One Pot Use of Combilipases for Full Modification of Oils and Fats: Multifunctional and Heterogeneous Substrates

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Abstract: Lipases are among the most utilized enzymes in biocatalysis. In many instances, the main reason for their use is their high specificity or selectivity. However, when full modification of a multifunctional and heterogeneous substrate is pursued, enzyme selectivity and specificity become a problem. This is the case of hydrolysis of oils and fats to produce free fatty acids or their alcoholsysis to produce biodiesel, which can be considered cascade reactions. In these cases, to the original heterogeneity of the substrate, the presence of intermediate products, such as diglycerides or monoglycerides, can be an additional drawback. Using these heterogeneous substrates, enzyme specificity can promote that some substrates (initial substrates or intermediate products) may not be recognized as such (in the worst case scenario they may be acting as inhibitors) by the enzyme, causing yields and reaction rates to drop. To solve this situation, a mixture of lipases with different specificity, selectivity and differently affected by the reaction conditions can offer much better results than the use of a single lipase exhibiting a very high initial activity or even the best global reaction course. This mixture of lipases from different sources has been called “combilipases” and is becoming increasingly popular. They include the use of liquid lipase formulations or immobilized lipases. In some instances, the lipases have been coimmobilized. Some discussion is offered regarding the problems that this coimmobilization may give rise to, and some strategies to solve some of these problems are proposed. The use of combilipases in the future may be extended to other processes and enzymes.

Keywords: lipases; combilipases; enzyme specificity; full modification; coimmobilization; cascade reaction
1. Introduction

1.1. Enzymatic Biocatalysis

Enzymes are extremely precise biocatalysts, exhibiting this precision in a chemo-, regio- and stereo- product selective manner when applied in biotransformations at lab or industrial scale, so that their use has been gaining a preponderant role in the last years etc. [1–10]. This may be coupled with high substrate specificity (e.g., stereospecificity) [11–22]. Additionally, the sustainability upgrade upon switching from chemical catalysis to biocatalysis is another aspect to be taken into account, as long as Biocatalysis and Green Chemistry present many common features [23–25]; in fact, considering the type of catalyst used, enzymes are obtained from easily accessible renewable sources are biodegradable and fundamentally innocuous and harmless, and their use generally avoids the need for toxic and expensive metals. From the point of view of the biocatalyzed process, reaction conditions are usually very mild (atmospheric pressure, room temperature), and many protection-deprotection steps can be avoided, therefore leading to more economical synthetic routes, also generating less waste than conventional processes [16,18].

Although enzymes are very precise performing their catalytic activity, it is also true that in many cases it is necessary to increase their activity versus industrially relevant substrates (in some instances far from the physiological ones) and/or stability for making them compatible with operational conditions, mainly at the industrial level [26]. For this aim, there are several accepted strategies. One simple strategy is to exploit Nature to obtain the enzyme that best fits the specific process development, with a great advancement in metagenomics tools [27–31].

The genetic improving of enzymes by means of rational (or semirational) design [32–37], directed evolution [4,38–45] or even de novo enzyme design [46–49] helped with machine-learning technologies [50–53], a very powerful (although rather complex) approach. The chemical modification of enzymes is also a usual technique to improve enzyme features [54–57]. Enzyme immobilization, that was initialized as a way to solve the problem of enzyme solubility, is also an extremely useful methodology for modulating enzymatic features such as activity, selectivity, stability, etc. [9,32,58–70]. As lipases are one of the most (if not the most) frequently used biocatalysts [71–76], it is not surprising to find a plethora of immobilization protocols described for these enzymes [77–82]. Interestingly, in many instances some strategies to improve enzyme features are combined. For example, immobilization is compatible with any other strategy [55,83–87].

One very interesting paper shows the integrated use of diverse techniques to get an enzyme with new properties. It is related to the creation of an enzyme bearing an ex novo-created active center, to generate the so called plurizymes, using protein modelling and site-directed mutagenesis [88]. The same research group, in a further paper, exemplified how the coupled utilization of several tools may drive to results beyond expectations. In a second step, using dynamic simulation, protein modelling and directed mutagenesis, the plurizyme second active center activity was improved [89]. Then, an irreversible covalent inhibitor was designed, bearing a catalytic metal complex. This was attached to just one of the 5εr located in the active centers, enabling a fully directed chemical modification of the plurizyme, and finally the artificial semimetal plurizyme was used in a cascade reaction involving both, the enzyme active center and the metal catalysts [89].

1.2. Enzymatic Full Modification of the Substrate

1.2.1. Modification of Monofunctional Substrates

When using monofunctional substrates, the enzyme must be selected to recognize and perform the reaction in an optimal way [90–92]. The situation is apparently simple—only one substrate and one product may exist in the reaction medium. However, even using monofunctional substrates, some changes in the reaction conditions may occur that can significantly affect the enzyme performance. For example, if the reaction is an ester hydrolysis performed without pH control, it is likely that the pH of the reaction medium decreases during reaction, and the intensity of this pH decrease will be related to the concentration of the substrate (Figure 1). The effect of pH should be
considered on enzyme properties, including activity, before selecting the optimal enzyme for the process [92–96]. A similar pH decrease is found in the hydrolysis of some amides, e.g., β-lactamic antibiotics, where the amino group has a significantly low pK value (under pH 5) [97–100]. If that is the case, it is also likely that the optimal enzyme under the initial conditions may not be the optimal under the final reaction conditions. In this situation, it is sensible to think that the selection of the “best” enzyme to catalyze this reaction may be more complex than it initially seems. The use of initial rates under initial conditions will provide incomplete information that can lead to erroneous conclusions when considering the full reaction course (Figure 1). Full reaction courses using the target substrate concentrations should be considered to actually identify the best enzyme for a specific process. In some cases, it is not unlikely that the use of several enzymes may be a more convenient strategy, one with optimal activity under the initial reaction conditions, another with optimal activity under the final reaction conditions. The amount and proportion of both enzymes should be optimized in each specific case (kinetics of the enzymes with the substrate, concentration of the substrate, initial pH value, etc.). However, we have been unable to find any example of this use of enzyme mixtures. The situation will be different when using a high concentration of buffer that can maintain the pH throughout the reaction (but this can complicate the final downstream of the product) or if the pH value is controlled by continuous titration (but the titrating agent may affect the enzyme, substrate or product stabilities) [60,61] (Figure 1).

![Figure 1](image-url)

**Figure 1.** Effect of the change in the pH value during the reaction on the selection of the optimal enzyme. Figure represents two theoretical enzymes, one with high activity at optimal pH (initial pH value) but a strong dependence on the pH, the other with a lower activity but active at acidic pH values (a). The (b) figure shows the theoretical reaction courses when the reaction was performed under a controlled pH value (solid line) or when the pH decreased along the reaction course (dashed line).

One condition that will always change during the process is the ratio between the concentrations of substrate and reaction product. Furthermore, in many instances, the enzymes may be inhibited by the reaction product [101–108]. That way, it is possible that one specific enzyme can exhibit an optimal performance in the absence of product or if the concentration of substrate is much
higher than the concentration of the product, but it may suffer a strong inhibition due to its presence, stopping the reaction long before reaching the total modification of the substrate even when this may be thermodynamically feasible [109] (Figure 2). Therefore, it is possible that under industrial conditions, the optimal enzyme may not be the best enzyme under initial conditions, but an enzyme with a compromise solution offering good activity (but lower initial activity than the “optimal” enzyme) and low product inhibition, giving more linear reaction courses (Figure 2), may be desirable. In fact, the situation may be so complex that the best overall enzyme may be different depending on the initial substrate concentration utilized, the exact reaction conditions, etc. Again, it is possible that optimal reaction courses (more linear and reaching higher yields) may be obtained by mixing diverse enzymes presenting different kinetic features. Once again, we have not been able to find any paper using enzyme mixtures clearly addressed to solve this problem.

![Figure 2](image-url)  
**Figure 2.** Effect of the product inhibition on the reaction courses using two hypothetic enzymes, one with a very high initial activity but showing a strong inhibition by the product, the other with a lower activity but without product inhibition.

It should be also considered that several changes in the reaction conditions may be simultaneously occurring, making it even more complex to find a really definitive optimal enzyme. However, the possibility of bearing in mind these changes along the reaction course is usually not considered in the selection of a biocatalyst for a specific process. In high-throughput screening, for example, which is normally used in directed evolution [110–112], analyzing the whole reaction course will add some difficulties in a screening that by definition must be very rapid [113–116]. That way, the selection of an optimal enzyme as catalyst for a specific process may not be as simple as it looks, and in some cases, it may be that there is no real “optimal” enzyme.

1.2.2. Modification of Multifunctional Substrates

There are many examples of homo-multifunctional substrates that are modified using biocatalytic approaches, such as polyhydroxy [117–128], polycarboxylic [129–131], and polycarboxylic compounds [132,133]. In some instances, the researcher intends to partially modify the substrate in a
selective way. Thus, a regioselective or enantioselective modification of the substrate is required [119,132–152] (Figure 3).

**Figure 3.** Schematic representation of enzymatic recognition capability for polyfunctional substrates: (a) regioslectivity (usually denoted as site selectivity) in transforming certain functional groups (FG, in blue) into a product (Pr in red) without altering others; (b) regioselectivity upon addition of an asymmetric reagent (R–H) to an asymmetric double bond; (c) prochiral discrimination by transforming only a functional group adjacent to the stereogenic center; (d) prochiral discrimination by transforming only a functional group directly attached to the pro-stereogenic center.

