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Review

Moving towards a Competitive Fully Enzymatic Biodiesel Process

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Abstract: Enzymatic biodiesel synthesis can solve several problems posed by the alkaline-catalyzed transesterification but it has the drawback of being too expensive to be considered competitive. Costs can be reduced by lipase improvement, use of unrefined oils, evaluation of soluble/immobilized lipase preparations, and by combination of phospholipases with a soluble lipase for biodiesel production in a single step. As shown here, convenient natural tools have been developed that allow synthesis of high quality FAMEs (EN14214) from unrefined oils in a completely enzymatic single-step process, making it fully competitive.

Keywords: biodiesel; lipase; enzyme improvement; crude oil; enzyme immobilization; transesterification; phospholipase; degumming

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1. Introduction

Bio-inspired processes or enzymatic reactions have a low environmental impact, reduce the amount of waste material and can minimize costs, thus serving the requirements to integrate environmental sustainability with economic growth and welfare. The global economic and energy crisis has given new impetus to the search for sustainable energy alternatives, and research in the area has significantly increased in the last years, including biodiesel as one of the targets. Biodiesel is defined as the mono alkyl ester of long chain fatty acids derived from vegetable oils or animal fats, for use in

compression-ignition engines [1]. Essentially all industrial biodiesel is produced nowadays using chemical catalysts [2]. The reaction for the chemical synthesis of biodiesel (transesterification) is the conversion of vegetable oils or animal fats into methanol (FAME) or ethanol (FAEE) esters. This reaction proceeds very efficiently in an alkaline medium by addition of sodium methoxide, sodium or potassium hydroxide, or by acid catalysis, usually with sulfuric acid [2]. However, this process requires efficiently pre-treated (degummed or refined) raw materials, is affected by the presence of water in the sample, and cannot make use of the free fatty acids (FFAs) contained by most crude oils [3], being thus difficult to achieve sufficiently high degrees of conversion [2,4]. Moreover, the chemically catalyzed process releases low value glycerol, and generates large volumes of effluents containing high quantities of catalyst and other chemicals. Therefore, chemical biodiesel may not accomplish the requirements for a clean biofuel production.

By contrast, the use of lipases for biodiesel synthesis (Figure 1) has become increasingly important [5–7]. Enzymatic production of biodiesel with lipases has received great consideration in recent years and is undergoing a rapid development [8,9].

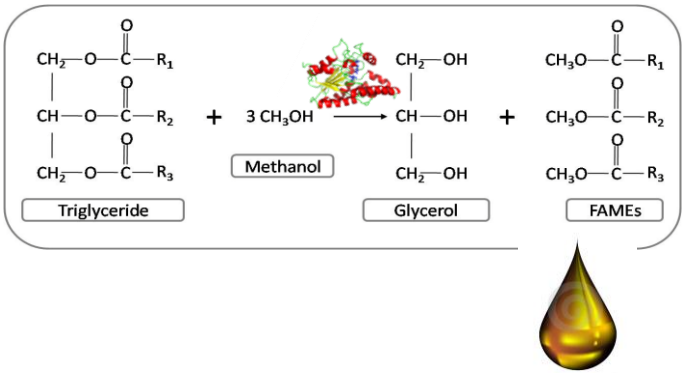

Figure 1. Schematic model of the enzymatic transesterification reaction for FAMEs synthesis from triacyl-glycerides.

Lipase-mediated transesterification can be carried out under mild reaction conditions to produce high quality biodiesel free of contaminants, favoring also an easy recovery of glycerol, without need for purification or chemical waste release [2,10]. In addition, the enzymatic process tolerates the common water content of oil and increases the biodiesel yield by avoiding the typical soap formation due to alkaline transesterification [11]. However, the enzymatic method has only scarcely been industrially adopted, mainly due to the still high costs of both, the immobilized enzymes traditionally used, and the raw materials [2,12].

2. The Enzymes

The enzymes directly involved in biodiesel synthesis are lipases (EC 3.1.1.1). These enzymes are versatile and powerful tools in biotechnology [13], capable of carrying out reactions involving insoluble lipid substrates [14,15], and preserving their catalytic activity in organic solvents [16]. Lipases can catalyze not only hydrolysis reactions, but also participate in various reverse reactions such as esterifications or transesterifications in low water systems or in anhydrous organic solvents [16,17]. Moreover, lipases can perform reactions with high specificity, regioselectivity and enantioselectivity,

becoming the most used enzymes in synthetic organic chemistry [13,18–20]. Therefore, there is substantial interest in developing appropriate lipase preparations for their use in biodiesel synthesis [21,22]. As an initial approach, the study of biodiversity is a valuable tool for the discovery of new lipolytic enzymes useful for biocatalysis [20,23,24]. However, performance of an enzyme that is active in a given reaction is not always sufficient for its application in an industrial process [13,25]. Thus, new strategies such as process modification or enzyme improvement are required for optimal yield [26]. Various strategies may be run in parallel to obtain the required biocatalyst in a defined process. For example, medium engineering or enzyme immobilization can be assayed [13]. Alternatively, the enzyme can be modified by either rational protein design [27] or by directed evolution [28,29], or both [30]. In this context, a previously isolated and characterized lipase, LipC from *P. aeruginosa* 42A2 [31] was improved for evaluation in biodiesel production, using rational design and saturation mutagenesis [32].

