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Strategies for the Immobilization of Eversa® Transform 2.0 Lipase and Application for Phospholipid Synthesis

Bruno R. Facin [1], Ernestina G. Quinto [2], Alexsandra Valerio [1], Débora de Oliveira [1], Jose V. Oliveira [1] and Gloria Fernandez-Lorente [2,*]

[1] Department of Chemical and Food Engineering, Federal University of Santa Catarina (UFSC), Florianópolis 88040-900, SC, Brazil; brfacin@gmail.com (B.R.F.); valerioalexsandra@gmail.com (A.V.); debora.oliveira@ufsc.br (D.d.O.); jose.vladimir@ufsc.br (J.V.O.)

[2] Laboratory of Microbiology and Food Biocatalysis, Institute of Food Science Research (CIAL, CSIC-UAM), Nicolás Cabrera, 9. UAM Campus, 28049 Madrid, Spain; ernestina.garcia.quinto@gmail.com

* Correspondence: g.f.lorente@csic.es; Tel.: +34-910017904

Abstract: Eversa® Transform 2.0 lipase (ET2) is a recent lipase formulation derived from the *Thermomyces lanuginosus* lipase cultivated on *Aspergillus oryzae* and specially designed for biodiesel production. Since it has not been available for a long time, research on the efficiency of this enzyme in other applications remains unexplored. Moreover, even though it has been launched as a free enzyme, its immobilization may extend the scope of ET2 applications. This work explored ET2 immobilization on octadecyl methacrylate beads (IB-ADS-3) and proved the efficiency of the derivatives for esterification of glycerophosphocholine (GPC) with oleic acid in anhydrous systems. ET2 immobilized via interfacial activation on commercial hydrophobic support Immobead IB-ADS-3 showed maximum enzyme loading of 160 mg/g (enzyme/support) and great stability for GPC esterification under 30% butanone and solvent-free systems. For reusability, yields above 63% were achieved after six reaction cycles for GPC esterification. Considering the very high enzyme loading and the number of reuses achieved, these results suggest a potential application of this immobilized biocatalyst for esterification reactions in anhydrous media. This study is expected to encourage the exploration of other approaches for this enzyme, thereby opening up several new possibilities.

Keywords: Eversa® Transform 2.0 lipase; biocatalyst; immobilization; esterification; solvent-free

Citation: Facin, B.R.; Quinto, E.G.; Valerio, A.; Oliveira, D.d.; Oliveira, J.V.; Fernandez-Lorente, G. Strategies for the Immobilization of Eversa® Transform 2.0 Lipase and Application for Phospholipid Synthesis. *Catalysts* **2021**, *11*, 1236. https://doi.org/10.3390/catal11101236

Academic Editor: Diego Luna

Received: 13 September 2021
Accepted: 11 October 2021
Published: 14 October 2021

1. Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are a class of enzymes able to perform a broad range of bioconversions owing to their substrate specificity, selectivity, high stability, and in many instances, high regio- and enantioselectivity [1–3]. This versatility, combined with the world's search for alternatives to supply the growing energy demand, has inspired the use of lipases as biocatalysts to produce biodiesel from a variety of fats and oils [4–6]. This insight led one of the most significant enzyme-producing companies, Novozymes, to launch a liquid lipase formulation named Eversa® Transform, as well as its second generation in 2016, Eversa® Transform 2.0 (ET2), which were designed especially for this propose [7]. Although ET2 has been mostly used in its free form as recommended by the supplier, proper immobilization may enhance a great deal of properties such as stability, activity, selectivity, or specificity, resistance to inhibitors or chemicals, and also applications as biocatalyst in other reactions [8,9]. Moreover, an immobilized derivative may present a new range of applications that the original biocatalyst could not perform itself, e.g., esterification of phospholipids in anhydrous systems. Phospholipid synthesis via enzymatic process usually occurs under milder conditions and, because biocatalysts present high stereoselectivity, it results in products that cannot be obtained through chemical methods [10].

Lipases are immobilized on hydrophobic supports by interfacial activation [11]. This immobilization allows the open form of lipases to be stabilized and, in some cases, it leads to hyperactivation [12]. In this technique, the orientation of lipases is very favorable to applications in reactions in an anhydrous medium, as the active center is protected and benefits the reaction [13]. In addition, the use of hydrophobic supports may be convenient for further applications in esterification reactions because they prevent the accumulation of hydrophilic compounds, such as water, in the biocatalyst [14–16]. Thus, a suitable immobilization protocol must be chosen depending on the reaction system in which the derivatives will be placed, since some strategies may have a disadvantage: enzymes can be desorbed under specific conditions [17,18].