For these homo-multifunctional substrates, the term regioselectivity describes the preference for reaction of a particular atom or group in a molecule that contains at least one different atom or group of the same type (Figure 3a). This type of regioselectivity is often referred to as site selectivity, in order to distinguish it from the capability of forming one regioisomer mainly from the other upon addition to a multiple bond (Figure 3b). The capability of recognizing prochirality upon converting only one of the groups adjacent to the stereogenic center (Figure 3c) or only one of the functional groups attached to a pro-stereogenic center (Figure 3d) is also noteworthy, in each case leading mainly to one enantiomer versus the other. In this case, together with a good enzyme activity, it is necessary for the enzyme to present the desired selectivity to give the target product, and the desired specificity; that is, the ability to recognize the initial substrate but not the first reaction product and to stop the reaction at this point [146,147,153–155]. An enzyme with full regio- and enantioselectivity towards the desired product and unable to recognize this product as substrate (or as inhibitor) will be the one that will give the maximum yield of the target product with minimal contamination of byproducts (other reaction products formed by modification of the target product, initial substrate) (Figure 3).

If the full modification of the multifunctional substrate is the objective of the process, enzyme specificity becomes a problem as it can limit the recognition of some of the partially modified substrates or intermediate products (Figure 4) and that way, that enzyme will be unable to provide the desired full modification of the substrate, giving only a partial yield. The problems in the selection of the best enzyme to catalyze the reaction that have been discussed above for the monofunctional substrates remain in this instance, but now it is necessary to consider how the enzymes recognize the different intermediate products (mono-modified, di-modified, etc., and all in different positions) [146,147,153–155] (Figure 4). This may be very complex if the number of possible intermediate products is large. Moreover, in many instances the modification will not be random, and each enzyme may have a different route in the full modification of the substrate, depending on the enzyme selectivity and specificity (Figure 5).
Figure 4. Effect of the enzyme activity (specificity) versus the different intermediate products in the modification of a trifunctional substrate. The figures show the expected reaction courses when a poorly active enzyme is able to recognize all intermediate products, or when the enzymes are unable to recognize some of these intermediate products, even being initially more active versus the initial substrate.

Figure 5. Ordered or random enzymatic modification of multifunctional substrate.

The difficulty in selecting an optimal biocatalyst may increase as it is possible that some enzymes that are not very suitable for the first modification of the starting substrate may be more active with progressively more extensively modified intermediate products [156–159]. The complexity of the catalysts selection may further increase if some of the intermediate products are chiral, as some enantiomers may not be recognized as substrates by some of the enzymes. In the
most extreme cases, some of these intermediate products may behave as an inhibitor of the enzyme (Figure 6).

Figure 6. Effect of enzyme specificity and product inhibition in the reaction courses of modification of multifunctional substrates. The figure shows one very active enzyme that cannot hydrolyze the last intermediate product, other that is less active but recognize all intermediate products and a last enzyme that not only cannot modify the last intermediate product, but that is also inhibited by it.

That way, once again the use of only the initial reaction rates provides fully incomplete information to select the optimal enzyme to be used in the process, making it necessary to evaluate full reaction courses to really determine the best enzyme for this process. In these instances, the combined use of several enzymes may be the best solution, as that way it is possible to use enzymes able to optimally hydrolyze each of the likely intermediate products, permitting it to always reach 100% of the conversion yield, maintaining high reaction rates [160,161] (Figure 7).
1.3. Lipases as Industrial Biocatalysts

Lipases are one of the most used enzymes in industry [9,79,81,162–164]. They belong to the class of hydrolases (EC 3.1.1.1) and their natural function is the hydrolysis of triglycerides to fatty acids and glycerol. Nevertheless, under non-aqueous conditions, lipases are able to catalyze a broad range of reactions such as esterification, transesterification, and interesterification or acidolysis [165–177]. Moreover, besides this broad range of reactions, lipases are able to recognize a vast diversity of substrates, being able to catalyze even promiscuous reactions [178,179].

This ability of lipases is perhaps based on their mechanism of action, called interfacial activation, makes the lipase active center very flexible [180,181]. The active center of most lipases is covered by a polypeptide chain called “lid”. The internal side of the lid is hydrophobic, interacting with the hydrophobic area surrounding the active center and isolating it from the aqueous medium. In the presence of a hydrophobic surface, such as a drop of oil, the lid opens exposing the active center and its hydrophobic neighborhood and this lipase is adsorbed and stabilized on that surface [182]. Induction of interfacial activation is not limited to oil drops and lipases can be adsorbed via its open form on any hydrophobic surface such as hydrophobic proteins, other lipases and hydrophobic supports [81].

Based on their versatility, lipases have been used in several areas such as food industry for flavor enhancement, synthesis of flavor esters and emulsifiers, modification of fats and oils; chemical industries for synthesis of esters, detergents, emulsifiers, biofuels (biodiesel), wastewater treatment; and pharmaceutical and cosmetics industries for resolution of racemic mixtures, etc. [183–191].

Figure 7. Solving the problem of product inhibition by using mixtures of several enzymes, one with high activity and high product inhibition, and the other with lower activity but not inhibited by the product.
2. Oils and Fats as Heterogeneous Substrates

Oils and fats are mainly composed of triglycerides, with some free fatty acids and very small amounts of mono and diglycerides. In this context, triglycerides may be considered as trifunctional and racemic (when presenting different acyl substituents) or prochiral substrates (when presenting monofunctional acyl substituents) (Figures 8 and 9) [192–195].

Figure 8. Diversity of reaction products in the first step of a lipase-catalyzed hydrolysis of a triglyceride possessing three identical acyl groups.

Figure 9. Diversity of reaction products in the first step of a lipase-catalyzed hydrolysis of a triglyceride possessing three different acyl residues (R1, R2 and R3), according to the lipase specificity (1-specific, 3-specific, 1,3-specific or 2-specific).
There are three ester bonds between glycerin and fatty acids. If the substituents in positions 1 and 3 are the same, they are prochiral substrates and after hydrolyzing position 1 or 3 an enantiomer of the diglyceride will be produced (Figure 8). If the substituents in these positions are different, they are already chiral substrates very likely in racemic form (Figure 9). That is, the lipase-catalyzed hydrolysis of a “pure” triglyceride may be initially a complex problem because the substrate may be a racemic mixture. Moreover, after the first modification, several diglycerides may be produced, having different enantiomers and regioisomers, and even free fatty acid composition (Figures 8 and 9). The final intermediate product, the monoglyceride, will have similar diversity in its composition (Figure 9).

In some instances, the goal of the research is not the full hydrolysis of the triglyceride, but to release only specific fatty acids to produce enriched solutions of the remaining glycerides or the free fatty acids [153–155,196–198]. This is the case when the nourishing value of triacylglycerols is the key point, as this property depends not only on the fatty acid composition, but also on their positional distribution. Therefore, lipases can be very useful for the preparation of novel structured lipids, possessing improved dietary or functional properties, such as low-caloric triglycerides [199], or for the enrichment of triglycerides with ω-3 poly-unsaturated fatty acids, such as eicosapentaenoic acid or docosahexaenoic acid [198,200–202].

In this case, enzyme specificity is the key for an optimal result, and the selection of the optimal enzyme will pursue the higher and faster accumulation of the target free fatty acid or the target glyceride [153–155,174,203–205].

However, a highly specific lipase becomes a serious problem when the objective is to have a full modification of this triglyceride, as the “substrate”, even just the main substrate in each reaction stage (Figure 10) (triglyceride, diglyceride or monoglyceride), will be different; additionally, if it is a hydrolytic process, the pH may be changing during the process (Figure 1). That way, selecting an optimal lipase for the full modification using a pure triglyceride becomes very problematic. Full reaction courses using the target concentration of the substrate should be studied to select the most adequate enzyme for that reaction. In this respect, the most active on the original triglyceride under the initial reaction conditions may be fully unsuitable to modify some of the final monoglycerides under a more acidic pH value. As stated above, the situation is more complex if some of the intermediate products are not good substrates for the enzyme but are good inhibitors: both reaction rates and reaction yields will be decreased (Figure 6).

Figure 10. Different components of a reaction, including the intermediate products, when an oil is hydrolyzed.
Internal acyl migration is undoubtedly the main side reaction which can be found when the regiospecific synthesis of structural triglycerides is intended. In fact, this process induces serious complications for obtaining pure regioisomers, either diglycerides [206] or monoglycerides [207], through different acyl-transfer processes, mainly trans- or interesterifications. It is known that the acyl migration rate depends directly on the reaction temperature (the lower the temperature, the higher reaction time required) [208], the pH value [153,209], the water activity (probably affecting the activation energy of the reaction by modifying the charge distribution of the transition state [210–212]) and on the type of solvent used (generally, polar solvents are described to reduce acyl migrations [213]).

The mechanism of these acyl migrations has remained controversial, but recently Mao et al. [214] have published a very interesting study, applying quantum chemical models using density functional theory at the molecular level. With this computational technique, these authors compared two possible situations, non-catalyzed and lipase-catalyzed acyl migration. In the first case, they considered three different pathways—concerted, stepwise or stepwise including a water molecule, as shown in Figure 11—concluding that the last one, a stepwise pathway with the aid of water, shows lower activation energy for the rate-limiting step (31.7 kcal/mol for path (c) versus 41.8 kcal/mol for (b)), although in any case non-catalyzed migration will proceed extremely slowly. Interestingly, they observed how the lipase-catalyzed migration, depicted in Figure 12, was much faster than any of the non-catalyzed migration pathways, describing how the rate-limiting step (the last one) implicating a water molecule shows an activation energy of 18.8 kcal/mol, which is very similar to the one (17.8 kcal/mol) experimentally measured [210].

Figure 11. The possible mechanisms of acyl migration in the non-catalyzed mechanism (NCM) scheme: (a) concerted pathway; (b) stepwise pathway; (c) stepwise pathway with the aid of water (adapted from Mao et al. [214]).
Figure 12. Postulated mechanisms of acyl migration in the lipase-catalyzed mechanism (LCM) scheme: (a) without and (b) with the aid of water (adapted from Mao et al. [214]).