2.1. Improvement of LipC Thermoresistance

The cold-activity shown by LipC [31] provides this lipase a valuable property that could be used in low–medium temperature operating systems (4–25 ℃). However, a higher thermostability of the enzyme would be required for long-term process in many applications, such as biodiesel production. For such purpose, a rational design strategy to improve LipC thermostability without compromising its cold-adapted properties was developed. In general, the structure of cold-adapted enzymes is more flexible compared to those of their thermostable homologues [33,34]. Thus, introduction of appropriate mutations at the structure sites displaying a high degree of flexibility [32,35] should provide higher compactness and thus more resistance to higher temperatures. As the 3D structure of LipC is not known, the choice of the appropriate sites on this lipase was performed by executing B-FITTER [36] on the known structure of homologous *P. aeruginosa* mesophilic LipA (55% protein identity) [37]. Most of the residues displaying a high degree of flexibility in the crystal structure of LipA [37] were also found in LipC, thus being good candidates for modification, and chosen to design focused saturation mutagenesis libraries. Following flexibility criteria, eight focused libraries (Figure 2) using different codon degeneracies were constructed involving one or two amino acid positions.

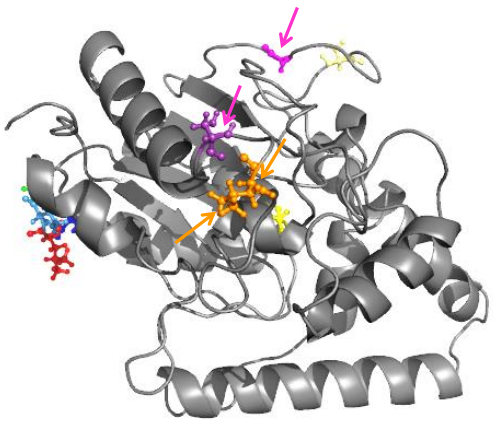

Figure 2. Structural 3D model of *Pseudomonas* sp. 42A2 LipC displaying the sites chosen for saturation mutagenesis. Site-directed saturation mutagenesis was performed on the highlighted residues after rational protein design. The two residues modified in thermostable mutant D2_H8 are depicted by arrows and colored in orange and purple.

A total of 3256 recombinant clones bearing activity on tributyrin agar plates at 4 °C were obtained and screened for activity prior and after heat shock (15 min, 60 °C). *Pseudomonas* PABST7.1 pBBlipCH, bearing wild type LipC and used as a control, showed an average 7.6% residual activity after the heat shock. Among the clones screened, five potential variants were obtained with higher residual activity at 4 °C than wild type LipC after 15 min treatment at 60 °C. Among them, variant D2_H8 displayed a residual activity after heat shock of 49%, seven times higher than that shown by wild type LipC after the same treatment, and was selected as the best thermostable variant. Analysis of the DNA sequence of modified *lip*C gene in variant D2_H8 revealed two substitutions in residues Leu^{288} and Ser^{291}, which were replaced by an alanine and a glutamic acid, respectively. The 3D model structure of variant D2_H8 was constructed and overlapped with that of wild type LipC, and their flexibility parameters were compared. No evident structural changes were detected between them in the conformation of the loop involving residues Leu288Ala and Ser291Glu, but flexibility was found to be slightly lower for mutant D2_H8 than for wild type LipC, showing global B-Factors (obtained from B-Factor putty in PyMol) of 25 and 28.9, respectively.

Re-evaluation of variant D2_H8, bearing two amino acid changes and showing higher thermal stability, revealed no significant changes in optimum temperature, still found between 4–20 °C. The substitutions occurred in this variant produced the desired modifications to make the enzyme more compact and thermostable, probably due to replacement of a serine, a hydrophilic amino acid by a glutamic acid, capable of forming salt bridges and to bind metal ions, causing charge changes in the environment of the region and probably allowing interactions with neighboring residues [38]. This mutant was the result of three levels of selection pressure applied during the screening: (i) selection of only active LipC mutants (tributyrin plates); (ii) selection of active mutants at 4 °C (temperature employed for screening assays); and (iii) selection of active mutants at 4 °C displaying an increased thermal stability. Taking into consideration that increased thermal stability and low optimum temperature may be regarded as apparently contradictory conditions for an enzyme [39], isolation of variant D2_H8 (named LipCmut), with a seven-fold thermostability increase over native LipC while maintaining the optimum temperature at 4–20 °C, allowed generation of a stable and biotechnologically well suited cold-active LipC variant bearing the expected properties, and also showing the capability of using longer carbon chain-length (C_{10}, C_{12}, C_{18}) substrates at higher temperatures, as previously reported [31]. To our knowledge, this was the first time that a cold adapted enzyme is converted into a thermostable variant maintaining its optimum activity at 4 °C.

2.2. Lipase Immobilization

Enzyme immobilization is a well-reported technology that allows application of enzymes in many biocatalyzed processes like in lipase-mediated biodiesel production [9,22,40]. In general, immobilization allows reuse of the biocatalyst, makes the product recovery easier, and frequently enhances enzyme resistance against inactivation by different denaturants, providing more stable and efficient catalysts [41,42]. Nevertheless, many immobilization procedures use sophisticated protocols for lipase entrapment on expensive supports [43], not always suitable for a real scaling-up, and causing an increase in the costs of industrial processes [44,45]. On the contrary, simple enzyme adsorption allows preparation and use of immobilized lipases under mild conditions without significant loss of activity [46], and the associated

process is very simple, with the carrier being easily recovered for repeated immobilization rounds [22,46]. Therefore, four non-commercial lipases from *Pseudomonas* species—LipA and LipC from *P. aeruginosa* 42A2 [31], LipCmut, the thermostable mutant of LipC previously described [47], and LipI.3 from *Pseudomonas* CR611 [21], which were produced in either homologous or heterologous hosts—were prepared, immobilized on different cheap supports, and examined for potential biodiesel production. The enzymes were obtained directly from crude cell extracts or from growth culture supernatants, and further immobilized on different carriers: Polypropylene polymers like Accurel EP100 or Accurel MP1000 [48,49] and a silica powder, Celite®545 [50] by simple adsorption. In this case, best immobilization was obtained on polypropylene supports: Accurel EP100 for LipA and Accurel MP1000 for LipC and LipCmut. Lip I.3, requiring a refolding step [21], was poorly immobilized on all supports tested (best results for Accurel MP1000). The behavior of all four immobilized lipases was tested for triolein transesterification, the best results being obtained for lipases immobilized on Accurel MP1000. Therefore, a new protocol was introduced for immobilized lipase production which does not require protein purification and uses crude enzymes immobilized by a fast adsorption technique on low-cost supports, making the method suitable for an eventual scaling up aimed at biodiesel synthesis. The low price of the supports tested and the simplicity of the procedure, skipping the tedious and expensive purification steps, will certainly contribute to cost reduction in biotechnological lipase-catalyzed processes.

