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# Immobilized Biocatalysts of Eversa® Transform 2.0 and Lipase from *Thermomyces Lanuginosus*: Comparison of Some Properties and Performance in Biodiesel Production

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**Abstract:** Eversa® Transform (ET), and the lipase from *Thermomyces lanuginosus* (TLL), liquid commercial lipases formulations, have been immobilized on octyl agarose beads and their stabilities were compared. Immobilized and free ET forms were more thermostable than TLL formulations at pH 5.0, 7.0, and 9.0, and the ET immobilized form was more stable in the presence of 90% methanol or dioxane at 25 °C and pH 7. Specific activity versus *p*-nitrophenyl butyrate was higher for ET than for TLL. However, after immobilization the differences almost disappeared because TLL was very hyperactivated (2.5-fold) and ET increased the activity only by 1.6 times. The enzymes were also immobilized in octadecyl methacrylate beads. In both cases, the loading was around 20 mg/g. In this instance, activity was similar for immobilized TLL and ET using triacetin, while the activity of immobilized ET was lower using *(S)*-methyl mandelate. When the immobilized enzymes were used to produce biodiesel from sunflower oil and methanol in *tert*-butanol medium, their performance was fairly similar.

**Keywords:** Eversa; lipase from TLL; interfacial activation; enzyme stability; enzyme specificity; biodiesel

## 1. Introduction

The climate change and the shortage of petroleum have made researchers look for liquid fuel substitutes. Biodiesel is one of these green and renewable energy alternatives. It is the short alcohol chain esters of free fatty acids, obtained from oils, fats, or their derivatives as raw materials [1–4]. These esters are nontoxic, biodegradable, and miscible with standard diesel. The usual production of biodiesel is via a transesterification catalyzed by alkaline catalysts, a very rapid and efficient process [1–4]. However, it requires free-acid oils, consumes high energy, and has some side reactions involving free fatty acids and glycerin. Because of the requirement of low acidity of the oil used as raw material, the use of low quality oils requires a previous step of esterification of free fatty acids by other techniques [5–7]. In this context, the tendency to avoid the use of edible oils in biodiesel production and use alternative sources is remarkable, like used frying oils [8,9], algae oils [10,11], distillates derived from oil industries [12–14], as some of these materials are very rich in free fatty acids. Acid catalysis does not suffer from these problems, being able to catalyze both esterification and transesterification reactions, but it is a much slower process and requires higher temperatures and the use of reactors that are resistant to acid corrosion [15–19].

In this context, lipases are receiving increasing attention as alternative catalysts in the production of biodiesel [20–23]. They can catalyze both transesterification and esterification reactions, allowing the use of very acid oils. Moreover, lipases do not produce side reactions of the fatty acid chains, producing glycerin of pharmaceutical quality as a final product. However, the lipase catalyzed biodiesel syntheses are slower and more expensive than those using alkaline or acid catalysis, due to the price and stability of the enzymes [20–24].

Enzyme immobilization is a tool that permits the easy reuse of the enzymes as well as control of the reactors and may be positive for the economic suitability of biocatalytic processes [25,26]. Moreover, a properly designed immobilization may have some additional advantages, as it may improve some enzyme features, such as stability, specificity, selectivity, together to reduce inhibitions or permit enzyme purification [27–35].

However, in the case of biodiesel production, an incorrect selection of the support may produce some important undesirable effects. For example, in both, esterification and transesterification, a hydrophilic compound is produced: water or glycerin. If the support is hydrophilic, these hydrophilic by-products tend to be accumulated on the enzyme environment inhibiting or inactivating the immobilized enzyme [36–39]. This fact occurs even when using molecular sieves to capture the produced water when it goes to the reaction medium, because if the volumetric activity of the biocatalysts is very high, the product diffusion limitation may favor the formation of a hydrophilic phase inside the particle of the immobilized biocatalyst. There are some solutions to avoid, or at least to mitigate, this problem. For example, the use of a very hydrophobic support is recommended to reduce the accumulation of these hydrophilic compounds into the biocatalyst particle [40–44]. Furthermore, the use of ultrasound, able to agitate even inside the biocatalyst particles, may avoid the formation of the hydrophilic compound phase in the support pores, while promoting a better mixture of substrates [45–49].

In any case, due to these problems of unsuitable immobilization support selection and the costs of the immobilization processes, and thanks to the relatively low price and high activity of some of the liquid lipase commercial formulations, there is a growing interest in the use of liquid enzyme formulations for the production of biodiesel [50–61]. In fact, Novozymes® has launched an enzyme formulation, currently called Eversa® Transform 2.0 (ET), which has been proposed to be used as a biocatalyst in the production of biodiesel in liquid form [62–68]. This enzyme is an industrial evolution of a traditional lipase, the one from *Thermomyces lanuginosus* (TLL) [69–74]. As the free lipase remains in the hydrophilic phase, this permits enzyme reuse after one biodiesel production cycle in some instances [75]. However, the use of free enzymes causes the user to not take advantage of enzyme immobilization to improve features of the enzyme [27–35].

Selection of an optimal lipase for the production of biodiesel may not be a very adequate strategy, as the oils are heterogeneous substrates composed of many different triglycerides that evolve during the reaction to many different di- or monoglycerides, and some of them may be bad substrates, even inhibitors, for a specific enzyme [42,46,76–80]. This fact has promoted the use of mixtures of lipases to produce biodiesel, mixtures that may be different liquid lipases, different coimmobilized lipases, different independently immobilized lipases, or even mixtures of the same lipase immobilized on different supports [80].

These combilipases usually give better results than a single lipase. However, in any case, ET has been successfully employed in many biodiesel processes [62–68].

The commercial immobilized preparation of TLL [81] is a biocatalyst that, in some instances, offers quite poor results when it is used in biodiesel production [82]. However, if the enzyme is immobilized on a very hydrophobic support, e.g., octadecyl metacrylate beads, their performance as a catalyst for this process becomes competitive with the other catalysis types (even surpassing the results obtained with acid or heterogeneous alkaline catalysts) [83,84]. The immobilization of lipases in hydrophobic supports is very efficient as it permits the one step immobilization, stabilization, purification, and hyperactivation of the lipase by involving the open form of the lipase [85,86]. As an additional advantage of this

immobilization strategy, the support may be reused when the enzyme is inactivated after the inactivated lipase desorption using detergents or caotropic agents [85]. Moreover, the immobilization mechanism permits to immobilize monomeric forms of the lipases in all cases, mainly when using low enzyme loadings in the support, simplifying the comparison of the features of different enzymes [85].