Acyl migration is a problem when a regioselective reaction is intended, but if a full modification of a triglyceride is pursued, it becomes an advantage [215–219] (Figure 13). This may somehow mitigate the effects of enzyme specificity, as this permits that the enantio- or regioisomers presented in the reaction may be evolving during the reaction in a spontaneous way giving some isomers that may be good substrates for the enzyme, enabling that strict 1,3 regioselective lipases can fully modify the triglycerides [215,220,221].

Figure 13. Full modification of triglycerides using a strict 1,3 lipase thanks to the acyl migration. The figure shows the diversity of reaction products in a 1,3-specific lipase-catalyzed hydrolysis of a
model triglyceride; the first step will produce a complex mixture of regio- and stereoisomers of diglycerides, while the second hydrolytic step will furnish a prochiral 2-acylglycerol. This one, after a lipase-catalyzed acyl migration, will lead to a racemic mixture of chiral monoacylglycerol, which eventually could be hydrolyzed to glycerol.

Moreover, neither natural oils nor fats are composed of a single triglyceride, they present many different free fatty acids, in different positions and giving different enantiomers [222–225] (Figure 14). That way, being a collection of many different triglycerides, any oil or fat is in fact a very complex and heterogeneous substrate. If the situation to choose a single optimal lipase to modify just a pure triglyceride was complex, the fact that a natural oil may present dozens or hundreds of different triglycerides makes the situation very difficult [222–225]. The best lipase for the main components of the oil may be strongly inhibited by other triglycerides, or by some of the produced diglycerides or monoglycerides. Moreover, it may be that this lipase cannot recognize some of the free fatty acids attached to the glycerin, preventing it reaching full oil conversion. That way, the lipase that gives the best initial rates could not reach full oil modification or slow down the reaction in the final stages (Figures 4 and 6). Moreover, in oil hydrolysis reactions, the control of the pH is not possible using a titrating reagent [226–228]. The addition of a titration agent can promote the formation of soaps. That way, a decrease of the pH value is expected during the hydrolysis reaction (Figure 1). Again, the selection of the best lipase should consider the full reaction course and may be very hard to find a single enzyme that has the best properties in the whole process.

Figure 14. Some examples of the structural diversity of triglycerides.

There are two cases where the full modification of all the glycerides contained in an oil or fat is desired. These are the hydrolysis of the substrates to transform all glycerides in free fatty acids [226,228] and the alcoholysis of the substrates to produce biodiesel [229–235].

2.1. Lipase Production of Free Fatty Acids via Hydrolysis of Oils and Fats

In oleochemistry, the main application of lipases is in the hydrolysis of vegetable oils to produce fatty acids [226,236,237]. Free fatty acids present a wide range of uses in food industry such as soap manufacturing or surfactants, and some biomedical applications [226,236,237]. The main chemical method of hydrolysis of fats and oils to produce fatty acids and glycerol involves high temperature and pressure presenting high yield. However, under these extreme conditions, oil and
fatty acids polymerization and formation of byproducts occurs, resulting in dark fatty acids and colored aqueous glycerol solutions [238]. Instead, using lipases for this process results in energy saving and minimization of thermal degradation of substrates and products, where the unsaturated fatty acids can be produced without oxidation [239].

However, enzymatic hydrolysis presents the disadvantage of enzyme specificity compared to chemical hydrolysis. Conventional chemical processes produce the full hydrolysis of the triglycerides, while using the enzymatic technology the final yield is limited by the regioselectivity or the substrate specificity of the used lipase. For example, Candida rugosa lipase produced by submerged fermentation was used for the hydrolysis of sunflower oil, resulting at the highest yield 39.5% of the original oil [240]. Fungal lipase from Aspergillus niger has been tested for castor oil hydrolysis and the best performance achieved was around 60% in 72 h [241].

Other problems that will decrease the full hydrolysis of oil by lipases are the production of mono and diglycerides and the inhibition caused by some of the fatty acids (Figures 2 and 6). The intermediate glycerides cannot be easily recognized by some lipases, while the accumulation of fatty acids in the reaction medium can produce product inhibition. Finally, during enzymatic oil hydrolysis, the reaction pH is generally uncontrolled to avoid saponification and some problems in the purification steps, being the final pH much more acid than the initial one. Thus, the reaction conditions will be heterogeneous and will be changing along the reaction course. Thus, it could be assumed that the full hydrolysis of these complex substrates, such as vegetable oils, could be better performed using a mixture of biocatalysts made up of different enzymes, with different specificities and activities [242] (Figure 6).

2.2. Biodiesel Production Using Lipases as Biocatalysts

2.2.1. Transesterification

Transesterification is the reaction between triglycerides (oils and fats) and alcohols to produce fatty acid alkyl esters and glycerol. When short chain alcohols are used, like methanol or ethanol, the resulting ester collection is called biodiesel [173,243]. Currently, the main synthetic approaches used for biodiesel production are alkaline-catalyzed and acid-catalyzed transesterification (with simultaneous esterification of free fatty acids) [244,245]. The technical issues associated with chemical transesterification, such as high energy requirements, difficult recovery of the catalyst and glycerol, and environment pollution, has attracted the interest towards the enzymatic process, using lipases as catalysts [246,247].

In the transesterification reaction catalyzed by lipases several factors affect the final reaction yield. First, the lipase source: fortunately there are lipases from very diverse origins, such as animal, plants and microbiological sources, and many of them have been tested in biodiesel production [246,248]. The main problem for each specific lipase is associated to the second factor in biodiesel production, which is the triglyceride source [249]. As mentioned before, oils and fats are very heterogeneous substrates (Figure 14). Therefore, lipase specificity will affect the enzyme activity over each substrate, thus, affecting the final reaction yield. For example, comparing soybean, sunflower and rice bran oils, and the three most used commercial immobilized lipases from Novozymes: Novozym 435 (an immobilized lipase B from Candida antarctica [250]); Lipozyme TL IM (an immobilized lipase from Thermomyces lanuginosus [251]) and Lipozyme RM IM (an immobilized lipase from Rhizomucor miehei [252,253]), the final yield changed for each lipase and each oil from 50% using Novozym 435 to 5 % using Lipozyme RM IM for sunflower oil [254].

Other aspects that change the final yield of lipase-catalyzed transesterification reactions are the alcohol source and the alcohol:oil molar ratio. As stated above, methanol and ethanol are the most used alcohols due to their low cost and the final properties of the produced ester as fuel. Nevertheless, some lipases are inhibited/inactivated by methanol or ethanol, and moreover, although the stoichiometric alcohol:oil molar ratio is 3:1, some excess of alcohol could be needed to displace the reaction towards the synthesis, as glycerin will remain in the medium as a competitor.
In this case, the choice of alcohol, as well as the lipase source, can affect the achieved yield and reaction course [254–257].

The combination of different lipases in the reaction, as will be discussed later, is an interesting way to reduce the reaction time in enzymatic transesterification and increase the final yield allowing the full alcoholysis of the triglyceride (Figure 6).

2.2.2. Hydroesterification

Another possibility to produce biodiesel by an enzymatic route is using a strategy called hydroesterification. This process involves a two-step mechanism where, firstly, the tri-, di- and monoglycerides are hydrolyzed to produce free fatty acids and glycerol, and in the second step, the purified free fatty acids are esterified using an alcohol, such as methanol or ethanol [258–263].

The hydroesterification allows the use of any fatty raw material (e.g., vegetable and waste cooking oils, animal fat, acid waste from vegetable oil production) independently from its acidity and water content [249]. It promotes an advantage over the single step transesterification process, which inevitably generates soaps in the presence of fatty acids, inactivating the catalyst, and making it difficult to separate the biodiesel from the glycerol which is recovered at a high level of purity because of the absence of alcohol and salts in the aqueous phase.

Although many researches used immobilized lipases for hydroesterification, recently, the use of liquid lipases has gained popularity [235,264–266]. The use of a liquid lipase instead of an immobilized lipase implies the presence of water in the process, thus, related to the first hydrolytic step. Moreover, water promotes alcohol dilution in the medium, reducing its denaturing effect on the enzyme and leading to the formation of a second liquid phase in the reaction, creating a hydrophobic interface that is known to activate many lipases [235,264–266].

3. Advantages of the Simultaneous Use of Several Lipases to Fully Modify Multifunctional Substrates: The Concept of Combilipases

In Section 2 of this review, the difficulties in finding a single lipase that can have optimal properties in the whole reaction course of multifunctional and heterogeneous substrates [222–225], such as oils or fats, has been outlined; enzyme specificity, a feature in many instances critical to preferring biocatalysis over conventional catalysis in many processes, becomes a problem in this instance. It is possible that conversion yields may be well under the thermodynamics of the process if the enzyme is unable to modify some of the initial glycerides or intermediate products (Figures 4 and 6). Moreover, it is very likely that the reaction rate may be much lower than the expected one in the final steps of the reaction, if the activity of the enzyme is not adequate versus some of the remaining glycerides in the reaction mixture or are strong inhibitors of the enzyme (Figures 2, 4 and 6). Some authors, utilizing the lipase regioselectivity to get just a partial modification of the triglycerides, propose the enzymatic production of a biodiesel-like product, called ecodiesel, containing esters of the free fatty acids and monoglycerides [267–272].

Considering the problems for the full modification of oils, the researchers have proposed the use of a mixture of several enzymes to catalyze these reactions as a solution [160,161]. These lipase mixtures have been named combilipases [242]. Although these combilipases may also have advantages in the modification of monofunctional substrates (as previously discussed) (Figures 1, 2, 4 and 6), we have found very few studies using combilipases apart from oil modification. However, we have found many examples using combilipases in full hydrolysis [242] or alcoholysis [273] of fats or oils. The way combilipases have been utilized includes: mixtures of free enzymes, mixtures of individually immobilized lipases, or coimmobilized lipases. Next, some examples will be detailed.