Therefore, in this paper, we have intended to immobilize the ET2 lipase on the commercial hydrophobic support Immobead IB-ADS-3. ET2 was immobilized on Immobead IB-ADS-3 via interfacial activation, and the resulting derivatives were evaluated for phospholipid synthesis. The proposed system has involved the synthesis of oleoyl-lysophosphatidylcholine (oleoyl-LPC) and dioleoyl-phosphatidylcholine (dioleoyl-PC) from glycerophosphocholine (GPC) by direct esterification with oleic acid. These phospholipids have been considered as important bioactive lipids involved in a large variety of both normal and pathological processes, particularly linked to the prevention of neurodegenerative diseases [19]. It is expected that this study may encourage other extensive works exploring this valuable biocatalyst in the near future.

2. Results and Discussion

2.1. Enzyme Fit to the Enzyme Activity Method

It is known that some lipases may have a vast hydrophobic region where the active site is placed. These hydrophobic regions have a pronounced inclination to stabilize among themselves, forming aggregates as dimers, for example [20]. This aggregation is greater with increased concentration of the enzyme solution, such as the concentrations employed to obtain biocatalysts with a high enzyme-loading capacity. The aggregation phenomenon leads to a deceleration of reaction kinetics and inaccurate measurement of the enzyme activity for concentrations above a linear range. After that range, the addition of larger quantities of enzyme does not increase enzyme activity and, consequently, activity per mg of enzyme is reduced [21]. Furthermore, as mentioned above, those aggregates have a direct influence on obtaining derivatives with high enzyme loading, as although the aggregate dismembers on a gradual basis, the immobilization speed decreases and progresses very slowly [22,23]. ET2 has very high enzyme activity (3919.6 $\pm$ 0.5 U/g in soybean oil hydrolysis [5]), and several dilutions were needed to find out that linear behavior at the spectrophotometer (Figure S1), i.e., enzyme concentration lower than 0.02 mg/mL (enzyme content 0.002 mg) and specific activity of 85.77 $\pm$ 1.22 U/mg in *p*-NPB hydrolysis. This study is rarely reported in similar works, especially for ET2. In addition to the fact that there is a small number of studies reporting its immobilization, this is the first one involving a biocatalyst with such a high loading capacity [7]. However, as most immobilization processes are monitored by the decrease of the supernatant enzyme activity over time, this insight becomes quite important; otherwise, it may lead to an inaccurate value of immobilization yield and hence to misinterpretations.

2.2. Immobilization of ET2 Lipase

ET2 became commercially available only in 2016 and, so far, almost all the works found in the literature involve its use for biodiesel production, as recently discussed in an excellent review [7]. As ET2 is an enzyme genetically modified for this purpose; its resistance and stability are far above the average in terms of temperature, solvent, and pH [24]. Thus, this biocatalyst may present interesting performance in other systems, since it is adapted for such purpose. Therefore, the possibility of enzyme immobilization on hydrophobic supports allows the use of ET2 for esterification in anhydrous systems

owing to the rearrangement in its molecular structure, which enables its active form to be stabilized [13,14,16].

Figure S2 shows the immobilization courses of ET2 on IB-ADS-3. Maximum enzyme loading (160.8 mg/g of support) was achieved at the highest concentration of enzyme solution (20 mg/mL) after 72 h of immobilization, which means 80.4% of immobilization yield. Table 1 summarizes the properties of the immobilized derivative (ADS3). The high enzyme loading achieved in this study was far from the commonly reported capacity for adsorption on most supports (<50 mg/g, enzyme/support) [25]. This is probably due to the high porosity of this support, which results in a large surface area. Similar support of octadecyl methacrylate (Purolite® Lifetech™ ECR8806M) was used for immobilization of ET2, TLL, and *Candida rugosa* (CRL) lipases, but the maximum enzyme loading achieved did not exceed 20 mg/g (enzyme/support) in any of the cases [26–28]. No other study was found in the literature reporting the immobilization of any enzyme on IB-ADS-3, which may open up possibilities for further research. ET2 was immobilized on the hydrophobic surface of the support via interfacial activation (Figure 1). This physical adsorption is related to the conformational modification of the enzyme, which exposes its hydrophobic region containing the active site [13]. This conformational transition is induced by the movement of the polypeptide chain (lid), which leads the lipase to acquire its active form, similarly to interfacial activation motivated by the hydrophobic substrate phase [29].