3. The Feedstocks

Nowadays, biodiesel is mainly produced by transesterification of edible oils such as soybean, rapeseed, sunflower or palm oil [51], which currently account for over 85% of production costs [52]. Selection of the appropriate raw material is of major importance for ensuring the feasibility of the process at industrial scale [53]. Thus, exploring the use of low-value or non-edible feedstocks (Figure 3) for biodiesel synthesis is a goal for innovation among biodiesel-producing partners. Besides waste cooking oils or animal fats, the biodiesel fuel market can also use unrefined oils [54,55] like crude soybean or canola oils (Figure 3).

Non-refined (crude) soybean oil is an alternative solution already investigated for enzymatic transesterification [55–57]. However, crude vegetable oils, also referred to as non-degummed oils, contain relatively high amounts of FFAs, plus certain impurities such as phosphorous compounds known as hydratable phospholipids or phosphatides, that may inhibit the transesterification reaction.

During an alkaline catalyzed chemical biodiesel production, FFAs react with the catalyst to produce soaps that induce formation of emulsions, making the final separation process very difficult. Furthermore, soaps can bind to the catalyst, and addition of extra catalyst is necessary. This is one of the main reasons why when waste oils are used as feedstocks, they require alternative and more complicated processing, since e.g., used cooking oil contains as much as 15% FFAs that would cause an important loss in the process yield [58]. Thus, the FFAs content in oil feedstocks should be as low as possible (less than 0.5%–2.0% *wt*) in alkaline-catalyzed transesterification reactions [59]. In contrast, enzyme catalysis is usually insensitive to high FFAs concentrations [60] as they can also be used as substrates and converted into fatty acid alkyl esters (FAAEs) through esterification reactions [61]. In this case, the enzyme-catalyzed esterification rate and yield depends on the lipase employed [62].

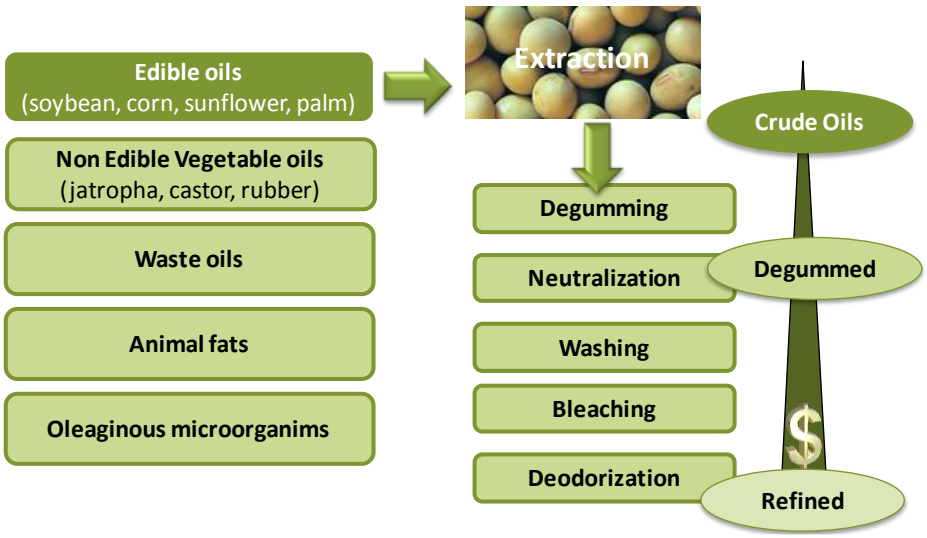

Figure 3. Putative feedstocks for biodiesel production. Edible oils must go through a refining process that significantly increases their costs. Refining is also a requirement for chemical biodiesel production but not for the enzymatic process, which can proceed even in the presence of FFAs and phosphatides. For other oils, only extraction would be required for the enzymatic process. Therefore, as shown here, the use of crude oils may contribute to high cost reductions, thus making the enzymatic process more economically competitive.

Concerning phosphatides, their content in e.g., crude soybean oils may vary from 0.5%–3% *w*/*w* [63], corresponding to a phosphorous content in the range 250–1200 ppm. These phospholipids can cause oil storage problems since they precipitate forming a gum, and produce water accumulation. Presence of phospholipids is thus a problem for alkaline biodiesel production, but not for the enzyme-catalyzed process, where, in general, it is not necessary to completely refine crude oils [64]. However, phospholipids have been described as inhibitory substances in transesterification reactions catalyzed by immobilized lipases. In that case, inhibition is due to formation of a film around the support of the enzyme that prevents proper interaction of the lipase with the substrate [56]. Therefore, for immobilized lipases, a degumming would be the only required pre-treatment for enzymatic transformation of vegetable oils into biodiesel, with no further refining steps being necessary [56]. The possibility of using unrefined, low price oils would result in a dramatic reduction of the global costs of enzyme-catalyzed biodiesel process because the yield loss from the refining/degumming is eliminated.