4. Use of Lipases from Microorganisms that Produce Several Lipases

Some microorganisms naturally produce several lipase forms, or produce some lipase modifications (e.g., glycosylation) that can alter the final lipase properties in a heterogeneous way. It should be stressed that lipase features may be easily altered by very small modifications. In many
instances, the enzymes are commercialized in the form of these lipase mixtures. Examples of lipase sources producing diverse lipases are Candida rugosa [274–276], Geotrichum candidum [277,278], Staphylococcus warneri [279], Penicillium simplissimum [280], and Aspergillus niger [281]. Porcine pancreatic extract presents also several lipase forms [282]. These lipase extracts have been used in some instances to produce biodiesel or free fatty acids. In these cases, the mixture of the different lipases has been used [280,283]. In some instances, this may be done on purpose; in some other cases the situation may be just by chance, due to the lack of knowledge on the existence of different lipase forms [284,285] (in some cases, some of the lipases may be presented in traces and remain unknown to the researcher) [286]. In these cases, the study took advantage of mixing enzymes with different features, but the amount and proportion of each of them may not be easy to alter and it would be a chance if the enzymes ratio is the optimal one in the studied process. The situation may be somehow similar to the use of commercial enzymes cocktails of some enzymes in cascade reactions, like glycosidases [287–292]. The research cannot easily alter the composition of it.

One option to control this phenomenon is to fraction all the lipase components. However, this may be too tedious and complex, although in some instances lipase fractioning may be achieved by the successive adsorption of the lipase extract on hydrophobic supports with different hydrophobicity [284,293–295]. In any case, it may be simpler to use lipases from different sources already in an isolated form than to purify and mix again the different components of a crude lipase extract, as there are no guarantees regarding the suitability of the mixture of the lipase fractions for the specific process that is under study. It is better to select lipases with the desired specificity properties than to use a natural mix of lipases crude extract. In most instances, even the researchers using these enzymes do not try to explain the results by the existence of several lipases forms.

Another possibility may be the control of the expression of one lipase and not the others, like in the case of G. candidum, which produces several lipases as a function of the free fatty acids used as lipase inducers [296]. Nevertheless, there are no systematic studies on how the changes of the lipases ratio forms alters the results in the use of these enzymes in the production of free fatty acids or biodiesel. That way, after drawing the attention to this possibility, we will not review this uncontrolled combilipase type.

5. Use of Lipases Mixtures in Liquid Formulations

In biodiesel production, there is a certain increase of the interest in using free enzymes as catalysts for this process [235,265,266,297–305]. This interest is based on the low prices of the lipases commercialized for this use and in the launching of new lipases specially commercialized for biodiesel production in liquid formulation (e.g., Eversa Transform launched by Novozymes) [306–308]. Moreover, in many instances the use of unsuitable supports raises problems that may be avoided using liquid formulations. In the synthesis of biodiesel, for example, Marty and coworkers have shown in many instances that glycerin (and water) can accumulate in the support producing an enzyme inhibition or inactivation [309–312]. Although this can be solved using very hydrophobic supports [309,313–315] or ultrasounds that can stir the biocatalyst particle from the inside and prevent the glycerin/water phase formation [316–318], some authors prefer to fully avoid the use of immobilized enzymes. In many instances, due to the non-aqueous nature of the medium, the enzymes will be used as aggregates [319–322] (that is, the problem of water/glycerin phase in the biocatalysts particle is not fully avoided, and enzyme aggregates may be hard to reproduce).

In the hydrolysis of oils, the use of inadequate immobilization systems may be also problematic. This reaction produced fatty acids, monoglycerides and diglycerides, with detergent-like and/or anion nature properties. They can promote the release of the enzyme from the supports when the employed immobilization strategy is the physical immobilization. This is the simplest enzyme immobilization strategy to produce an immobilized enzyme biocatalyst [323–325]. This may be solved using intermolecular crosslinking strategies or heterofunctional supports [81,326,327].

In aqueous media, lipases may have a tendency to form lipase–lipase aggregates [328–331], or be adsorbed on any hydrophobic molecule of the crude protein solution [295,332]; however, the
presence of oil drops and all the detergent-like products should greatly reduce the risks of this enzyme aggregation (Figure 15). In any case, immobilization will be advantageous for a simpler enzyme reuse and a general improvement of the enzyme features [60–63]. Nevertheless, there are many examples of using combilipases in liquid form for both applications.

Figure 15. The tendency of lipase to give lipase–lipase dimers may be avoided by the addition of detergents or the interfacial activation on drops of biodiesel.

5.1. Hydrolysis of Oils and Fats Using Combilipases in Liquid Form

In a first example, lipase D from Rhizopus delenar, lipase N from Rhizopus niveus and lipase G from Penicillium sp. were used in the hydrolysis of soybean oil. These enzymes, not used in combination, gave free fatty production yields of 44%, 42% and 7.2%, respectively, after 10 h [333]. The authors showed that the use of combilipases formed by lipases G and N or lipases G and D, permitted to reach a hydrolysis yield of 95%–98% under similar conditions. This occurred even though lipase G was the least effective enzyme in the process [333], suggesting that it was able to eliminate some glyceride that could reduce the reaction rate for more efficient enzymes D and N.

More recently, lipases from different A. niger strains (named A, B, C and D) were used in the hydrolysis of soybean oil [334]. This way, different forms of the lipase produced by the fungus could be obtained and assayed in this reaction. After optimization by a three-factor mixture design and triangular surface, it was decided that a combilipase using 31.2% of lipase B and 68.8% of lipase D exhibited the optimal properties in this reaction, indicating a synergistic effect of the combilipases. This was attributed to the different fatty acid specificities of the two lipases (Figures 4 and 6), although different pH activity/stability profiles could be also relevant [334] (Figure 1). Again, initial reaction rates of lipase B were significantly lower than the activity of lipase D. Curiously, the mixture of lipases A and B gave lower reaction rates than the individual enzymes, suggesting a mutual inhibition of some of the reaction products of each enzyme [334].

In a previous paper, lard has been hydrolyzed as a source of free fatty acids using the lipases from R. miehei and Penicillium cyclopium [335]. Using the lipases in an individual way enabled a hydrolysis reaction yield of 39.9% using the lipase from R. miehei, while the lipase from P. cyclopium gave a yield of only 8.5%. The use of a combilipase of both enzymes permitted to reach a yield of
78.1%. If this was assisted with 5 min ultrasound treatment before the reaction started, reaction yields became 97% [335]. This exemplified again how the use of two lipases, even if one of them was much less effective in the overall reaction than the other, may be a very interesting way to improve the reaction performance, based on the combination of enzymes specificities.

5.2. Use of Combilipases in Liquid Form in Biodiesel Production

In a first report, lipases from R. miehei and P. cyclopium (expressed in Pichia pastoris) were utilized to catalyze the methanolysis of soybean oil in aqueous medium [336]. The lipase from R. miehei yielded 68.5% of biodiesel conversion, but when supplemented with lipase from P. cyclopium, the yields were above 95%. Again, this effect was explained by the use of lipases with different specificities. In another paper, a very “complex” combilipase has been used to produce biodiesel from nonedible oils adding methanol in a stepwise way to prevent enzyme inactivation. [337]. The authors mixed lipases from Candida rugosa, Pseudomonas cepacia, Rhizopus oryzae and porcine pancreas type II, together with Novozym 435. This permitted it to reach conversion yields of 93% that were improved to 97% by adding 10 wt% of silica gel to eliminate water from the system. However, they did not compare the results with individual lipases or less complex combilipases. In another paper, rapeseed oil deodorizer distillates were used as raw material to produce biodiesel using lipases from C. rugosa and R. oryzae, giving 92.63% after 30 h and 94.36% after 9 h of reaction, respectively [338]. The use of a mixture of both enzymes increased the biodiesel yield to 98.16% in 6 h (after optimization via response surface methodology). Another study shows how an oil rich in phospholipids and free fatty acids from Chlamydomonas sp. JSC4 (a microalga) were used to produce biodiesel [339]. A combilipase mixing lipases from Candida cylindracea and T. lanuginosus lipase gave a high yield (over 95%). Yields were similar to those obtained using a lipase from Fusarium heterosporum expressed in Aspergillus oryzae and used as a whole-cell biocatalyst, but the reusability of this last was higher.

Phospholipids may be a problem in biodiesel production, for this reason a degumming step using a phospholipase is used in many instances. In a first example, the reactions were performed in two steps, degumming and transesterification, using crude canola oil [340]. In the first step, degumming was performed using phospholipase A2 reducing the phospholipid content 60-fold. In the second step, lipases of R. oryzae and C. rugosa were utilized to produce biodiesel. Using the individual enzymes, the yields were 68.56% and 70.15%, respectively. Using a 1:1 mixture of these enzymes, the yield increased to 84.25% [340]. In another paper, both degumming and biodiesel production reactions were performed in just one pot, using a combilipase composed of lipase and phospholipase. The researchers utilized crude soybean oil, which also requires an additional pretreatment for gums removal if it is utilized for biodiesel production [341]. The authors proposed using combilipases mixing a lipase (Callera Trans L), two phospholipases (phospholipase A1 Lecitase Ultra and phospholipase C Purifine) and a lyso-phospholipase, achieving the degumming and transesterification in a single pot. The yield of biodiesel was higher than 95%, avoiding the inhibitions caused by the phospholipids and converting part of the phospholipids into biodiesel, and the phosphorus content was lowered from 900 ppm to <5 ppm [341]. In another paper, the lipase AY from C. rugosa was selected among six lipases by their high activity, and utilized in biodiesel production, but it only gave 21.1% biodiesel yield from oil containing phospholipids [342]. The combination of this lipase with some of the other lipases was assayed, and the best solution was the combination with Callera Trans L. Optimizing the methanol stepwise addition yielded more than 95% biodiesel in 6 h.