Table 1. Summary of immobilized derivative properties.

Derivative Cod.	Support	Matrix	Group	Particle Size (μm)	Buffer	Enzyme Loading (mg/g Support)	Immob. Yield (%)
ADS3	IB-ADS-3	Methacrylate	Octadecyl	150–300	100 mM sodium phosphate pH 7.0	160.80	80.40

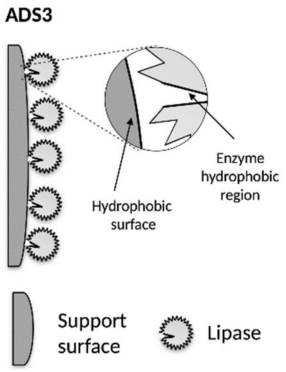

Figure 1. Schematic representation of Eversa®Transform 2.0 lipase (ET2) immobilization.

An inconvenience for any immobilized enzyme by physical adsorption is the possibility of immobilization reversibility, consenting the enzymes to be desorbed from support under mild conditions, especially with detergent or ionic solution [30]. This way, it becomes crucial to understand the reaction system in which the derivatives will be performing. Anhydrous reaction systems usually avoid such inconvenience and could be a great choice to prove the enzyme derivatives obtained from this protocol.

2.3. Esterification of GPC in Anhydrous System

The ADS3 derivative was proved for esterification of GPC (Figure 2). Figure 3 shows the kinetics for the two possible products for this esterification reaction: oleoyl-lysophosphatidylcholine (oleoyl-LPC) and dioleoyl-phosphatidylcholine (dioleoyl-PC). ET2 lipase has a preference for the *sn*1 position on the GPC molecule, which is more reactive. However, it is also able to reach the *sn*2 position with less selectivity, resulting in a rate of dioleoyl-PC formation that is much slower than the one for oleoyl-LPC formation

(Table 2). In fact, the selectivity of most lipases is described as *sn*1,3. However, it can be modified by different factors such as immobilization, reaction medium, or reaction conditions [31]. The kinetic profiles for the GPC esterification can be seen in Figure 3A,B, based on quantification by TLC and HPLC, respectively. The iodine staining method employed in the TLC technique has high sensitivity and provides evidence of the reaction progress even with small quantities of the product, which indicates that monitoring the conversion progress by TLC may be a simple, fast, and reliable alternative for qualitative purposes [32]. As mentioned above, the reaction products were also quantified by HPLC (Figure 3B), and the data profiles were very similar to those obtained by TLC.

Table 2. Summary of initial reaction rate (v) for the GPC esterification products.

Product	Initial Reaction Rate (v) (mg/mL.h)	
	0% Butanone	30% Butanone
Oleoyl-LPC	3.30	4.11
Dioleoyl-PC	0.26	0.35

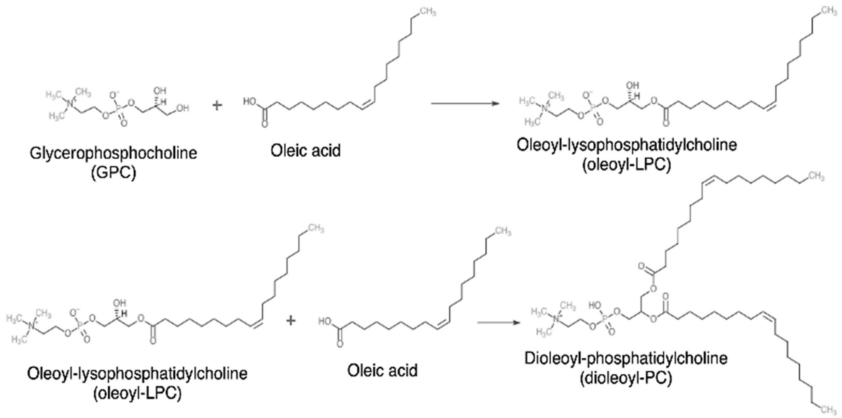

Figure 2. Scheme for esterification reaction of Glycerophosphocholine (GPC) with oleic acid.