4. Lipase-Catalyzed Biodiesel Synthesis

Many lipases from different sources have been studied, improved and tested for biodiesel synthesis [51]. In general, this process has been performed using immobilized lipases [9]. However, the latest results promote the use of soluble enzyme preparations to reduce the cost of the enzymatic preparations and to make the whole process more competitive with respect to the chemical systems. Some examples of immobilized and soluble lipase preparations evaluated for FAMEs synthesis are presented below.

4.1. Evaluation of Soluble and Immobilized Lipases

The soluble and immobilized preparations of LipA, LipI.3 and the cold-adapted lipases LipC and its thermo stable variant LipCmut described in Section 2.2 were evaluated for biodiesel synthesis. Although cold adapted enzymes can provide significant savings due to the possibility of performing processes at much milder conditions, the use of psychrophilic lipases is a rare phenomenon among previously described industrial processes [65]. For evaluation purposes, these lipases were over expressed and produced in recombinant hosts using a low-cost preparation protocol to further reduce costs of eventual scaling-up. The enzymes were obtained from crude cell extracts or from growth culture supernatants without any further purification step. When required, they were immobilized by simple adsorption on Accurel EP100, Accurel MP1000 and Celite®545 [66], and several conditions of transesterification were tested to evaluate FAMEs production using water-degummed soybean oil.

Prior to FAMEs synthesis, the immobilized and soluble forms of the lipases were assayed for activity, thermoresistance, and methanol tolerance. After 1 h incubation between 4 and 37 °C, the cold adapted lipases LipC and LipCmut maintained more than 65% activity [47], while, as expected, thermoresistant LipCmut displayed a significantly higher resistance at 50 °C, showing good tolerance to the methanol concentrations tested. Reactions for FAMEs synthesis were carried out at different ratios of water and methanol to set up the conditions for the highest yield. A great FAMEs increase with the amount of water content in the reaction was observed. Immobilized LipCmut reached the highest production, with 25% FAMEs release using 7.5% MeOH and 10% water. For reactions carried out with the lipases in soluble form, lower FAMEs synthesis was achieved (5.4% for LipC and 11.6% for LipCmut) at their best reaction conditions. Nevertheless, when the loading of soluble enzymes in the reaction was increased to 10% (*v*/*v*), FAMEs production was comparable to that obtained for the immobilized forms. Thus, these results open up the possibility to perform transesterification reactions using soluble enzymes and at low temperatures, a procedure that could help to reduce the costs of enzymatic biodiesel processes. No matter the moderate efficiency of LipC and LipCmut preparations for FAMEs production, it must be highlighted that in the literature very few works can be found related to biodiesel synthesis using psychrophilic lipases [65]. Use of cold-adapted enzymes in biodiesel production would allow reducing the reaction temperature, with significant economical savings, and could contribute to the design of innovative synthesis systems for alternative chemicals production.

4.2. Testing Soluble Lipase Callera Trans L

Although the use of immobilized lipases for FAMEs production has been extensively reported [67,68], they show several drawbacks: high costs of the enzyme, use of expensive supports, and the immobilization process itself. For instance, the price of immobilized Novozym 435 is very high and would require many re-uses (typically, more than 100 cycles) to become cost-efficient for bulk biodiesel production. On the contrary, soluble lipases can be produced and sold at a much lower price (30–50 times lower; P. M. Nielsen, personal communication) and can also be re-used for several times after recovery from the water phase, which makes them cost-efficient compared to immobilized enzymes. Moreover, soluble lipases can also be used under mild reaction conditions, have a faster reaction time, display higher conversion rates than immobilized enzymes [69], and allow, without any FAMEs yield loss, the

presence of water in the process, which is required for oil degumming or during downstream steps [57].Therefore, the use of soluble lipases on low cost, unrefined oils, will launch the enzymatic FAMEs synthesis process into the range of the most competitive chemical systems for fuel production [70].

Up to date, however, soluble lipases have scarcely been tested in transesterification [70–72] due to their water requirement. But in fact water would not inhibit transesterification if used at low concentrations, as it is essential to maintain the specific tridimensional structure of the lipase, especially if used in soluble form [58]. Accordingly, a new liquid lipase (Callera Trans L, Novozymes) was tested to obtain FAMEs from crude, non-degummed soybean oil. A successful conversion into FAMEs (96% *w*/*w*) was obtained after 24 h reaction at 35 ℃ in 200 rpm agitation in the presence of water (3%–15%). Moreover, a general good reduction of free fatty acids in the final product was produced, especially in the two lower (3%–5%) water systems, where Callera Trans L produced an exceptional 96.3% and 95.6% FAMEs release, a transformation never found before for non-degummed oils.

To evaluate the mode of action of Callera Trans L, variation of FFAs, FAMEs and acyl-glycerides during the reaction was determined by acid titration, GC and HPLC. A clear increase of FFAs during the first 3 h was observed, suggesting that the reaction takes place through a first step of enzyme-catalyzed triacyl-glycerides (TAGs) hydrolysis that releases FFAs (Figure 4). As the reaction continues, the concentration of FFAs decreases, suggesting that once the acyl-glycerides have been hydrolyzed, the released FFAs are further esterified with methanol by the enzyme into the corresponding FAMEs. In that case, FFAs would be considered the intermediates of the reaction [70], which probably proceeds through an initial hydrolysis reaction where TAGs are hydrolyzed to DAGs, MAGs and FFAs, followed by the subsequent esterification of the FFAs derived from the complete acyl-glycerides hydrolysis, as previously suggested [70]. Evidence for this mode of action was obtained through HPLC analysis where TAGs are hydrolyzed by Callera Trans L into DAGs, MAGs and FFAs during the first 5 h of reaction at all water concentrations. This hydrolysis is also favored by the low initial methanol concentration, added step-wise during the reaction. The FAMEs appeared during the first 5 h (Figure 4) are probably due to a true transesterification activity of the enzyme, whereas at longer reaction times, when TAGs have almost disappeared, esterification activity is predominant and FAMEs formation derives from the FFAs generated by the complete hydrolysis of TAGs, DAGs and MAGs. This effect was particularly evident at 3%–5% water concentrations, where FAMEs production by Callera Trans L was more effective (Figure 4).