5.3. Other Uses of Combilipases in Liquid Formulations

In some instances, the objective of using combilipases is not the production of biodiesel or free fatty acids, but to produce special triglycerides. During the process, the nucleophile substrate will be changing, at the start it will be glycerin, then monoglycerides, later diglycerides, to finally get the target product, the triglyceride. That way, the production of these complex triglycerides may be carried out using combilipases.
For example, triglycerides were produced via esterification of glycerin and a concentrate of enriched conjugated linoleic acid using the lipases from *Alcaligenes* sp., *Penicillium camembertii* and *R. miehei* [343]. Using only the lipase from *R. miehei*, yields were just under 65%. The lipases from *Alcaligenes* sp. and *P. camembertii* alone gave yields of only 3%. However, the combined use of both enzymes permitted it to obtain a yield of 83%. Moreover, the combined use of the lipase from *R. miehei* and lipase from *Alcaligenes* sp. permitted it to obtain a yield of 82% and the reactions proceeded three times faster than when using the lipase from *R. miehei* alone [343].

When the aim of the process is to stop the reaction in an intermediate state, using mixtures of lipases may be risky. However, considering the heterogeneity of oils, even in this situation the use of a mixture of lipases may be advantageous and could enable a higher yield, if the enzymes activities versus the target intermedium product are low. For example, a study was intended to produce monoglycerides via glycerolysis of beef tallow or palm oil [344]. Many commercial enzymes were assayed, but in the context of this review we will remark that a mixture of lipases from *P. camembertii* and *Humicola lanuginosa* gave a yield of approximately 70 wt% monoglyceride, more than either enzyme alone. However, the mixture of lipases from *P. camembertii* and *Ps. fluorescens* or *R. miehei* gave similar values to the ones obtained employing individually the lipases from *Ps. fluorescens* or *R. miehei* [344].

The use of mixtures of lipases to get specific structured lipids may not be a good idea, but in order to get a general mixture modification between two oils again may be beneficial by combining different specificities. In an example of this research, lipases from *Rhizopus* sp. and Lipozyme TL IM were utilized in individual form or as a mixture in the interesterification between Amazonian patuá oil and palm stearin [345]. This reaction is quite complex, as it involves the hydrolysis of both oils and the further esterification of the released fatty acids to the glycerides, expecting the interchange of the free fatty acids in the final triglycerides [346–349]. The lipase from *Rhizopus* sp. modified the sn-1,3 positions of the triacylglycerol, yielding an oil richer in saturated fatty acids in the sn-2 position. The lipase from *T. lanuginosus* showed no regioselectivity in this reaction, there was no alteration in the distribution of unsaturated and saturated fatty acids in the triacylglycerol, there was only a replacement of fatty acids at the same position in both oils. The use of both enzymes showed the combination of both situations, but no synergetic effects were detected [345,349].

6. Use of Individually Immobilized Lipases

In most industrial applications, enzymes must be immobilized to facilitate their recovery and reuse [350,351]. This was the first objective of enzyme heterogenization, as enzymes were initially quite expensive biocatalysts. The decrease in the price of enzymes (very clear in the area of lipases) makes this initial objective not so necessary at present. In fact, Novozymes has launched some new lipase products recommending its use in non-immobilized form to save the enzyme immobilization costs, as for example Eversa Transform catalyst to be used in biodiesel production [306–308]. Other authors remarked the possibilities of using free lipases in these reactions [235,265,266,297–305]. However, a proper immobilization may have more additional advantages than just facilitating enzyme recovery, and even more so in the case of lipases [60–63,352–356]. A proper immobilization may increase enzyme stability by rigidifying the enzyme structure, by partitioning deleterious compounds from the enzyme environment, or by stabilizing a more favorable enzyme conformation of the lipase. Moreover, lipase immobilization may improve enzyme activity for different reasons, e.g., by producing more active lipase forms, by avoiding enzyme distortion under harsh conditions if the immobilization has provided some enzyme structural rigidification, or by reducing enzyme inhibition [62] (Figure 16).
Figure 16. Effect of the stabilization of the enzyme after immobilization on enzyme activity in the presence of inhibitors or distorting conditions.

Immobilization may also alter lipase selectivity or specificity or produce enzyme purification [60–63,352–356] (Figures 17 and 18). That is, enzyme immobilization may be advantageous by very different reasons. Moreover, even if enzyme disposal may be economically feasible after just one reaction cycle, free lipases, as interfacially active molecules, can give rise to some problems in the purification steps, e.g., stabilizing emulsion of hydrophobic substances [357] (Figure 15). That is, even if the costs of the enzyme loss may be economically acceptable, the advantages of a proper enzyme immobilization may be too relevant to discard this powerful tool to improve the enzyme. This great potential of enzyme immobilization has promoted the continuous growth in the number of scientific publications in this apparently old-fashioned topic [64,358].
Figure 17. Effect of the immobilization support on the conformation of the immobilized enzyme. Tuning lipase activity, selectivity, specificity and stability.

Using most enzymes, an intense multipoint covalent attachment is the best way to improve enzyme stability [62]. This process may not be simple, requiring a suitable active group in the support and a proper immobilization protocol (that in many instances may have several steps) [359]. In the case of lipases, all these advantages may be obtained by a very simple immobilization strategy. Using lipases, the best protocol to have an improved biocatalyst is just a simple physical adsorption of the enzyme on the support: the interfacial activation of the lipases versus support hydrophobic surfaces [360]. This protocol produces the one-step immobilization/purification/stabilization/hyperactivation of the lipases, as immobilization involves the stabilization of the open form of the lipases [81] (Figure 18). This stabilized open form of the lipase is very stable [361–363], even more so than the lipases immobilized via multipoint covalent immobilization [364,365]. The method has other additional advantages, such as its simplicity, high immobilization rate and high stability of the supports that can be stored for long times without any risks of alteration. Novozym 435, the most used commercial lipase biocatalyst, is prepared using this immobilization strategy [250].
Figure 18. (a) Interfacial activation of lipases on hydrophobic supports at low ionic strength: stabilized, purified and hyperactivated enzyme form. (b) Standard immobilization course of lipases on hydrophobic supports. Solid triangles: Suspension. Solid Squares: Reference. Solid circles: Supernatant.

Although lipase immobilization on hydrophobic supports may be achieved under a wide range of conditions, it has been recently shown that the immobilization medium conditions may greatly alter the properties of the immobilized lipases, at least when using some lipases [366–368]. This may be considered as an advantage, as it permits the modulation of the enzyme properties using a single immobilization support [61], or as a problem, as this means that changes in the immobilization medium composition may produce biocatalysts with different catalytic properties (activity, specificity, stability), and this may be in some instances hard to control (Figure 19).
Lipase immobilization via interfacial activation is reversible [81,325]. That way it permits the reuse of the support after enzyme inactivation, but it also raises the main problem of this lipase immobilization strategy: the lipase may be released during operation, under drastic conditions (high temperatures, organic cosolvent presence) or in the presence of detergent-like substrates or products [154,323]. This enzyme release may be avoided using heterofunctional supports [155,369–377] or by physical or chemical crosslinking of the immobilized enzymes [154,327,378–380] (Figure 20). That way, many examples of combilipases are based on mixtures of immobilized lipases.

**Figure 19.** Effect of the immobilization conditions on hydrophobic supports on the conformation of the immobilized enzyme. Tuning enzyme activity, selectivity, specificity and stability.
Figure 20. Preventing the risk of lipase release when immobilized on hydrophobic supports by (a) use of heterofunctional acyl-reactive groups supports or (b) physical intermolecular crosslinking with ionic polymers.

6.1. Use of Mixtures of Immobilized Lipases

6.1.1. Hydrolysis of Oils and Fats Using Individually Immobilized Combilipases

Commercial immobilized lipases Lipozyme TL IM and Lipozyme RM IM were used in the hydrolysis of soybean oil, comparing the use of individual enzymes and combilipases [160]. Optimal results were obtained utilizing a mixture of 65% Lipozyme TL IM and 35% Lipozyme RM IM, with higher reaction rates and yields (95%) than using the individual enzymes. Later on, this research group used the mixture of three commercial immobilized lipases, adding to the previous ones, the biocatalyst Novozym 435 [242]. Although Lipozyme TL IM was the most active biocatalyst and Novozym 435 was the least active one, the combination of 80% of Lipozyme RM IM and 20% of Novozym 435 gave better activity and yields of the use of just Lipozyme TL IM.

6.1.2. Use of Individually Immobilized Combilipases in Biodiesel Production

The use of immobilized lipases in biodiesel production is perhaps the first application of combilipases. S. W. Kim and co-workers were pioneers in this concept and a very active group in this area. In a first paper, they showed that using immobilized lipases from R. oryzae or C. rugosa in the production of biodiesel using soybean oil, the yields were 70% (after 18 h) or 20% (after 30 h) [161]. Using of a mixture of both immobilized lipases, yields became 99% after 21 h of reaction. Later, they optimized the process, using a 75% (mass) of the immobilized lipase from R. oryzae, reaching a biodiesel yield of 98% in only 4 h [381]. In a further research, they studied this process in batch or continuous way (stepwise addition of methanol was used in the batch process) [382]. The batch process gave 98.33% after 4 h. The continuous process design required considering mass transfer problems. After optimization, a conversion yield of biodiesel of 97.98% after 3 h was achieved [382]. Later, they analyzed this use of these combilipases in supercritical carbon dioxide [383]. After optimization, the batch process gave a biodiesel conversion yield of 99.13% after 3 h; that was improved to 99.99% after 2 h when 90 mmol methanol was used in a stepwise reaction.
Later, other research groups also used this concept. For example, a combilipase composed of a mixture of Novozym 435 and Lipozyme TL IM, was utilized as catalyst in the production of biodiesel from methanol and stillingia oil [384]. The objective of this research was to analyze the effect of the presence of some solvents to improve the solubility of methanol and the produced glycerol. The authors used a relation of 1.96% Novozym 435 and 2.04% Lipozyme TL IM regarding the oil weight. Optimal results were obtained using a mixture of 60% acetonitrile and 40% t-butanol (v/v) as a reaction medium. After optimization, a more than 95% biodiesel yield was obtained [384]. Later, this research group utilized Novozym 435 and Lipozyme TL IM to produce biodiesel from methanol and lard, optimizing the reaction by response surface methodology [385]. The best results were obtained using a combilipases formed by 49/51 (Novozym 435/Lipozyme TL IM) total lipases (w/w). After 20 h of reaction, a biodiesel yield of 97.2% was obtained [385]. Combilipases composed by Novozym 435 and Lipozyme TL IM were also utilized to produce biodiesel from methanol and waste cooking oil using tert-butanol as solvent [386]. After optimization, the biodiesel yield was up to 83.5%. They later studied the possibility of using ionic liquids as reaction medium [387]. They selected 1-ethyl-3-methylimidazolium trifluoromethanesulfonate, and the combilipases produced a biodiesel yield of 99% in these conditions. The combilipase was more active in this ionic liquid medium than in solvent-free or using solvents such as tert-butanol or isooctane media [387].