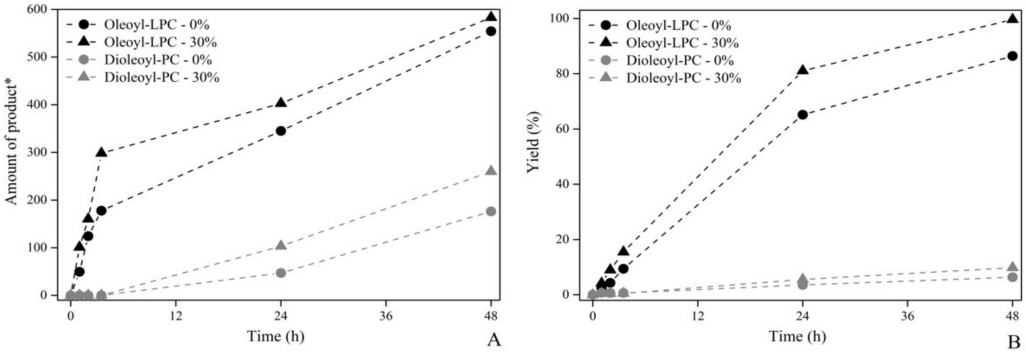

Figure 3. Kinetic profiles for the Glycerophosphocholine (GPC) esterification with oleic acid catalyzed by Eversa® Transform 2.0 lipase immobilized on Immobead IB-ADS-3 (ADS3) by (**A**) Thin-Layer Chromatography (TLC) and (**B**) High-Performance Liquid Chromatography (HPLC). Dotted lines are presented to improve visualization. Arbitrary scale.

Regarding the results, both kinetics (0% and 30% butanone) showed similar final conversions. The reaction with 30% of butanone was faster, with initial reaction rates higher than those found for the solvent-free system: 24.5% for oleoyl-LPC formation and 34.6% for dioleoyl-PC formation. Nevertheless, the outcomes for the absence of butanone were quite exciting. Initially, poor results were expected for this condition since

the butanone was just only added as a solvent to resolve the GPC solubility issue [10]. However, this enzyme seems to have no major problems with this and produced excellent results. It is an interesting fact because it allows us to think of a solvent-free system for the GPC esterification, which would be more attractive from the perspective of green chemistry.

2.4. Stability Study in GPC Esterification

For the study of operational stability, two points of the kinetic reaction were analyzed: 24 and 48 h of reaction. With 24 h of reaction, the formation of oleoyl-LPC, in particular, could be assessed since the amount of dioleoyl-PC until that moment was still low. Dioleoyl-PC could be analyzed for 48 h of reaction. The resulting data can be seen in Figure S3.

In general, ADS3 was stable in the solvent-free system (0% butanone), and only a significant decrease in oleoyl-LPC synthesis was detected after 9 days of incubation (23.7%), as shown in Figure S3A. However, for the system with butanone, the results were quite different, and a minor decrease was readily detected in oleoyl-LPC synthesis in the first days of incubation. There was a reduction of 37.4% after 9 days of incubation (Figure S3A). For the dioleoyl-PC synthesis (Figure S3B), the decrease was higher than the one found for oleoyl-LPC. Indeed, it is known that organic solvents can inactivate lipases through several mechanisms [33,34]. Thus, some deviations in the enzymatic activity were already expected, although the enzyme immobilization process may offer some advantages [22,24].

2.5. Successive Reuse Cycles for GPC Esterification

ADS3 proved to be very stable for the proposed system and was then applied to a study of successive reuse cycles for GPC esterification with oleic acid. Importantly, oleoyl-LPC synthesis (Figure 4) has remained constant between reuses, and only a minor decrease was detected for the solvent-free system (0% butanone). However, the same cannot be said about dioleoyl-PC synthesis. A high decrease in dioleoyl-PC synthesis was found for both systems, and it was slightly more marked for the system with 30% butanone. Enzymatic leaching could be an explanation for this reduction in ADS3 activity. However, no activity was detected in the washing water fraction after immobilization, which would indicate this propensity. Furthermore, the esterification system proposed for evaluating the enzyme derivative is anhydrous, and it does not contain significant detergents or ionic compounds that cause this phenomenon. It implies that the activity of the derivative was reduced owing to some inactivation or diffusion issues, and it would take longer to reach the first conversion yields.