Thus, this paper intends to compare different biocatalysts prepared using TLL or ET to discriminate the real functional differences between the original enzyme and the new industrial proposal. First, the performance of both immobilized enzymes was compared by immobilizing them in octyl agarose beads. Agarose has some advantages to immobilize enzymes and octyl agarose is among the most used supports to immobilize lipases [85–87]. However, the relevance of water in the support structure means that agarose is not recommended for use in biodiesel production.

For this reason, the enzymes have been also immobilized on octadecyl methacrylate beads, a support produced by Purolite® [88] that is valid for use in any anhydrous media [89,90]. The hydrolytic activities versus different esters of both immobilizates were evaluated to check their substrate specificities. Subsequently, the immobilized enzymes were modified with polyethylenimine (PEI), a strategy that permits the reduction of enzyme release from the support [91–94], analyzing how this affected the hydrolytic activity of both immobilized enzymes. Finally, the performance of both immobilized enzymes was compared as catalysts in the production of biodiesel from methanol and sunflower oil.

## 2. Results and Discussion

### *2.1. Immobilization of TLL and ET on Octyl Agarose*

The specific activity of the free TLL versus *p*-nitrophenyl butyrate (*p*NPB) is around 185 U/mg under the assay conditions, while that of ET is almost 350 U/mg. The explanation for this result may be complex using free lipases. It may be due to a real lower activity of TLL versus *p*NPB, but it may also be explained by differences in the enzyme purity, a higher tendency to form lipase aggregates by TLL [95–97], some effects of the components of the crude extract on the lipase activity, etc. Figure 1 shows that the enzyme purity was quite high in both commercial lipase preparations, but the band of ET was more diffuse than that of TLL, suggesting a higher diversity of glycosylation degree on ET formulation. In any case, significant differences in enzyme purity may be discarded as a reason for the different specific activity. Moreover, the same molecular weight was determined for both enzymes.

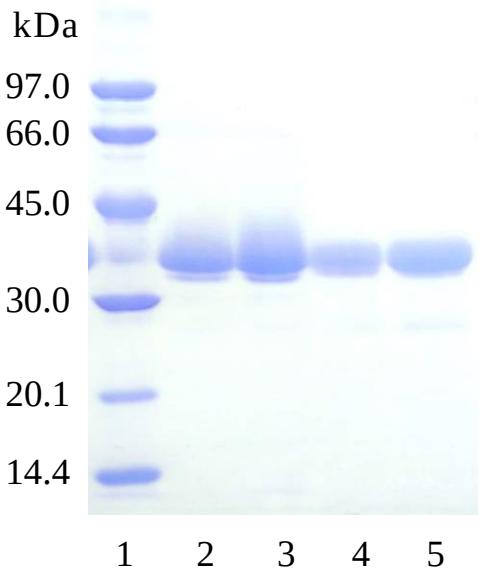


**Figure 1.** SDS-PAGE analysis of different *Thermomyces lanuginosus* (TLL) and Eversa® Transform (ET) preparations. All the samples were prepared at 0.35 mg protein/mL. Lane 1: low molecular weight markers; lane 2: ET-octyl; lane 3: free ET; lane 4: TLL-octyl; lane 5: free TLL. Experiments were performed as described in the Methods section.

Figure 2 shows the immobilization courses of TLL on octyl agarose beads using different enzyme loadings, offering different results as described before [98]. Immobilization of lipases on octyl agarose frequently promotes an increase of the enzyme activity versus *p*NPB [85,86]. The specific activity of the free enzyme versus *p*NPB greatly increased upon immobilization in a more significant extension when a lower load of enzyme was employed. Thus, TLL activity increased by 2.5-fold using 0.2 mg TLL/g of support or by just 1.3 using 1 mg/g of support. This decrease in specific activity of the immobilized enzyme when increasing the enzyme loading may correspond to an increase in the substrate diffusional problems caused by the higher volumetric activities [99]. However, the total volumetric activity of the biocatalysts increased when the load increased, from around 90 ± 9 U/g using 0.2 mg TLL/g to 240 ± 19 U/g.

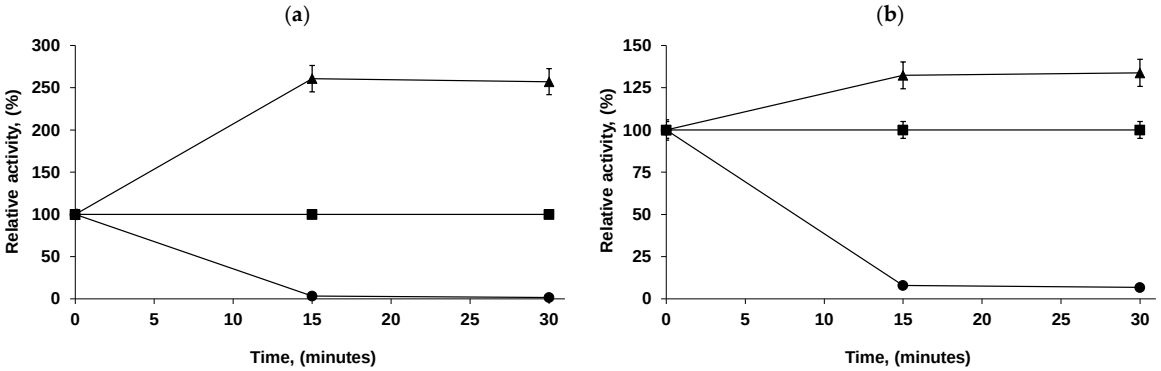


**Figure 2.** Immobilization courses of TLL on octyl agarose beads using enzyme loadings of (**a**) 0.2 mg/g or (**b**) 1 mg/g. Other specifications are described in the Methods section. Solid squares: reference; filled triangles: suspension; filled circles: supernatant.

After ET immobilization on octyl agarose (Figure 3), the observed hyperactivation was similar using enzyme loads of 0.12 or 0.2 mg ET/g (1.6-fold), suggesting that it was not determined by substrate diffusional limitations. This value was significantly lower than in the case of TLL. Using an enzyme load of 1 mg/g, even a slight decrease in enzyme activity could be observed (to 81%). This activity decrease could be attributed to an increase in the substrate diffusional limitation problems due to the higher volumetric activity of the ET biocatalysts (the activity of this enzyme is very high versus *p*NPB). In fact, the biocatalyst loaded with 1 mg ET/g of support presented an activity of around 275 ± 24 U/g of biocatalyst, while the biocatalyst having 0.2 mg/g biocatalyst presented an activity of 108 ± 10 mg/g of biocatalyst.