In another paper, five immobilized lipases were employed to produce biodiesel using ethanol and palm oil in a solvent-free system [388]. The best results obtained using the individual enzymes were obtained using the Lipase AK from *Ps. fluorescens*, but they were improved using a combilipase of this immobilized enzyme and Lipase AY from *C. rugosa*. Using a continuous packed-bed reactor, yields over 67% of biodiesel were obtained [388]. In another paper, Lipozyme TL IM and Lipozyme RM IM were utilized in biodiesel production using ethanol and soybean oil [160]. Using the central composite design and the response surface methodology, the reaction was optimized. The best results were obtained using 80% of Lipozyme TL IM and 20% Lipozyme RM IM, reaching a yield of 90% (more than doubling the results using only Lipozyme RM IM and 15% higher than employing only Lipozyme TL IM) [160]. In another paper, different lipases were utilized as catalysts in the synthesis of biodiesel from the crude oil extracted from spent coffee grounds, and they found that the biocatalyst with better performance was Novozym 435 (conversion of 60% in 4 h) [389]. After optimization (including oil purification), the conversion yields were improved to 88% in 24 h. Mixing Novozym 435 with Lipozyme TL IM, yields were improved and reaction rates enhanced [389]. In another paper, olive and palm oils were utilized to produce biodiesel using ethanol as alcohol using Novozym 435, Lipozyme TL IM and Lipozyme RM IM [273]. Optimization showed that the best results were reached using combilipases, and that the optimal composition of the combilipases depended on the substrate. Using olive oil, the optimal combilipase was composed of 58.5% of Novozym 435, 29.0% of Lipozyme TL IM and 12.5% of Lipozyme RM IM. This permitted it to reach a 95% biodiesel conversion in 18 h of reaction, while the best individually immobilized lipase (Novozym 435) gave only 50%. The composition of the optimal combilipases was very different when the oil changed. That way, using palm oil, the optimal combilipase did not have Novozym 435, but 52.5% of Lipozyme TL IM and 47.5% of Lipozyme RM IM. This gives an 80% biodiesel conversion in 18 h, while the results obtained when using the best individual enzyme for this oil, Lipozyme TL IM, was a biodiesel yield of only 44% [273]. One contribution used a combilipase composed of used and discarded immobilized lipases from *C. rugosa*, *Ps. cepacia*, *R. oryzae* and lipase from porcine pancreas type II, and Novozym 435, as catalysts of the biodiesel productions from nonedible oils [337]. Stepwise addition of 6 mmol of methanol to 1 mmol of oil permitted to reach a 93% biodiesel yield, and adding silica gel the yields increased to 97% biodiesel. In a further research effort, lipase B from *C. antarctica* and lipase from *R. miehei* were covalently immobilized onto epoxy-functionalized silica and utilized to produce biodiesel from methanol and wasted cooking oil [390]. The combilipase formed by both immobilized lipases was used, and response surface methodology and a central composite rotatable design were utilized to optimize the process. The best combilipase was composed by a 75% of immobilized lipase B from *C. antarctica*
and a 25% of the immobilized lipase from R. miehei; the best results were obtained using 10% t-butanol to oil (10 wt%) and silica gel (yields were 91.5%).

The use of a plug-flow, packed-bed continuous reactor and tert-butanol as solvent was analyzed utilizing a combilipase composed of commercial immobilized lipases and two different oils [391]. The optimal combilipases varied depending on the oil. When employing wasted cooking oil, the combilipase was formed by Novozym 435 (35%), Lipozyme TL IM (40%) and Lipozyme RM IM (25%). When using soybean oil, the combilipases was formed by Novozym 435 (50%), Lipozyme TL IM (22.5%) and Lipozyme RM IM (27.5%). The presence of glass beads facilitated the flow of this viscous substrate solution in the mixture of different biocatalysts, prepared using different supports [391].

In another paper, homemade biocatalysts were prepared to produce biodiesel from ethanol and macauba pulp oil [392]. To reach this goal, lipases from Burkholderia cepacia and T. lanuginosus were covalently immobilized on desilicated and thiol-modified ZSM-5. The highest yields (just under 95%) and reactions rates (48 h of reaction) were obtained by the use of the immobilized combilipases [392].

The use of ultrasound in the biodiesel production using combilipases has been studied. For this purpose, methanol and waste frying oil or soybean oil were used as substrates and Novozym 435, Lipozyme TL IM and Lipozyme RM IM were used as biocatalysts [393]. The best combilipase composition was designed by a statistical design of three factors. Ultrasound stirring and these optimized combilipases permitted it to get a biodiesel yield of about 90% when using soybean oil and 70% using the wasted oil after 18 h of reaction [393]. The same group used ultrasound stirring in the biodiesel production from methanol and soybean oil catalyzed by individual immobilized enzymes or the combilipase composed by the mixture of these immobilized enzymes, studying the effects of pulse conditions and ultrasonic amplitude [394]. The best results were obtained using an optimum combilipase formed by 10% Lipozyme TL IM, 15% Lipozyme RM IM and 75% Novozym 435, time pulse of 15 s, duty cycle of 50% and ultrasonic amplitude of 30%. The presence of tert-butanol did not improve the yields under ultrasounds stirring, while it did under mechanical stirring, suggesting that ultrasonic technology was enough to eliminate the diffusional problems. Under optimal conditions, the proposed combilipase produced 75 % ethyl esters while the best individual lipase gave only 55% in 5 h [394].

In another paper, oil from Isochrysis galbana was used to produce biodiesel utilizing commercial lipase from Ps. cepacia and commercial lipase B from C. antarctica [395]. The enzymes were immobilized on aminated SBA-15 mesoporous silica groups. Using wet extracted oil, the individual immobilized biocatalysts gave an 85.5% yield using the lipase B from C. antarctica and 87% using the immobilized lipase from Ps. cepacia (commercial Novozym 435 gave just under 70%). The use of a combilipase composed of immobilized lipase B from C. antarctica and immobilized lipase from Ps. cepacia (in a relation 1:3) permitted to reach a yield of 97.2% [395].

Ethanolysis of soybean oil was intended using free lipases from T. lanuginosus and porcine pancreas, with very poor results using the individual enzymes [396]. Mixing equal activity proportions of both enzymes, the yields increased 5 or 100 folds, but yields were still quite low (around 20% wt). The lipases were immobilized using the cross-linked enzyme aggregate (CLEA) technique [70,397–399] (Figure 21) yielding biocatalysts with 119 (lipase from T. lanuginosus) and 89% (lipase from porcine pancreas) of expressed activity. The combilipase formed by similar activities of both CLEAs permitted to reach a yield of 90.4 (wt.%), while the individual CLEAs gave a yield of 84.7 wt% using porcine pancreas lipase CLEA or 75.6 wt% using T. lanuginosus CLEA [396].
Display of enzymes on the surface of cells is an immobilization technique with growing popularity nowadays [400–403]. This technique for production of immobilized enzymes has been employed to separately express and display the lipase B from *C. antarctica* and the lipase from *R. miehei* on *P. pastoris* [404]. These biocatalysts were employed to produce biodiesel in tert-butanol and isooctane cosolvent media using statistical optimization. The use of a combilipase with the two displayed lipases (in different yeast cells) gave an ester yield higher than 90% in 12 h, higher than the use of the individual biocatalysts [404].

The synergy between immobilized lipase from *R. oryzae* and Novozym 435 in biodiesel production was showed in another research, increasing the yield by 30% compared to the results obtained using the immobilized lipase from *R. oryzae* [405]. After optimization, a biodiesel yield of 98.3% in 21 h was achieved. The authors also showed that the combination of Novozym 435 with other lipases with a similar regioselectivity to the lipase from *R. oryzae* showed similar synergies [405]. Later, the same group used rapeseed oil deodorizer distillate as raw material to produce biodiesel. This is a complex mixture of glycerides and free fatty acids, rich in phytosterols [406,407]. This makes the situation even more complex, as now the lipase must be selected to efficiently catalyze both esterification and transesterification reaction, making the concept of combilipases even more interesting. That way, biodiesel was produced using this substrate and methanol, achieving the one-pot esterification of the free fatty acids and the transesterification of the glycerides in a solvent-free system [408]. As catalysts, Novozym 435 and immobilized lipase from *Ps. cepacia* G63 were employed. The use of combilipases composed by both immobilized enzymes gave better results than the individual enzymes, and it gave an ester yield over 95% under optimal conditions. The process did not affect the phytosterols [408]. In a somehow similar situation, soybean oils with acid contents ranging from 8.5 to 90 and ethanol were used to produce biodiesel using Novozym 435, Lipozyme TL IM and Lipozyme RM IM [409]. Although Novozym 435 and Lipozyme RM IM were efficient in the decrease of oil acidity, a synergistic effect occurred when a combilipase using Novozym 435 and Lipozyme TL IM was used, doubling the ester production using an oil with an acidity value of 90.

