafuente, R. Evaluation of different lipase biocatalysts in the production of biodiesel from used cooking oil: Critical role of the immobilization support. *Fuel* **2017**, *200*, 1–10. [\[CrossRef\]](#)
123. Bahri, S. Lipolytic activity and chilling requirement for germination of some almond cultivars. *Afr. J. Biotechnol.* **2012**, *11*, 14096–14101. [\[CrossRef\]](#)
124. 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. [\[CrossRef\]](#) [\[PubMed\]](#)

125. Wood, A.N.P.; Fernandez-Lafuente, R.; Cowan, D.A. Purification and partial characterization of a novel thermophilic carboxylesterase with high mesophilic specific activity. *Enzym. Microb. Technol.* **1995**, *17*, 816–825. [[CrossRef](#)]
126. 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](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.