Although the *p*NPB activity of free ET was clearly higher than that of free TLL, the lower hyperactivation of ET caused the final immobilized biocatalysts activities to become similar when using the same enzyme loading.

Figure 1 shows that after immobilization, the diffuse band from ET remained that way. As almost no other protein bands could be visualized, that is, the commercial enzyme is already quite pure, purification obtained by the immobilization may be not pointed [85,86].

### *2.2. Stability of Immobilized ET- or TLL-Octyl*

The stabilities of both enzymes were analyzed by incubation at high temperatures in aqueous media at pH 5.0, 7.0, and 9.0 (Figure 4). The stabilities of the immobilized enzymes were higher than that of their free enzyme counterparts, and ET-octyl was the most stable preparation. The differences in the stabilities of free and immobilized enzymes were lower using TLL than using ET, that is, the observed stabilization of ET was higher after enzyme immobilization than that obtained using TLL at all studied pH values. It should be considered that the tendency of lipase to form dimers involving the open forms of two lipase molecules could apparently increase the stabilities of the free enzymes [95–97].

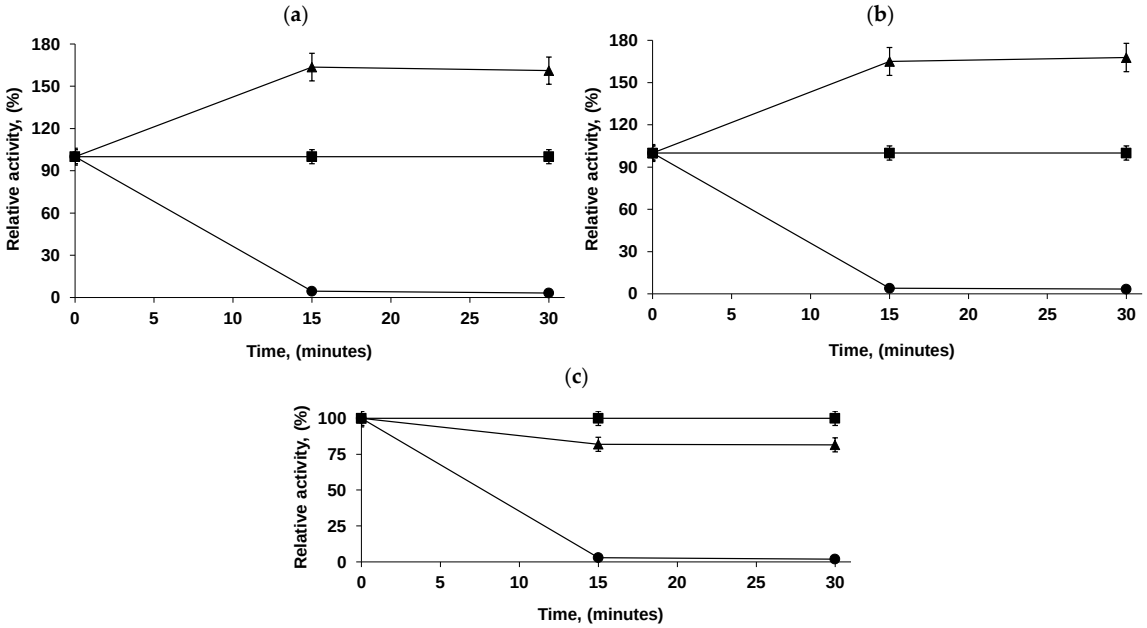


**Figure 3.** Immobilization courses of ET on octyl agarose beads using different enzyme loadings: (**a**) 0.12 mg/g, (**b**) 0.2 mg/g, and (**c**) 1 mg/g. Other specifications are described in the Methods section. Filled squares: reference; filled triangles: suspension; filled circles: supernatant.

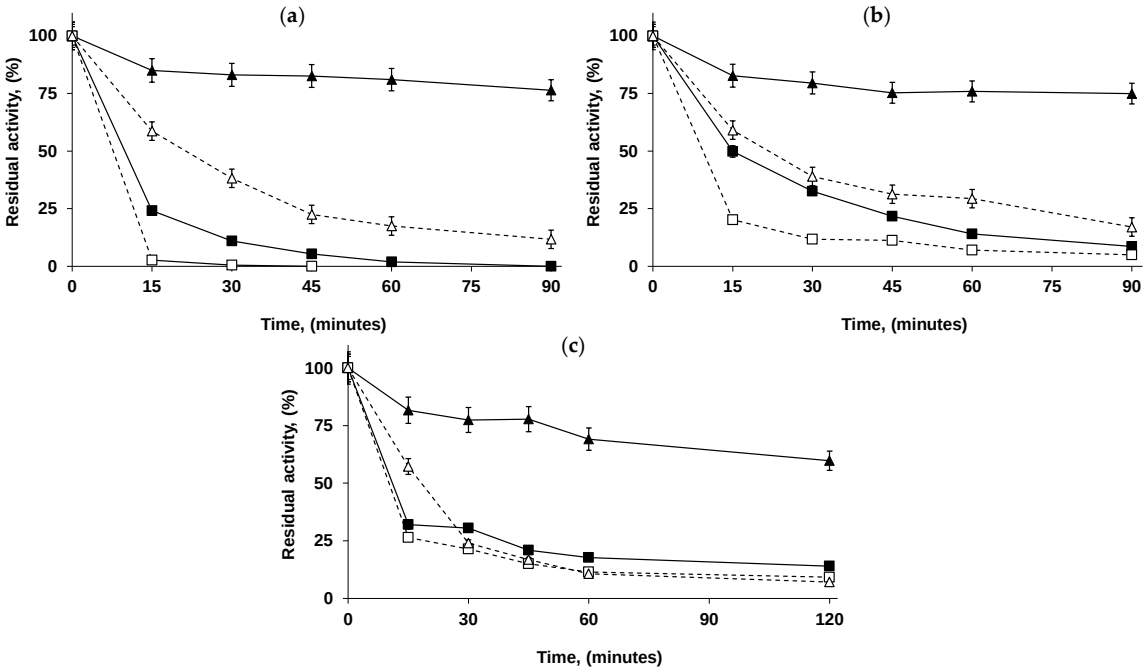


**Figure 4.** Inactivation courses of different enzyme preparations. The biocatalysts were incubated under different conditions (**a**) in 50 mM of sodium acetate at pH 5.0 and 72 °C, (**b**) in 50 mM of Tris at pH 7.0 and 72 °C, or (**c**) in 50 mM sodium carbonate at pH 9.0 and 70 °C. Other specifications are described in the Methods section. Continuous lines and filled squares: TLL-octyl; continuous lines and filled triangles: ET-octyl; dotted lines and empty squares: free TLL; dotted lines and empty triangles: free ET.