### 6.1.3. Other Uses of Immobilized Combilipases
As in the case of the free enzymes, interesterification is one of the reactions where immobilized combilipases have been utilized. For example, the enzymatic interesterification of coconut oil and palm stearin was performed using Novozym 435, Lipzyme TL IM and Lipzyme RM IM [410]. Some dual combilipases, such as mixtures of equivalent amounts of Novozym 435 and Lipzyme TL IM or Novozym 435 and Lipzyme RM IM, presented a significant synergistic effect as well as an enhanced degree of interesterification. The authors show that the carrier material may play an important role. Combindilipases formed by immobilized lipases and non-immobilized lipase from Ps. fluorescens enhanced the activity of the free enzyme. A combilipase formed by 70% free lipase AK mixed with 30% of any of the immobilized lipases more than doubled the theoretical activity. The coimmobilization of the free lipase on the support was proposed to explain this effect, and that was shown by a reaction catalyzed by free Lipase AK-and an immobilized but inactivated lipase preparation [410]. However, this experiment could be explained by some role of the support where the lipase is immobilized on the reaction (e.g., perhaps facilitating the acyl migration), not necessarily by the immobilization of the enzyme, that may be hard during the reaction in such a complex medium (Figure 15). In another paper, extra virgin olive oil, tripalmitin, arachidonic acid and docosahexaenoic acid were utilized to produce structured lipids with high palmitic acid content at the sn-2 position enriched with arachidonic acid and docosahexaenoic acid [411]. This means that interesterification and acidolysis occurred and the researchers, among other possibilities, used a combilipase formed by Novozym 435 and Lipzyme TL IM to analyze if some synergistic effect could be found. In parallel, Novozym 435 was used to catalyze the interesterification reaction in a first step and Lipzyme TL IM was utilized to catalyze the acidolysis in a second step (using a sequential design). All products presented more than 50 mol % palmitic acid at the sn-2 position, but the use of one-pot approach and the combilipase made the reactions faster [411].

Novozym 435 and Lipzyme RM IM were used in the enzymatic synthesis of kojic ester via esterification of kojic acid and oleic acid [412]. This is a reaction where just a single modification of each substrate is intended, phenol hydroxyl group of kojic acid is not very reactive. After optimization, the best results were found using equal amounts of both immobilized lipases (ester yields were 70%). This is one of the few examples of the use of combiobiocatalysts in the case of monofunctional substrates, and may be a consequence of some of the changes in the reaction conditions explained in the Section 1.2.1 [412,413].

In another example, isosorbide diester plasticizer was synthesized using immobilized lipase from Yarrowia lipolytica Lip 2, Lipzyme RM IM or Novozym 435 [414]. The most efficient enzymes, immobilized lipase from Y. lipolytica or Lipzyme RM IM, did not produce the S-isomer. To avoid this limitation, the researchers used a combilipase mixing one of those immobilized enzymes with Novozym 435, greatly increasing the ester yields.

6.2. Use of Mixtures of the Same Lipase Immobilized Following Different Protocols: A Special Combilipase

Lipases, perhaps due to the flexibility of their active center and their mechanism of action, are among the enzymes whose properties may be more easily modulated via different strategies [415–420] as well as via immobilization [421–424] (Figure 17). It has been widely showed that changes in the immobilization protocol or the physical or chemical modification of the immobilized lipases may greatly alter the enzyme features [61]. Using the same immobilization mechanism, e.g., the interfacial activation of the lipase versus hydrophobic support surfaces [81,360], it has been shown that the change of the support features greatly affects the final enzyme specificity, activity and stability [425–427]—even immobilization of the same enzyme using the same hydrophobic support, but just changing the immobilization conditions gives very different enzyme properties [366–368] (Figure 19). In fact, it has been recently shown that the lipase from T. lanuginosus immobilized on a hydrophobic support under certain conditions was a strict 1,3 selective enzyme, being unable to hydrolyze 2-monoglycerides, while the enzyme immobilized under other conditions can hydrolyze 2-monoglycerides, and that also depended on the immobilization support [428,429].

In this context, Godoy and coworkers immobilized several lipases on Lewatit®VPOC1600 and Purolite®ECR1604 and used the biocatalysts to produce biodiesel from ethanol and palm olein [430].
Immobilizing the same lipase on these two supports, they found that the support affected the lipase performance. For example, using the lipase from *T. lanuginosus*, yields went from 78.2% (using Lewatit®VPOC1600) to 70.3% (using Purolite®ECR1604) [430]. This showed that the immobilization support affected the properties of the lipase as catalyst of biodiesel production, an already known fact [165,431,432] (Figure 17). The mixture of the individually immobilized biocatalysts produced better results than the use of each independently immobilized enzyme catalysts. Moreover, very interestingly, the authors of this paper showed that using the mixture of both biocatalysts of the same enzyme, the yields were better than using the best biocatalysts, and increased to 86.1% [430]. That way, immobilization following different protocols should produce enzymes with different catalytic properties, and we can call these “combilipases”. We have not found any other paper showing this fact.

7. Use of Coimmobilized Lipases

7.1. Coimmobilization of Lipases: Advantages, Problems and Proposed Solutions

Enzyme coimmobilization means the immobilization of different enzymes on the same particle (Figure 22). This coimmobilized enzymes are frequently used in cascade reactions, mainly because they provide a kinetic advantage, as the in situ production of the intermediate products allows expressing the activity of the intermediate enzymes from the beginning of the reaction, saving the lag time usually found in these cascade reactions [433–441] (Figures 22 and 23).

![Figure 22. Schematic representation of the use of coimmobilized and independently immobilized enzymes in a cascade reaction.](image-url)
The full modification (hydrolysis or alcoholysis) of oils and fats may be considered a cascade reaction, as it involves three consecutive modifications of the triglyceride (Figures 4, 6 and 8–10). If the use of mixtures of lipases improves the reaction course, this means that some of the glycerides (substrate or intermediate products) are not good substrates for the lipase that performs the reaction best with the main substrate components (Figures 4 and 6). The fact that perhaps all the substrate modifications may be catalyzed by a single biocatalyst is not enough to discard the possibility of some advantage of lipase coimmobilization, as some of the glycerides (initial substrate or intermediate products) may behave as inhibitors of the main enzyme, and its rapid elimination by other lipase will permit the expression of the maximum activity by the main enzyme.

However, coimmobilization of enzymes, and of lipases, has some problems [60]. The first one is the necessity of immobilizing all enzymes following the same protocol. Fortunately, for lipases, interfacial activation using hydrophobic supports is an almost universal and very good immobilization protocol that may be applied to most lipases [81]. The second problem is the possibility that the different lipases may present very different stabilities. This makes discarding all immobilized enzymes necessary when just one has been inactivated [60,442] (Figure 24). Using reversible immobilization methods, such as interfacial activation, the support may be recovered, but only in a very lucky case the most stable enzyme will remain on the support when the inactivated one is released from it. However, at least this will permit the reuse of the support (Figure 25). That means that coimmobilization of several enzymes should consider not only the advantages, but also the problems of the coimmobilization [287].
Figure 24. The problem of covalently coimmobilizing enzymes with different stabilities: both immobilized enzymes must be discarded when the least stable enzyme is inactivated.

Figure 25. The problem of coimmobilizing two lipases with different stabilities via interfacial activation on hydrophobic supports: when the least stable enzyme is inactivated, both enzymes may be released from the support enabling the reuse of the support.

Recently, some solutions have been designed to solve this latter problem, making the reuse of the most stable lipases after inactivation of the least stable ones possible. We will briefly present these strategies at the end of this section.

7.2. Use of Coimmobilized Combilipases in Biodiesel Production

We have found uses of coimmobilized combilipases only in biodiesel production. As in the case of the use of combilipases by mixing immobilized enzymes, S. W. Kim’s group initialized and spearheaded the use of coimmobilized combilipases in the production of biodiesel. In a first paper, a continuation of a previous paper where the use of non-immobilized lipases from *R. oryzae* and *C. rugosa* for degumming by the action of phospholipase A2 had given good results in the production
of biodiesel from crude canola oil, which had 100–300 ppm of phospholipids was presented using coimmobilized enzymes. To this goal, the enzymes were coimmobilized on silica gel [340]. After optimization, the ester yields reached a value of almost 90%. Next, they performed a study comparing a combilipase of immobilized enzymes and coimmobilized combilipases in the transesterification of soybean oil and methanol at two different pressures [443]. At atmospheric pressure, the initial reaction rates of both combilipases decreased when the methanol concentrations increased. However, under supercritical fluid conditions, the initial reaction rate of both combilipases (individually immobilized or coimmobilized) increased until methanol concentration became double the concentration of oil. Results pointed out that the coimmobilized combilipase had higher initial reaction rate, but the negative effects of methanol on enzyme stability were also higher than using the mixture of immobilized lipases [443]. Later on, after optimizing the coimmobilization process, the coimmobilized combilipase was used in two different reactors [444]. A continuous packed-bed reactor and a batch system with stepwise methanol feeding were utilized. In the last system, around 99% yield after 3 h of reaction was obtained and remained over 90% after 30 reuses. In a last paper from this group, the same lipases were coimmobilized on activated carbon modified with aminopropyltriethoxysilane and glutaraldehyde [445]. After optimization of the coimmobilization, the coimmobilized combilipase was used to produce biodiesel with very high yields using algal oil (93.8%), waste cooking oil (95.7%) and soybean oil (98.5%) after only 4 h of reaction.

Other research groups also used coimmobilized combilipases in the production of biodiesel. For example, lipase B from C. antarctica and lipase from R. miehei were coimmobilized on epoxy-functionalized silica gel using different enzyme ratios [446]. The transesterification of palm oil with methanol to produce fatty acid methyl esters catalyzed by these biocatalysts was optimized by response surface methodology and a composite rotatable design. The best ratio between both enzymes was 2.5:1 (lipase B from C. antarctica: lipase from R. miehei), giving an ester yield of 78.5% [446]. In a continuation of the work discussed in Section 6.2, lipases from T. lanuginosus and R. miehei were coimmobilized via interfacial activation on Lewatit®VPOC1600 and Purolite®ECR1604 [430]. The biocatalysts were used in ethyl ester production using palm olein as substrate. The authors described that coimmobilization improved the results of the use of mixtures of independently immobilized lipase (see Section 6.2). The support also has a great effect; the results obtained using Lewatit®VPOC1600 were better than those using Purolite®ECR1604. The best results were obtained with coimmobilized lipases on Lewatit®VPOC1600, biodiesel yield increased from 81.8% to 89.5% compared to the respective mixture of individually immobilized enzymes [430].