These results show not only the ability of soluble Callera Trans L for FAMEs synthesis but also its double hydrolysis and esterification activities in low water systems. The use of a soluble instead of immobilized lipase resulted in a significant simplification of oil transesterification and is the background for the new, cost-effective process developed by Novozymes using liquid lipase Callera Trans L, which is now being used at several production plants for biodiesel synthesis from different feedstocks [73]. In the first quarter of 2014, both Blue Sun Biodiesel in St. Joseph, Mo., and Viesel Fuel LLC in Stuart, Fla., announced the full-scale production of biodiesel based on lipase as catalyst.

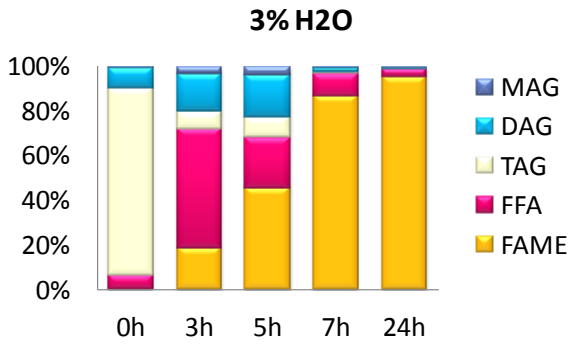

Figure 4. Mode of action of lipase Callera Trans L when used in a low water system (3% water). From the HPLC analysis of the reaction products, it can be concluded that an initial hydrolysis releases FFAs, MAGs and DAGs from the original oil (mostly TAGs). All these products are good substrates for Callera Trans L, which then proceeds to FAMEs formation through esterification of the FFAs with methanol. Although most of the reaction occurs through hydrolysis followed by esterification, the results indicate that some transesterification occurs as well since the beginning of the reaction, where the enzyme uses the TAGs present in the oil for FAMEs synthesis. Reaction conditions were: crude soybean oil (7.8% FFAs; 690 ppm P); 1.5 equivalents. MeOH added at t = 0, 3 h, 5 h, 7 h; 1% *w*/*w* Callera Trans L; T = 35 °C; mixing: 200 rpm; and reaction time: 24 h.

5. Degumming

As mentioned in Section 3, crude, non-degummed oils contain impurities like phosphorous compounds (phosphatides), which have to be removed for efficient transesterification. Oil degumming is a requirement to obtain refined, edible oils, but it is also essential for biodiesel production using immobilized lipases [56]. Moreover, according to the legal specifications, phosphorus concentration in final biodiesel must be reduced to less than 10 ppm (EN14214:2003; ASTM D6751:2012). For biodiesel synthesis, a high content of phospholipids in the feedstocks means a concomitant loss of yield in FAMEs production, as the fatty acids enclosed into the phospholipid molecules are not accessible to the lipase for transesterification, and the gums entrap oil when it is separated. In fact, degumming represents a 2.5% loss of total oil; being the current market price $1,100 per ton, this corresponds to a loss of $27.5 per ton of oil. Depending on the raw materials used, degumming becomes a requirement for biodiesel synthesis to achieve phospholipids removal and to reduce the final phosphorus content below the specified limits. In addition to an extra tank, the degumming process involves the use of acids and high temperatures, all factors boosting the process costs and health risks.

Phospholipids are commonly removed by previously developed refining/degumming processes of physical or chemical nature. In the 1990s, the first industrial enzymatic oil-degumming process was launched, the Lurgi's Enzymax®, based on phospholipase conversion of non-hydratable phospholipids (NHPs) to hydratable lyso-phospholipids [74]. NHPs, which consist on phospholipids combined with metal cations such as Fe, Ca or Mg (Figure 5), can be chemo-enzymatically converted into hydratable compounds and simply removed by water washing and centrifugation [75].

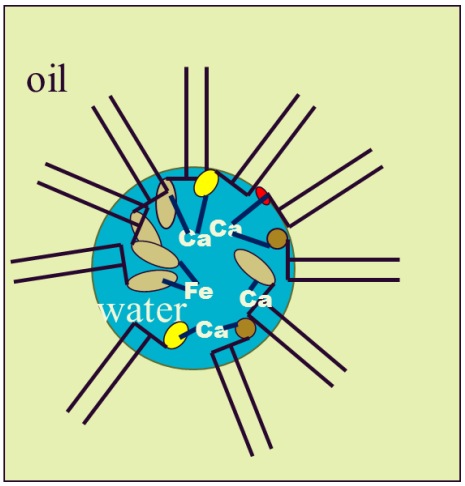

Figure 5. Schematic representation of phospholipids combined with metal cations as can be found in most unrefined, non-degummed oils. In order to disrupt the phospholipid micelles for gums removal, ions must be chelated, a process that involves acid treatment with high-shear mixing of the oil at high temperatures. Once the micelles are disrupted, the phosphorous group can be removed to produce DAGs with a more hydrophobic moiety, adequate for enzymatic FAMEs synthesis.