ET-octyl presented similar stabilities at pH 5.0 and 7.0 (TLL-octyl is more stable at pH 5.0). At pH 9.0, the lower stability of both enzymes caused it to decrease by 2 °C—the inactivation temperature necessary to achieve some reliable results.

In any case, even free ET was more stable than the immobilized TLL, and these differences increased when comparing both immobilized preparations. The higher stabilities of ET-octyl when

compared to the stabilities of TLL-octyl were observed at all studied pH values, more intensively at pH 9.0 and 7.0 than at pH 5.0, but very significant in all cases.

This clearly shows a first difference between both enzymes, both immobilized via its open form on the same support, under low loading to prevent enzyme–enzyme interactions, washed to eliminate any additive, and comparing only monomeric forms of both enzymes: ET is much more stable than TLL at all studied pH values.

Phosphate anions have been described to produce a deleterious effect on lipase stability, mainly when immobilized on octyl agarose supports [100]. The exact mechanism of this effect remains unknown, but it has been shown that it is mainly specific for lipases, at pH 7.0, and immobilized via interfacial activation on octyl agarose [101]. For this reason, we decided to evaluate the effect of this anion on the stability of TLL and ET formulations (Figure 5). The effect of sodium phosphate was more significant using TLL-octyl, which became less stable than free TLL. This is due to the fact that this anion had no effect on the free TLL stability, but it is negative for the stability of the immobilized enzyme. In the case of ET, the negative effect on enzyme stability was not very significant and affected in a similar way both the free and the immobilized enzyme formulations.

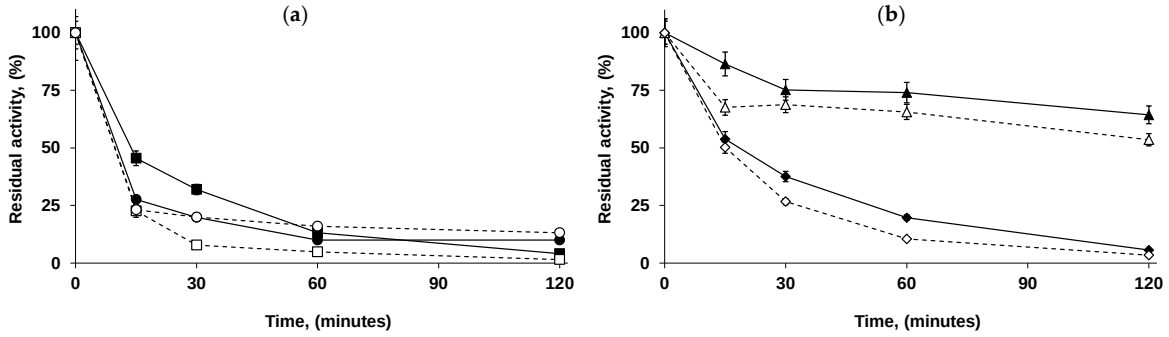


**Figure 5.** Effect of sodium phosphate in the inactivation courses of different enzyme formulations TLL-octyl, ET-octyl, free TLL, and free ET in the presence of phosphate anions. The biocatalysts were incubated in 100 mM of Tris-HCl or 100 mM of sodium phosphate at pH 7.0 and 72 °C. (**a**) TLL formulations inactivations, (**b**) ET formulations. Other specifications are described in the Methods section. Continuous lines and filled symbol: 100 mM Tris-HCl at pH 7.0; dotted lines and empty symbol: 100 mM sodium phosphate at pH 7.0; squares: TLL-octyl; circles: free TLL; triangles: ET-octyl; rhombus: free ET.

That way, another difference between TLL and ET is their sensibility to this deleterious anion and the way that the immobilization affected it.

Next, the stability of the immobilized biocatalysts in 90% (*v*/*v*) of different solvents was analyzed (Figure 6). In this case, only the immobilized enzymes were compared as the free enzyme precipitation caused by this drastic medium could make an interpretation of the results very complex. ET-octyl was clearly more stable than TLL in the presence of 90% (*v*/*v*) methanol. Thus, after 6 h, it still retained over 75% while TLL-octyl only retained 37%. After 1750 h of incubation, ET maintained more than 30% of the initial activity while TLL only maintained 10% of the initial activity. In 90% acetonitrile, both immobilized enzymes were very stable, and even a slight increase in activity after 30 h (slightly higher analyzing ET-octyl) was detected. After 1750 h, both biocatalysts still presented higher activity than the initial one. In 90% dioxane, ET-octyl slightly increased the activity while TLL-octyl maintained the initial activity after 30 h almost unaltered. Then, progressively, the activity of both biocatalysts decreased, but ET was quite more stable, maintaining over 90% of the initial activity after 1750 h, while TLL maintained only 20%. Thus, it can be stated that both immobilized enzymes were quite stable in the presence of 90% acetonitrile. The stability was decreased in the presence of 90% dioxane, while in methanol the stability was the lowest one, but ET-octyl was significantly more stable than TLL-octyl. As ET has been designed to be a good biocatalyst in the synthesis of biodiesel, and methanol is one of the components of the reaction mixture, it could explain the higher stability of ET in this medium.

However, the ET-octyl stability in the presence of this organic solvent was much lower than using the lipase B from *Candida antarctica* [102]. Thus, the comparison of both immobilized enzyme properties suggested some differences on their stabilities.