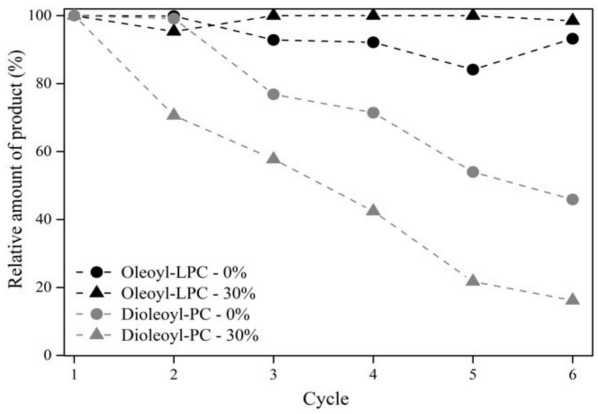

Figure 4. Kinetic curves for successive reuse cycles for Glycerophosphocholine (GPC) esterification with oleic acid catalyzed by Eversa® Transform 2.0 lipase immobilized on Immobead IB-ADS-3 (ADS3). Dotted lines are presented to improve visualization.

3. Materials and Methods

3.1. Materials

A commercial solution of lipase Eversa® Transform 2.0 (ET2) from *Aspergillus oryzae* (protein content 35 mg/mL) was provided by Novozymes (Araucária, PR, Brazil). Immobead IB-ADS-3 was purchased from Chiral Vision (Den Hoorn, Netherlands). Oleic acid was purchased from Sigma-Aldrich (Sigma-Aldrich, Darmstadt, Germany). *Sn*-glycero-3-phosphocholine (GPC) was purchased from Bachem AG (Bachem AG, Bubendorf, Switzerland). Protein concentration was determined by Bradford with bovine serum albumin as a standard [35].

3.2. Lipase Immobilization

ET2 was immobilized in Immobeads IB-ADS-3 (methacrylate beads with octadecyl groups) by hydrophobic interaction (via interfacial activation), using 5–200 mg of enzyme per g of wet support. Briefly, 4 g of wet support was added to 40 mL of enzyme solution with appropriate concentrations (i.e., 0.5 to 20 mg/mL). A total of 10 mM sodium phosphate buffer pH 7.0 was used as a solvent/diluent. Aliquots were taken at pre-established times to evaluate immobilization progress. The decrease of enzyme content in the supernatant was measured by the respective decrease in enzyme activity by employing *p*-NPB. A blank, containing soluble ET2 under identical experimental conditions, was prepared to confirm the stability of the enzyme under the study immobilization conditions. At the end of the immobilization process, the derivatives (ADS3) were filtered, washed with distilled water, and stored wet at 4 °C [12]. The washing water fraction was subjected to measurement of enzymatic activity, but it did not present significant values. The resulting derivatives were resuspended in distilled water for enzyme activity measurement and exhibited 100% of recovered activity, related to the free enzyme. All assays were carried out in triplicate.

3.3. Determination of Enzyme Activity

Enzymatic activity was measured spectrophotometrically with *p*-Nitrophenyl butyrate (*p*-NPB) as a substrate [36]. In a quartz cell, 20 µL of 50 mM *p*-NPB was added into 2.5 mL of 25 mM sodium bicarbonate buffer pH 8.5. Then, 100 µL of enzyme solution in different concentrations was added, and the reaction was monitored spectrophotometrically at 345 nm (isosbestic point of *p*-NPB) for 2 min. The increase in absorbance by the release of p-nitrophenol in the hydrolysis of *p*-NPB was measured, and a unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the production of 1 µmol of *p*-nitrophenol per minute under experimental conditions. All assays were carried out in triplicate.

3.4. Esterification of GPC in an Anhydrous System

The major reaction of GPC esterification with oleic acid in an anhydrous system was investigated. First, the ADS3 derivatives were dried by successive washing with acetone/water (progressive acetone concentration). Then, 300 mg of GPC was added to 5 mL of oleic acid solution (0 or 30% butanone *v*/*v*). After a brief period of homogenization (15 min under the following operational conditions), 0.2 g of ADS3 was added, starting the reaction. Molecular sieves were added to absorb the water generated during the esterification reaction. The reaction was carried out at 40 °C in a horizontal shaker at 150 rpm. Aliquots were taken at particular times to monitor the reaction progress. All assays were carried out in triplicate.