The easily hydratable phospholipids are phosphocholine (PC), phosphatidylinositol (PI) and lysophosphatidylcholine (LPC), whereas phosphatidilethanolamine (PE) and phosphatidic acid (PA) are generally considered as NHPs [76]. The proposed industrial enzymatic degumming [63] involves a first step of citric acid treatment with high-shear mixing at high temperatures to chelate the metals and to open the phospholipid micelles (Figure 5). After cooling down, degumming can be completed with a final phospholipase treatment. For this purpose, different types of phospholipases can be applied depending on the acyl ester bond they hydrolyze in the phospholipid molecule [77]: Phospholipase A_1 (PLA_1), which catalyzes the hydrolysis of the fatty acid at position sn-1 in the phospholipid molecule releasing a lyso-phospholipid (Lyso-PL) and a free fatty acid (FFA), has already been applied to the degumming process [63,77,78] also in combination with phospholipase C (PLC), which cleaves the phosphorus–oxygen bond between glycerol backbone and phosphate, releasing a diacylglycerol (DAG) and the phosphate ester group [79]. Since phospholipase-aided degumming requires the presence of water in the reaction mixture and Callera Trans L can efficiently be used for FAMEs synthesis in low-water containing systems, a more cost-effective biodiesel production process could be performed by combination of enzymatic degumming and transesterification of crude feedstocks using phospholipases and Callera Trans L (Section 4.2) in a *single-pot* reaction.

5.1. Combined Enzymatic Degumming and Transesterification

The possibility of using efficient soluble lipases like Callera Trans L allows combination of the required steps for enzyme-catalyzed synthesis of biodiesel from crude oils: Phospholipase-catalyzed oil degumming followed by lipase-mediated synthesis of FAMEs. The use of water, required in the degumming step, would not inhibit transesterification with Callera Trans L, as water is tolerated by this enzyme [57]. To search for such a more sustainable and competitive process, two phospholipases (PLA_1

and PLC), a lyso-phospholipase (LPLL-2) and their combinations with liquid lipase Callera Trans L were tested to unify the enzymatic oil degumming with the transesterification (Figure 6), aimed at producing a final biodiesel that meets the phosphorus legal limits. The two processes were studied combined or separately using crude soybean oil to define the optimal conditions for each parameter and enzyme.

As stated in Section 5, citric acid is used as helper in degumming for disruption of phospholipid micelles due to its chelating action towards binding ions [63,78]. Therefore, influence of citric acid on lipase activity was studied. A clear negative effect of citric acid on transesterification was observed, with maximum FAMEs production of 67%, too low for a competitive biodiesel process. On the other hand, the effect of methanol on phospholipases was tested. For these assays, the degumming process included phospholipases and the conventional citric acid step, carried out on crude soybean oil with or without addition of methanol. Higher phosphorus values were found in the oil phase for all enzyme mixtures when assayed in the presence of methanol, and the same results were obtained when control samples containing only crude soybean oil were assayed with (575 ppm P) and without methanol (64 ppm P). This suggests a direct action of methanol on removal of the phospholipids from soybean oil and shows that phospholipases are not inhibited by this solvent. In fact, for crude soybean oil, when methanol was present, the oil phase was completely clear and no sediment (gums) could be observed, contrarily to what happened to acid degummed or untreated samples, which displayed some turbidity or a sediment, respectively [80]. Therefore, methanol can solubilize the phospholipids of crude soybean oil [81], constituted mainly by PC (and to a lower extent by PE), described as easily hydratable phospholipids and also soluble in short-chain alcohols like ethanol [82]. The evidence of solubilization of phospholipids by methanol is of great importance in the process. It means that gums can be removed with no requirement for a conventional acid treatment, thus making the released phospholipids more available for enzymatic treatment, that is, methanol seems to break apart the micelles, contributing to the release of extra FFAs (Figure 6). Therefore, presence of methanol may allow skipping the acid degumming step without any loss of performance and it can concomitantly provide more FFAs to the reaction for a higher FAMEs yield.

Since citric acid has a negative effect on transesterification, and taking into consideration the role of methanol in phospholipid solubilization, enzymatic degumming with phospholipases was further coupled to transesterification in the same batch, using Callera Trans L best operating conditions (24 h incubation, 35 °C, 250 rpm, 24 h), with no need for a conventional citric acid treatment. Performance of different types of phospholipases with Callera Trans L for FAMEs production and phosphorous decrease was tested (Table 1). The high P concentration (823 ± 56 ppm) found in the oil phase when only transesterification, used as a control, was run is remarkable. In this case, the phosphorus content was approximately the same as that of raw material (823 ppm), evidencing that Callera Trans L does not have any phospholipase activity. The transesterification yield of Callera Trans L reached >95% FAMEs production when phospholipases were added to the reaction mixture, where concomitantly a dramatic phosphorus content decrease, below 10 ppm, was found (Table 1). A higher FAMEs production was obtained in samples containing combinations of phospholipases, probably due to a synergic effect between phospholipases themselves and the lipase. Therefore, phospholipase activity leads to a gain of oil useful for lipase-mediated transesterification. This means that beyond the reduction of phosphorus in the final biodiesel, phospholipases contribute to the release of FFAs from phospholipid molecules, which can then be used by the lipase for increased FAMEs synthesis (Figure 6). When a lyso-phospholipase (LLPL-2) was added to help PLA_1 in reducing the final content of phosphorus, the P values in the oil

phase were even lower (Table 1) due to the mode of action of LLPL-2, which releases the FFAs located at position sn-2 after PLA_1 hydrolysis on the phospholipid molecule. After the combined action of PLA_1 and LLPL-2, a glycerol-phosphatide would presumably be released which, being more polar than a lyso-phospholipid, would migrate to the glycerol phase, thus reducing the P content in the FAMEs phase. Higher reductions of phosphorus were observed when PLA_1 was combined with PLC, which directly removes the phosphodiester group, releasing a diacyl glyceride (DAG), known to be also a good substrate for Callera Trans L transesterification [57].