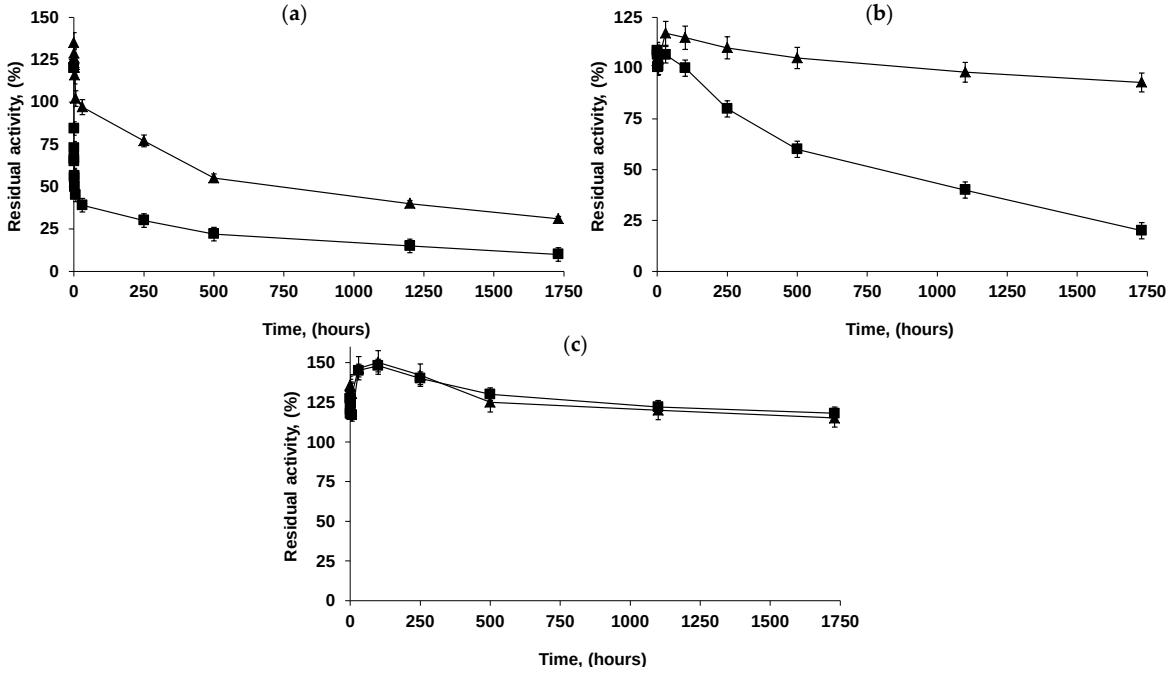


**Figure 6.** Inactivation courses of different immobilized lipases in the presence of 90% (*v*/*v*) organic co-solvents at pH 7.0 and 25 °C. (**a**) Methanol, (**b**) dioxane, or (**c**) acetonitrile. The aqueous phase was 100 mM Tris. Other specifications are described in the Methods section. Filled squares: TLL-octyl and filled triangles: ET-octyl.

Next, the enzymes were immobilized on octadecyl methacrylate beads to check the performance on biodiesel production, the real target of this new enzyme formulation.

### *2.3. Immobilization of TLL and ET on Octadecyl Methacrylate Beads (ECR8806M)*

Our objective was to prepare fully loaded biocatalysts of both enzymes to perform a fair comparison of them in the biodiesel synthesis; in fact, we offered an enzyme load that overpassed that of the support to ensure the full loading of the support. This support was very similar to that used in previous papers [82,84,88–90], but with approximately double the particle size (300–710 μm) (Purolite® commercial information). Table 1 shows the textural properties of the support, with a surface area of more than 23 $m^2$/g and 38 nm average pore diameter under dried conditions, which is more than enough to immobilize this enzyme.

**Table 1.** Textural properties of the different biocatalysts used in this paper, prepared using octadecyl methacrylate beads (ECR8806M). The experiments were performed as described in the Methods section.

| Support Modification | Specific Area ($m^2$/g) | Empty Volume ($cm^3$/g) | Pore Diameter (nm) |
|---|---|---|---|
| Empty support | 23.50 ± 1.15 | 0.180 ± 0.009 | 38.30 ± 1.92 |
| Support-TLL | 20.10 ± 0.99 | 0.130 ± 0.007 | 22.50 ± 1.05 |
| Support-TLL-PEI | 17.10 ± 0.80 | 0.090 ± 0.004 | 18.3 ± 0.85 |
| Support-ET | 17.20 ± 0.82 | 0.110 ± 0.004 | 20.80 ± 0.98 |
| Support-ET-PEI | 16.50 ± 0.76 | 0.09 ± 0.003 | 18.80 ± 0.91 |

Figure 7 shows the immobilization courses. In these instances, due to the high enzymes load, the activity of the suspension is not supplied as it will be subjected to very intense diffusional limitations.

The immobilization yield, offering 50 mg TLL/g of support after 2 h, was slightly less than 45%. Using 40 mg ET/g of support, the immobilization yield was slightly over 50%. That is, both biocatalysts presented very similar amounts of enzyme (20–22 mg/g of support), and this was considered the maximum loading of this support using these enzymes. Table 1 shows that the textural properties of the immobilized biocatalysts differed for those of the naked support, with a reduction of the pore diameter very similar for both enzymes (to 22.5 nm using TLL or to 20.8 using ET). The small difference in the pores diameter for both biocatalysts may be caused by differences in the glycosylation of the enzymes. In any case, this reduction of the pore diameter of support pores after immobilization confirms the enzyme filling the pores.

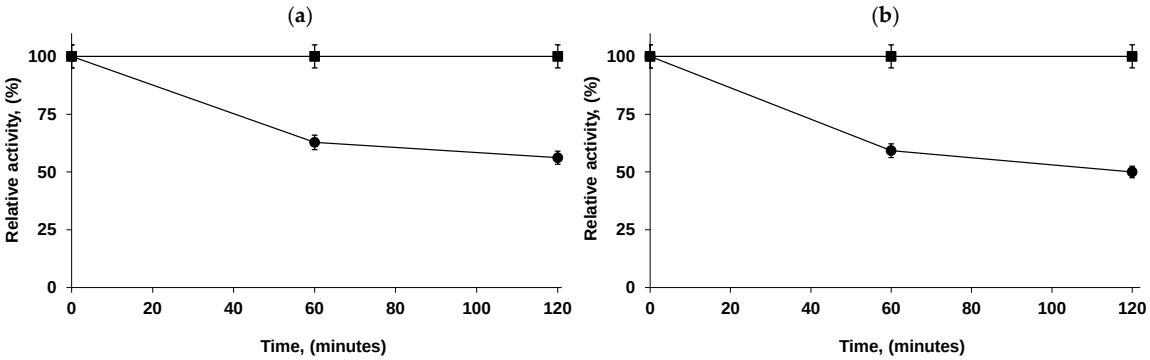


**Figure 7.** Immobilization courses on octadecyl methacrylate beads (ECR8806M) of (**a**) TLL (50 mg/g) and (**b**) ET (40 mg/g). Other specifications are described in the Methods section. Solid squares: reference and solid circles: supernatant.