Protein-coated microcrystals are not a much-utilized immobilization strategy [389]. This immobilization technique consists in the use of water-soluble, micron-sized crystalline particles coated with the target enzyme. The biocatalysts are prepared in a one-step rapid dehydration process [447–449]. This strategy was used to prepare a coimmobilized combilipase coated microcrystals including lipase B from C. antarctica and lipase from R. miehei, using K2SO4 as the core of the particles, giving similar results to the best results obtained using the commercial immobilized enzymes (83% conversion in 48 h) [389].

Finally, the display of enzymes on the surface of surface of cells [400–403] has been employed to co-express and co-display lipase B from C. antarctica and lipase from T. lanuginosus on the surface of P. pastoris cell as biocatalyst for biodiesel production [450]. This permitted a 95.4% ester yield and a good operational stability.

7.3. Preparation of Coimmobilized Combilipases to Reuse the Most Stable Enzymes

As it has been explained in Section 7.1, enzyme coimmobilization has some drawbacks (Figures 24 and 25). These problems have not been considered in any of the above uses of combilipases. If they are not considered in the preparation of a biocatalyst, coimmobilization may afford more problems than advantages [60,287]. One of the points is that coimmobilization is only reasonable when that has some clear advantage over individual enzyme immobilization, e.g., in cascade reactions [60,287] (Figures 22–25). However, in some instances, the problems are ignored and several
enzymes are coimmobilized, even stating that the intention is not to produce a biocatalyst to catalyze cascade reactions in one pot, but to produce the so-called “multipurpose biocatalysts” [32,451].

This is the case of the preparation of a combiCLEA containing lipase, α-amylase, and phospholipase A2 [452]. The CLEA immobilization strategy is simple, but even using this strategy the optimal precipitant, crosslinking agent nature and concentration, feeding protein, etc., may be different for each specific enzyme [397–399] (Figure 21). Furthermore, it still has the problems of the coimmobilization and none of the gains, the recommendation should be to immobilize each enzyme in an individual way under optimal conditions [60,287].

This reuse of the most stable enzyme is usually ignored in most papers, where only in many few instances the stability of the different coimmobilized enzymes is even presented. However, the problem was clearly exemplified when lipase B from *C. antarctica* immobilized on octyl agarose was coated with polyethylenimine (PEI) and the lactase from *A. niger* was coimmobilized on it via ion exchange [442] (Figure 26). The lipase was much more stable than the lactase, in a way that remained fully active when the lactase was almost fully inactivated. However, thanks to the different immobilization strategies employed for each enzyme, the inactivated lactase could be released to the medium after its inactivation without affecting the activity of the immobilized lipases. Just by incubation at high ionic strength to release the inactivated lactase, the immobilized lipase could be reused for many cycles involving lactase inactivation/lactase desorption/PEI recoating of immobilized lipase/new batch of lactase immobilization [442] (Figure 26). The reuse of a support may not compensate the costs of the recycling process, but in this case, by just an incubation at high ionic strength, the immobilized lipase could be reused for many cycles, and this may have a higher economical interest. The lipase coating with PEI produced an increase in lipase activity and stability [378,380], and even the treatment with glutaraldehyde to prevent the PEI release during lactase desorption produced some positive effects [379], making this coimmobilization strategy very suitable [453].

![Figure 26. A strategy to coimmobilize two enzymes that permit the reuse of the most stable one combining immobilization via interfacial activation of the most stable enzyme, immobilized enzyme coating with ionic polymers and ion exchange immobilization of the least stable enzyme.](image)

When the researchers analyzed the stabilities of some of the most used lipases, they found a great variety in lipase stabilities, and in fact, some lipases immobilized on octyl agarose via interfacial activation [360] remained fully active under conditions where other lipases were fully...
inactivated [454,455]. Thus, the utilization of different strategies to prepare a coimmobilized combilipase biocatalyst to reuse the most stable lipases has sense. First of all, it was shown that the use of PEI as coupling agent permitted to prepare multilayers of the same lipase [456,457] or of different lipases [458] (Figures 27 and 28). A problem found when using some lipases was that the enzymes already immobilized on enzyme-PEI composites the enzyme was released when treating with PEI to immobilize a new lipase layer, requiring to treat the biocatalyst with glutaraldehyde to prevent enzyme release via covalent enzyme–polymer crosslinking [457]. Apparently, this may not look a coimmobilized combilipase when using the same lipase. However, the strategy presented finally three different immobilized lipase forms. The lipase in the bottom layer was the lipase immobilized via interfacial activation on octyl agarose, modified with PEI and glutaraldehyde, the second lipase layer (and all intermediate lipase layers) was the lipase immobilized via ion exchange, modified with glutaraldehyde and PEI, while the last layer, if desired, could be immobilized via ion exchange but without any other further modification [457,458] (Figure 27). As explained above, the use of mixtures of the same lipase immobilized following different protocols permitted to have better results than the use of single immobilized catalysts [430], and we can call this “combiplase”, as we have different lipase forms [61].

**Figure 27.** Preparations of a multilayer biocatalyst with the same enzyme employing PEI and glutaraldehyde as glue. The result is a combilipase bearing three lipase forms.

This enzyme layer by enzyme layer strategy, when immobilizing different enzymes, permitted to control the spatial distribution of the different enzymes, until five different lipases were immobilized using different spatial distribution, with very different impact on the final biocatalysts activity versus different substrates [458] (Figure 28).
Figure 28. Coimmobilization of several enzymes with controlled spatial distribution using a multilayer strategy.

Following the same strategy utilized to coimmobilize lipases and lactase [442] (Figure 26), several very stable lipases (lipases A and B from *C. antarctica* and lipase from *T. lanuginosus*) were immobilized on octyl-divinylsulfone agarose, treated with PEI and coimmobilized with several less stable enzymes (lipase from *R. miehei* and Lecitase Ultra) via ion exchange [459]. The most stable lipases could be reused for several cycles of stress inactivation of the least stable lipases/release of these inactivated enzymes/recoating of the immobilized stable enzymes with PEI/immobilization of a new batch of the non-stable lipases [459] (Figure 26).

Another strategy that permitted the reuse of the most stable lipase [454], this time using the advantages of the lipase immobilization via interfacial activation for all lipases [81], is based in the use of heterofunctional supports [359], using supports with hydrophobic acyl chains to get the interfacial activation of the lipase [360] and reactive groups able to give the covalent lipase immobilization [369]. The event that permits the first immobilization of the lipase on these supports is the lipase interfacial activation, and later, the formation of some enzyme-support covalent bonds may occur [369] (Figure 20). The most stable lipase is immobilized on the heterofunctional support, after getting some enzyme-support covalent bonds, the other groups in the support are destroyed, and the least stable enzymes may be immobilized just via interfacial activation [454,455]. This permitted the release of the least stable lipases after its inactivation by incubation in the presence of detergents [81], and enabled the reuse of the most stable lipases, that are covalently immobilized. Several lipases with similar stabilities may be immobilized using the same immobilization strategy. The main problem of this strategy is the release of all detergent molecules from the immobilized lipase biocatalysts [454].

However, these biocatalysts have not been assayed in the production of biodiesel or free fatty acids, all the utilized coimmobilized combi-biocatalysts have not considered these problems of the enzyme stability of the different biocatalysts.
8. Conclusions

This review shows how combilipases may have a great potential in the development of modifications of heterogeneous substrates. In fact, it looks an obvious solution to optimize this kind of processes. The improvements in free fatty acids or biodiesel production have been clearly illustrated because it is unlikely that a single lipase can modify all the different triglycerides in an oil, and less considering the partial glycerides produced during the reaction. Although we have been only able to find one case, the use of this combilipase concept may also have extension to monofunctional substrates, as the reactions conditions will change always during the reaction and this can be beneficial for some enzymes and negative for some others.

As expected, the use of immobilized enzymes offers some advantages compared to the use of the free enzymes, as recycling is simpler, the enzymes become more stable, and diverse reactor configurations may be utilized. Nevertheless, immobilization must be carefully designed, if we can really improve most enzyme features and not just facilitate enzyme recycling, immobilization advantages may benefit from their costs or derived problems. Moreover, although this has been only shown in one example, the preparation of combilipases using just one lipase but immobilized using different protocols may become an easy way to take the advantages of this idea. Our expectations are that this idea may be disseminated rapidly and many examples will be available in the future.

Another different point is the use of coimmobilized lipases. Considering just the problems derived from coimmobilization, it is necessary to consider if the gains are higher than the losses. Fortunately, lipases have been the model enzymes to design some strategies that solve the problem of enzymes bearing different stabilities. There are already strategies that permit to reuse the most stable enzymes after inactivation of the least stable enzymes. In fact, it has been showed that a combilipase may be built by immobilizing different layers of the same enzyme submitted to different modifications.

The authors of this review foresee that the use of combilipases, and combienzymes in general, will be more generalized in the near future, as the number of problems that this can solve in a reaction are many, although the use of several enzymes may complicate the design of the processes. Very likely, even some relatively simple processes will benefit from the use of mixtures of enzymes, having different responses to changes in the conditions of the medium, inhibition, specificity or selectivity. This is a reality in the case of oil and fats modification and combilipases, but we are convinced that the concept of optimal enzyme for a given process may be changed in the near future for the concept of optimal combienzyme. The formulation of these combienzymes may be in free, immobilized or coimmobilized enzymes forms, but also in combinations of different formulations.

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