Table 1. FAMEs release (%) and P (ppm) content of reactions performed in a single batch with the enzyme combinations stated. Crude oils were incubated with the enzymes for 24 h at 35 ℃ and 200 rpm agitation. All enzymes were added in soluble form providing an average 3% water in the final mixture. Methanol was added following a continuous gradient of 0.4 mL/h for 10 h, to a total amount of 1.5 molar equivalents.

	Soybean Oil (1% FFA; 900 ppm P)		**Corn Oil (6% FFA; 62 ppm P)**		**Canola Oil (1% FFA; 250 ppm P)**	
Enzyme Combination *	FAMEs	P	FAMEs	P	FAMEs	P
Callera	85.2	823	93.4	7.5	98.7	145
PLA_1 + Callera	98.2	8.0	94.5	7.3	98.3	142
PLC + Callera	90.8	12.8	95.7	9.2	98.2	130
PLA_1 + LLPL-2 + Callera	97.8	6.0	96.2	9.3	98.9	145
PLA_1 + PLC + Callera	96.6	4.6	95.5	7.5	99.1	130

* Callera: Lipase Callera Trans L® (Novozymes A/S); PLA_1: Phospholipase A_1 (Lecitase® Ultra, Novozymes A/S), releases fatty acids from position sn-1; PLC: Phospholipase C (Purifine, Verenium), removes the phosphate group; LLPL-2: Lysophospholipase (Novozymes A/S), releases fatty acids from position sn-2.

Figure 6 shows a schematic representation of the successful, completely enzymatic process that has been developed, resulting in a more economic and eco-friendly biodiesel production. Combination of crude soybean oil degumming and transesterification in a unique step is possible by using phospholipases and liquid lipase Callera Trans L. In the combined process, an important cost reduction can be achieved: In addition to the $27.5 per ton savings in the case of crude soybean oil, costs can also be substantially lowered by avoiding the extra tank commonly required for oil degumming pre-treatments, by using mild temperatures (35 ℃ or below) and a soluble lipase. Moreover, citric acid treatment can be skipped, thus increasing the savings of the whole process. Therefore, the developed method bears the conditions for being easily scaled-up and meets the requirements of a sustainable and competitive process.

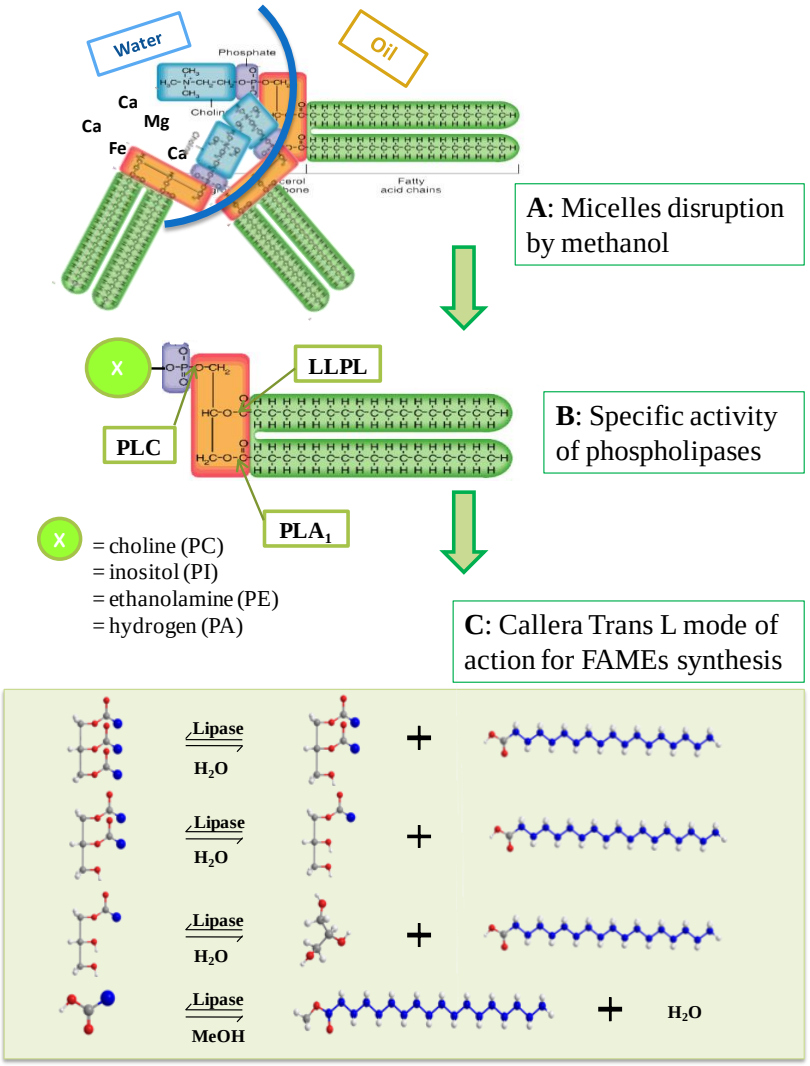

Figure 6. Schematic representation of the combined enzymatic degumming-transesterification reaction performed using phospholipases and lipase Callera Trans L for a more competitive FAMEs synthesis from soybean oil. As shown in the combined process developed here for soybean oil, the acid treatment can be skipped as methanol, one of the substrates of the reaction, can disrupt the micelles and dissolve the gums (**A**). Further phospholipase treatment contributes to remove the phosphate group (PLC), whereas the fatty acids at positions 1 and 2 can be released by a PLA_1 and LLPL-2, respectively, (**B**) to provide extra FFAs, which will then be converted into FAMEs by Callera Trans L esterification activity (**C**).