### *2.4. Activity Versus Different Substrates in Hydrolytic Reactions of The Lipases Immobilized on Octadecyl Methacrylate Beads*

Next, the activity of these immobilized enzymes was evaluated versus *(S)*-methyl mandelate and triacetin (Table 2). The values were not very different using triacetin as substrate, 321.06 U/g ± 30 for TLL or 327.31 ± 33.75 for ET. However, TLL biocatalyst was more active than ET using *(S)*-methyl mandelate: 2.1 U/g versus 1.54 U/g. This suggested that both enzymes presented slight differences in enzyme specificity, being less effective ET in hydrolysis of complex substrates such as *(S)*-methyl mandelate than TLL, while being very similar in hydrolysis of triacetin.

**Table 2.** Octadecyl lipase biocatalysts activities in hydrolysis of several esters. Other specifications are described in the Methods section.

| Biocatalyst | Activity Versus Triacetin (U/g) | Activity Versus *(S)*-Methyl Mandelate (U/g) |
|---|---|---|
| TLL | 321.06 ± 30.52 | 2.08 ± 0.12 |
| TLL-PEI | 257.21 ± 24.05 | 1.92 ± 0.05 |
| ET | 327.31 ± 33.75 | 1.54 ± 0.04 |
| ET-PEI | 262.46 ± 20.98 | 1.52 ± 0.07 |

### *2.5. Effect of The Modification With PEI on The Activities of The Lipases Immobilized on Octadecyl Methacrylate Beads*

Modification with PEI of the enzymes immobilized by interfacial activation may have some advantages. In fact, it has been proposed to coat Novozym 435 with PEI with some positive results in the biodiesel production process by these biocatalyst [103,104]. Moreover, this treatment, in some instances, has increased lipase activity [93,105–107]. However, in this case, this treatment promoted a slight decrease in the activity of both immobilized biocatalysts. This is in disagreement with previous reports using octyl agarose CL-4B and TLL, perhaps due to the larger size of the biocatalyst particle and the smaller pore diameter [87]. In the case of TLL, the activity versus triacetin decreased to 80%,

while it decreased only to 92% using *(S)*-methyl mandelate. In the case of ET, there was no decrease on enzyme activity using *(S)*-methyl mandelate but a similar activity decrease using triacetin (Table 2). The higher effect of the PEI coating on the biocatalysts activity using triacetin, in contraposition to other previous reports [93,105–107], may be related to i) the very high loading of the enzyme preparations that greatly increases the volumetric activity, ii) to a decrease in the pore diameter caused by the PEI coating of the enzyme that decreases the diffusion rate of the substrate, and iii) to the increase of the pathway tortuosity for the substrate, increasing its difficulty to reach the enzyme-active center (Figure 8) [108–111]. In fact, Table 1 shows that after modification of the biocatalysts with PEI, the pore diameter decreased to around 18.5 nm for both biocatalysts. However, the effect of the PEI coating of the biocatalysts using *(S)*-methyl mandelate, where these derivatives exhibited much lower volumetric activity, was almost negligible That is, the PEI coating presented similar effects on octadecyl-ET and octadecyl-TLL biocatalysts.

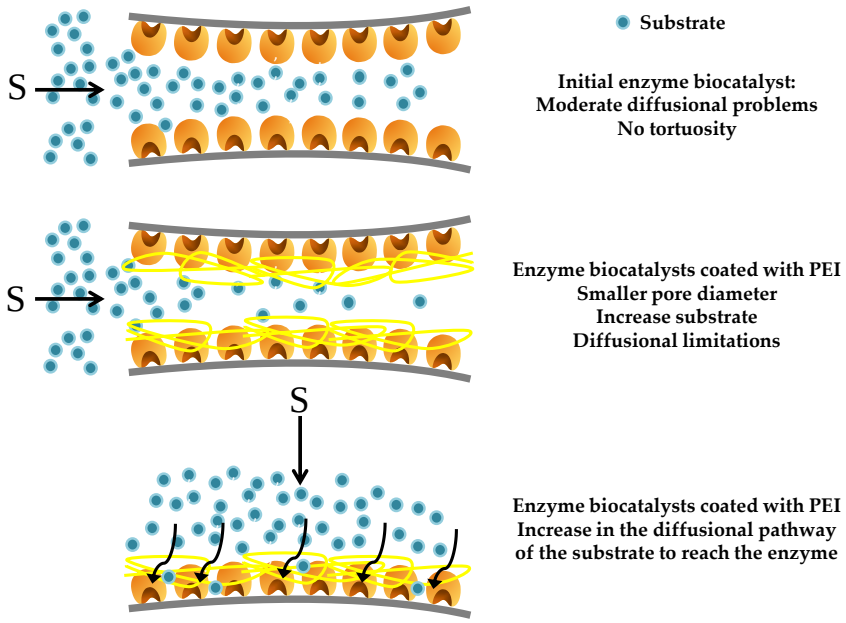


**Figure 8.** Schematic representation of the problems generated by the polyethylenimine (PEI) coating in the expressed activity of the biocatalysts.

### *2.6. Synthesis of Biodiesel Catalyzed by Immobilized TLL and ET*

Finally, the performance of both immobilized enzymes was analyzed in the production of biodiesel using methanol and sunflower oil. As reference, commercial Lypozyme® TL IM was studied. *tert*-butanol was added to the reaction medium as co-solvent. This was selected in order to have a good substrate mixture and to reduce the diffusion limitations, even though it has been shown that it was not positive for the synthesis of biodiesel with a similar TLL biocatalyst [82]. Figure 9 shows that both TLL and ET home-made immobilized biocatalysts offer very similar reaction courses, and that both are more rapid than that observed using the commercial enzyme. That is, the expected advantage of ET versus TLL immobilized on the same support cannot be observed in the biodiesel production under the studied conditions.

The coating of the ET biocatalyst with PEI produced a slower initial rate and a slow second phase of the reaction when compared to the biocatalysts not coated with PEI, suggesting that this treatment was not positive for the performance of the immobilized enzyme in this reaction under these conditions.