3.5. Thin-Layer Chromatography and High-Performance Liquid Chromatography of the GPC Esterification Products

Thin layer chromatography (TLC) was performed on silica plates (DC-Alufolien Kieselgel 60 F254, Merck, Darmstadt, Germany). The aliquots were diluted in butanone (1:1 *v*/*v*), and 5.0 µL was applied to a silica plate. The run was carried out with a mixture of methanol-chloroform-ammonium hydroxide (65:25:4 *v*/*v*). Visualization of the esterification products

was performed by exposure to iodine vapor until the corresponding bands appeared [37]. The reaction products were also determined by high performance liquid chromatography (HPLC) (SP 100 coupled with a SP 8450 UV detector, Spectra Physics, Santa Clara, CA, USA) using LiquidPurple Sil 5 µm (250 × 4.6 mm) with a column pre-filter (disc and seal 0.5 µm). Products were eluted at a flow rate of 1 mL/min with methanol/2-propanol (95:5 v:v). UV detection was performed at 205 nm. Retention times for the reaction products were 4.9 min for dioleoyl-PC and 5.7 min for oleoyl-LPC. All assays were carried out in triplicate.

3.6. Stability of the Derivatives in the Presence of Butanone

To study the operational stability of this system, 0.1 g of ADS3 was added to 5 mL of oleic acid solution (0% and 30% of butanone v/v). The vessels were incubated at 40 °C in a horizontal shaker at 150 rpm, and after particular periods (0, 1, 3, 7, and 9 days of incubation), 60 mg of GPC was added, starting the reaction. Molecular sieves were added to absorb the water generated during the esterification reaction. Aliquots were taken at particular times to monitor the reaction progress. All assays were carried out in triplicate.

3.7. Successive Reuse Cycles on the GPC Esterification

In a vessel, 60 mg of GPC was added to 5 mL of oleic acid solution (0% and 30% of butanone v/v). After a brief period of homogenization, 0.4 g of ADS3 was added, starting the reaction. Molecular sieves were added to absorb the water generated during the esterification reaction. The reaction was carried out at 40 °C in a horizontal shaker at 150 rpm for 24 h. At the end of each reaction cycle, the system was drained, and a new cycle began with the addition of new GPC and oleic acid solution. Aliquots were taken at particular times to monitor the reaction progress. All assays were carried out in triplicate.

4. Conclusions

In this study, the strategy of hydrophobic interaction by interfacial activation was used for ET2 immobilization in Immobead IB-ADS-3. By handling lipase aggregation during the immobilization process, the enzyme derivative ADS3 could be obtained with very high enzyme loading capacity (up to 160 mg/g, enzyme/support). ADS3 showed high enzyme activity and was able to provide excellent results for GPC esterification with oleic acid in anhydrous and solvent-free systems. ADS3 also proved to be stable in 30% of the butanone and solvent-free systems, keeping 62.6% and 76.3% of its initial capacity in oleoyl-LPC synthesis after 9 days of incubation, respectively. Although slightly more stable in the solvent-free system, ADS3 showed the inverse profile for the reaction rates. In the butanone system, stability was lower while the initial reaction rates were higher, probably owing to the increase in solubility. In the study of successive reuse cycles for GPC esterification, ADS3 achieved 63% of yield after six reaction cycles. Therefore, it can be concluded that ADS3 may be an excellent possibility for biocatalysis and reactions in anhydrous or solvent-free systems. Moreover, the high enzyme loading achieved in this work could be exceptionally attractive from an economic point of view.

Supplementary Materials: The following materials are available online at https://www.mdpi.com/article/10.3390/catal11101236/s1, Figure S1: Hydrolytic activity versus the mass of enzyme curves found for *p*-NPB hydrolysis using commercial Eversa® Transform 2.0 lipase; Figure S2: Immobilization kinetics of Eversa® Transform 2.0 lipase on Immobead IB-ADS-3; Figure S3: Kinetic curves for the stability of Eversa®Transform 2.0 lipase immobilized on Immobead IB-ADS-3 (ADS3) on the Glycerophosphocholine (GPC)esterification with oleic acid under different concentrations of butanone. (A) after 24 h of reaction and (B) after 48 h of reaction.

Author Contributions: B.R.F. designed and wrote the manuscript. E.G.Q. performed experimental work. G.F.-L. and A.V. provided guidance on manuscript organization and edited the manuscript. J.V.O. and D.d.O. revised the language and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are grateful for the financial support from Brazilian agencies CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) (CAPES-PRINT, Project number 88887.508974/2020-00). This study was also funded by Plan Nacional (Spanish Ministerio de Ciencia) Grant number RTI2018-093583-B-I00.

Data Availability Statement: Data are contained within the article.

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

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