5.2. Other Feedstocks Evaluation

To evaluate performance of the degumming-transesterification combined process, Callera Trans L and phospholipases were tested in a single step procedure for FAMEs production and P removal starting from other oils commonly used for chemical biodiesel production but more restrictive for an enzymatic process, like corn oil and canola oil. The corn oil here evaluated was obtained after dry corn milling from a biorefinery–bioethanol plant. When tested for combined enzymatic biodiesel synthesis it showed a slightly lower FAMEs production compared to soybean oil. Optimum operational conditions were found with the combination PLA_1 + LLPL-2 + Callera, with a 96.2% production of FAMEs (Table 1).

Transesterification was not complete, probably due to the composition of corn oil, rich in sterols and waxes, which are hardly processed by the lipase for methyl esterification. Like for soybean oil, phospholipases helped to increase the transesterification yield, with final phosphorus values below 10 ppm, even when phospholipases were not present in the reaction mixture. This was probably due to the low initial content of phosphorus in corn oil (62 ppm), mostly constituted by hydratable phospholipids, which can be easily removed by simple washing with the water present in the system. However, it must be highlighted that when a phospholipase is present in the reaction mixture, phospholipids are hydrolyzed releasing FFAs, and more useful oil is available for lipase methylester formation.

When crude canola oil was tested, a high transesterification rate was achieved. However, the final phosphorus content was not reduced below 130 ppm. In this particular batch of canola oil phospholipids have a very high content of Ca^{2+} and Mg^{2+}, which make it especially difficult to degum. In some cases, crude canola oil is considered one of the most difficult oils for degumming because it is mostly constituted by NHPs, barely attacked by phospholipases without the aid of an acid treatment. Moreover, it requires a strong chelating agent such as citric acid at higher concentrations (0.1%) to achieve a suitable phosphorus reduction. Such conditions can hardly be reached with milder citric acid concentrations or even with phosphoric acid [83], thus making it difficult to reach the required phosphorus content in the final biodiesel.

In order to confirm the predicted activity of the phospholipases tested in the combined degumming-transesterification process, and to exclude the possibility that the phospholipids contained in the original raw material had migrated to the glycerin phase or to the interphase as entire molecules without any gain of oil, the phospholipids content of both, the oil and glycerin phases obtained from the combined reactions were analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS-MS). Among the compounds putatively released by phospholipases, glycerol and other polar molecules containing the remaining phosphorus, like the glycerolphosphatide and the phosphodiester group should be found in the glycerin phase, thus justifying the good reduction of P achieved in the final FAMEs phase for samples treated with the enzyme combinations assayed.

5.3. Phospholipase Activity in the Combined Process

Comparing the oil phase of control samples containing only Callera Trans L, where only transesterification occurs, with those resulting from reactions containing both, the lipase and PLA_1 (PLA_1 + Callera), intact phospholipids containing phosphocholine completely disappeared, indicating that phospholipase A_1 activity almost completely removed all phosphocholine from the oil phase (Figure 6). Interestingly, also lysophosphatidylcholine, apparently contained in the raw oil from the beginning, disappeared from the oil phase after PLA_1 + Callera treatment, suggesting that PLA_1 might display an extra activity causing removal of the fatty acid of the LPC molecule and not only the one at position sn-1, as would be expected. This extra activity, not described before for a PLA_1, could be due to the long reaction time used in the combined process: Generally phospholipases are used for 2–6 h, whereas they were acting for 24 h in the developed process.

When the final glycerine phase of all combined reaction samples was analyzed for phosphorous compounds, the predicted mode of action of the phospholipases could be confirmed (Figure 6). As expected, phosphocholine was found only in the glycerin phase of the reactions where PLC was

present (PLC + Callera and PLA_1 + PLC + Callera), proving the specific activity of this phospholipase for the phosphodiester bond. Instead, as a result of PLA_1 activity, lyso-phosphocholine could only be detected in very small amounts in the glycerine phase, suggesting a kind of instability of the LPC molecule. In fact, glycerol-phosphocholine, resulting from the combined activity of PLA_1 + LLPL-2 + Callera, should be present in the glycerin phase due to its polarity. But it was also found in the oil phase, supporting the hypothesis of an extra activity of PLA_1 causing additional removal of the fatty acid at position sn-2, or being due to a kind of instability of the LPC molecule. LLPL-2 activity was confirmed in sample PLA_1 + LLPL-2 + Callera by the finding of glycerol-phosphocholine in the glycerine phase. Parallel analysis by UPLC/MSMS of the phosphatides found in the oil and glycerin phases resulting from the combined reactions performed turned out to be a powerful tool for the study of such a complex system, allowing dissection of the activity of each enzyme, and confirming the importance of phospholipases in the combined degumming-transesterification process.

6. Conclusions

In order to reduce the costs of the industrial enzymatic biodiesel production process, several recombinant and improved lipases were prepared and tested in their immobilized and soluble forms for FAMEs synthesis. Cost reduction was also achieved by using crude (non-degummed) soybean oil as a cheaper raw material. Use of liquid lipase Callera Trans L in its soluble form instead of the common immobilized preparations in biodiesel production can provide numerous and sustainable benefits: besides reducing the costs derived from enzyme immobilization, the lipase can be used in combination with other enzymes such as phospholipases for gums removal, thus allowing the use of much cheaper, non-refined oils. The possibility to perform degumming and transesterification in a single tank denotes a great efficiency increase in the new era of enzymatic biodiesel production at industrial scale.

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Author Contributions

Silvia Cesarini participated in the design of the study, carried out the experiments, organized and interpreted the data, and drafted the manuscript. F. I. Javier Pastor contributed to the manuscript draft discussion. Per M. Nielsen coordinated and designed part of the study, critically interpreted the data and

revised the manuscript. Pilar Diaz coordinated the project, contributed to the critical discussion and data interpretation, and prepared, revised and corrected the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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