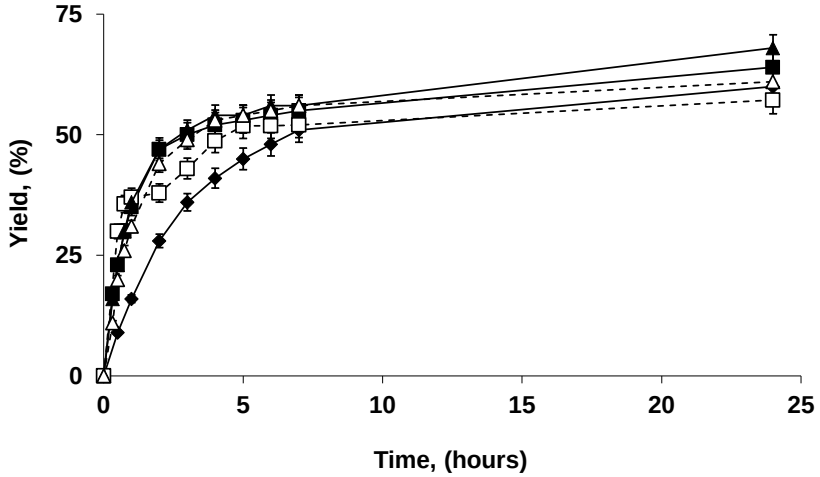


**Figure 9.** Synthesis of biodiesel from sunflower oil and methanol catalyzed by different lipase biocatalysts. The experiments were performed as described in the Methods section. Rhombus, continuous line, filled symbol: commercial Lipozyme TL IM; filled squares, continuous line, TLL immobilized on octadecyl metacrylate, empty squares, dotted line: immobilized TLL coated with PEI; filled triangles, continuous line, ET immobilized on octadecyl metacrylate, empty triangles, dotted line: immobilized ET coated with PEI.

## 3. Materials and Methods

### *3.1. Materials*

Soluble lipase from *Thermomyces lanuginosus* (Lipozyme® TL 100 L; TLL, 19.5 mg of protein per mL), Eversa® Transform 2.0 (27.5 mg of protein per mL), and commercial TLL immobilized Lipozyme® TL IM were kindly donated by Novozymes Spain (Alcobendas, Spain). The protein concentrations were determined by the Bradford method [112]. Octyl Sepharose™ CL-4B gel beads were bought from GE Healthcare (Madrid, Spain). Lifetech™ ECR8806M (octadecyl methacrylate beads) were kindly supplied by Purolite® ECR Enzyme Immobilization Resins (Llantrisant, UK). Polyethylenimine, branched (PEI) (MW 25,000), triacetin, *p*-nitrophenyl butyrate (*p*NPB), and *(S)*-methyl mandelate were purchased from Sigma Aldrich (Alcobendas, Spain). The commercial oil was from Koipesol (San Sebastian, Spain). FAMEMIX No. 37 standard mixtures of FA and FA methyl esters were purchased from Supelco (Bellefonte, PA, USA). All solvents were of HPLC grade (Scharlau, Spain). All other reagents were of analytical grade.

### *3.2. Methods*

#### 3.2.1. Determination of Enzymatic Activities

Hydrolysis of *p*NPB

A total of 50 µL of substrate (50 mM) dissolved in acetonitrile was added to 2.5 mL of 25 mM sodium phosphate at pH 7.0 and 25 °C and the reaction was started by adding 50 µL of the enzyme sample (solution or suspension). Their activities were measured by the variance in absorbance at 348 nm (isosbestic point of *p*NPB), ε under these conditions is 5150 $mol^{-1}cm^{-1}$ produced by the release of *p*-nitrophenol during 90 s, under magnetic stirring and thermostatization system. One unit of activity (U) was defined as the amount of enzyme that hydrolyzes one µmol of *p*NPB per minute under the conditions described previously.

Hydrolysis of Triacetin

The reaction was initialized by adding 0.025 g of biocatalysts to 8 ml of 50 mM of triacetin dissolved in 50 mM sodium phosphate at pH 7.0. The reactions were performed at 4 °C in order to decrease the biocatalysts volumetric activity and decrease the substrate diffusion problems. Under these reaction conditions, the enzyme product, 1,2 diacetin, suffers acyl migration, and a mixture with 1,3 diacetin is obtained [113]. The conversion degree was determined by HPLC (Kromasil C18 column of 15 cm × 0.46 cm) using a solution of 15% acetonitrile-85% Milli-Q water as mobile phase with a flow rate of 1 mL/min. The detection was performed at 230 nm and the retention times were 4 min for diacetins and 18 min for triacetin. Conversions between 15% and 20% were used to calculate initial rates.

Hydrolysis of (*S*)-methyl Mandelate

The reaction was initialized by adding 0.05 g of biocatalysts to 1 mL of 50 mM of *(S)*-methyl mandelate dissolved in 50 mM sodium phosphate at pH 7.0 and 25 °C. The conversion degree was determined by HPLC (Kromasil C18 column of 15 cm × 0.46 cm) using a solution of 35% acetonitrile-65% Milli-Q water with 10 mM of ammonium acetate at pH 2.8 as mobile phase with a flow rate of 1 mL/min. The detection was performed at 230 and the retention times were 2.5 min for the acid and 4.2 min for the ester. Conversions between 15% and 20% were used to calculate initial rates.

#### 3.2.2. Immobilization of TLL and ET on Octyl Agarose Beads

The support loadings were 0.2 or 1 mg of enzyme/g of support for TLL and 0.12, 0.2, or 1 mg of enzyme/g of support for ET. A total of 1 g of octyl agarose beads was added to 10 mL of enzyme solution of TLL or ET in 5 mM sodium phosphate at pH 7.0 and 25 °C with gentle stirring. Activity of supernatant and suspension were followed using the *p*NPB assay. After immobilization the suspension was filtered and the biocatalysts were washed with distilled water, filtered, and stored at 6–8 °C.

#### 3.2.3. Wetting of the Octadecyl Methacrylate Beads (ECR8806M)

Octadecyl methacrylate beads are very hydrophobic, making it difficult for water to penetrate their pores. Therefore, the support was subjected to a pre-treatment to remove the air and fill the pores with water. A total of 1 g of the support was suspended in 10 mL of methanol for 1 h. Subsequently, 10 mL of distilled water were added, thus obtaining a 50% water/50% methanol solution. After 15 min of gentle stirring, the support was vacuum-filtered and washed 5 times with excess distilled water. Finally, the support was stored at 6–8 °C with distilled water to avoid possible dehydration.

#### 3.2.4. Immobilization of TLL and ET on Octadecyl Methacrylate Beads (ECR8806M)

The lipases were immobilized using an excess enzyme that doubled the maximum load capacity of the support to ensure complete coverage of the support with the enzymes (50 mg of enzyme/g of support for TLL and 40 mg/g for ET). A total of 1 g of octadecyl methacrylate beads was added to 10 mL of enzyme solution of TLL or ET in 5 mM sodium phosphate at pH 7.0 and 25 °C with gentle stirring. Activity of the supernatant was followed using the *p*NPB assay, suspension activity was not measured due to the high substrate limitations problems that make the measurements unreliable. After 2 h the suspension was washed with distilled water, filtered, and stored at 6–8 °C.

#### 3.2.5. Coating of Immobilized Enzymes with PEI

The immobilized enzymes were treated with a solution 10% (*w/v*) of PEI at pH 7.0 and 4 °C during 16 h under gentle stirring. Afterwards, the biocatalysts were washed thoroughly with distilled water, filtered, and stored at 6–8 °C.

#### 3.2.6. Thermal Inactivations

The immobilized biocatalysts suspensions and the enzymes in their free form solutions were incubated in 50 mM sodium carbonate at pH 9.0 and 70 °C, 50 mM of Tris at pH 7.0 and 72 °C, or 50 mM of sodium acetate at pH 5.0 and 72 °C. The possible effects of phosphate anions on the stabilities of the free or immobilized formulations of TLL and ET were analyzed. Periodically, samples of the inactivation suspensions were withdrawn and their activities were determined employing *p*NPB as substrate. Residual activities were given as the percentage of the initial activities.

#### 3.2.7. Stability of The Biocatalysts in The presence of Organic Co-solvents

Immobilized enzymes were incubated in mixtures of 90% (*v*/*v*) acetonitrile, 1,4-dioxane, or methanol, and 10% (*v*/*v*) 100 mM Tris-HCl at 25 °C and pH 7.0. Periodically, samples of these inactivation suspensions or solutions were taken, and their activities were determined employing *p*NPB as substrate. Residual activity was defined as the percentage of initial activity.

#### 3.2.8. SDS-PAGE Analysis

SDS-polyacrylamide electrophoresis gel was carried out following the indications from Laemmli [114]. After diluting the protein samples in rupture buffer (4% SDS and 10% mercaptoethanol) to a final concentration of 0.35 mg of protein/mL, the suspensions or solutions were boiled for 8 min. Then, 15 µL aliquots of supernatant were injected to perform the experiment. The samples were run at 100 V. Gels were revealed employing Coomassie brilliant blue.

#### 3.2.9. Textural Features of the Different Supports

The textural characterization of the supports was performed by using Mercury Intrusion Porosimetry (MIP) [115]. Around 0.1 g of solid sample, after eliminating the water by incubation overnight at 60 °C, was precisely weighed into a sample container and located in a low-pressure porosimeter (Pascal 140, Thermo Scientific). Next, the dried sample was outgassed to a vacuum of 0.1 kPa and submerged in mercury. The pressure over the mercury was then gradually augmented from the vacuum to 400 kPa, and the intrusion data were recorded as a function of the pressure at each moment. Subsequently, the pressure was reduced to atmospheric pressure, and the sample container was withdrawn and weighed before being located in the high pressure porosimeter (Pascal 240, Thermo Scientific) in which the pressure was increased from atmospheric pressure up to 200 MPa. The combined pressure/volume data were transformed into a cumulative pore volume versus pore diameter curve by employing the Washburn equation [116] with the suggested values of the contact angle (141) and surface tension (484 dyne $cm^{-1}$) for mercury [117]. Thus, initializing the experiments under vacuum conditions and increasing the pressure to 200 MPa, the textural features of the solids over a range of approximately 120 µm down to 7.5 nm were obtained. The analysis of the data indicates the cumulative pore size distribution, pore volume, skeletal densities, and bulk. Considering a cylindrical non-intersecting pore model, the intrusion data may also be used to calculate the surface area of the solids by addition of the surface areas of the pore walls at each incremental pressure. However, only the data below 1000 nm were used for the calculation of the pore volume due the solid intraparticulate porosity within the support material as the low pressure data was a consequence of the interparticulate porosity of the solids aggregates. The first upward deviation in the cumulative curve is called the "threshold diameter" and is the limiting diameter below where the porous structure of the support materials becomes accessible when using the data below 1000 nm. The peak maxima indicate the sizes of the most frequent pores in the material from the derivative of the cumulative intrusion curve.

#### 3.2.10. Synthesis of Biodiesel

The production of biodiesel from sunflower oil and methanol was carried out in 20 mL glass bottles (3 cm id, 4 cm height). Prior to a reaction, all of the agents and solvents used were dried overnight

with a molecular sieve. The hydrolysis of the oil was carried out as follows: to a total mass mixture of 4 g (oil + alcohol) with a molecular ratio of 3:1 alcohol to oil, hexadecane (10% *w/w*, internal standard), the biocatalyst (5% *w/w*), and 1.6 g of *tert*-butanol (40% *v/v*). The flasks were introduced into a Batch reactor at 40 °C with orbital shaking at 200 rpm, and samples were periodically withdrawn for analysis by gas chromatography. Thus, the aliquots taken (200 μL) were dissolved in 1.8 mL of chloroform, shaken, and filtered through a 0.45 μm nylon syringe filter to remove the immobilized enzyme.

Analysis of Reaction Products by Gas Chromatography

Aliquots of 400 μL were collected of the sample during the course of the reaction, which were subsequently dried by nitrogen and transferred to a vial containing 2.5 mL of hexane. These samples were dried by adding anhydrous sodium sulfate for 2 h; prior to analysis, these samples needed to be filtered to remove sodium sulfate with a syringe filter.

Complete Derivatization of Triglycerides

To determine the total fatty acid residues of the oil, 200 μL of an oil solution in chloroform (20 mg/mL) were methylated with the addition of 1 mL of 0.2 N HCl-methanol. This mixture was heated to 60 °C for 4 h to subsequently add 200 μL of water and 1 mL of n-hexane. This mixture was stirred, observing a phase separation from which the upper oil phase (where the fatty acids were found) was extracted. The phase thus extracted was dried by adding sodium sulfate for 2 h and filtered with a syringe filter to remove the sodium sulfate, thus being prepared for gas chromatography analysis.

## 4. Conclusions

TLL and their industrially evolved ET have some significant differences, such as the higher stability of ET under different conditions and its lower sensibility to the presence of phosphate anions. As expected, the sizes of both enzymes are very similar. Maximal enzyme loading on methacrylate octadecyl beads (ECR8806M) was also quite similar. Regarding the activities of these two immobilized lipases, there are differences suggesting some differences in enzyme specificity (comparing activities versus *p*NPB, triacetin, and *(S)*-methyl mandelate), but these are not very significant (e.g., maximum difference is 1/3 of higher activity using TLL versus the mandelic acid). Surprisingly, the performance of both enzymes is pretty similar in the production of biodiesel when the industrial evolution of ET was mainly directed to improve its performance on this process although in liquid form. This may be due to the very good features of TLL immobilized on octadecyl methacrylate beads (ECR8806M) as catalysts of this process. In any case, following the different features of TLL and ET, a likely continuation of this research may be the development and optimization of combilipases, using mixtures of these two enzymes [80].

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