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INSIGHTS IN THE BIOCATALYZED HYDROLYSIS, ESTERIFICATION AND TRANSESTERIFICATION OF WASTE COOKING OIL WITH A VEGETABLE LIPASE<!–<ForCover>Mateos PS, Navas MB, Morcelle SR, Ruscitti C, Matkovic SR, Briand LE, INSIGHTS IN THE BIOCATALYZED HYDROLYSIS, ESTERIFICATION AND TRANSESTERIFICATION OF WASTE COOKING OIL WITH A VEGETABLE LIPASE, *Catalysis Today*, doi: 10.1016/j.cattod.2020.09.027</ForCover>–>

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INSIGHTS IN THE BIOCATALYZED HYDROLYSIS, ESTERIFICATION AND TRANSESTERIFICATION OF WASTE COOKING OIL WITH A VEGETABLE LIPASE

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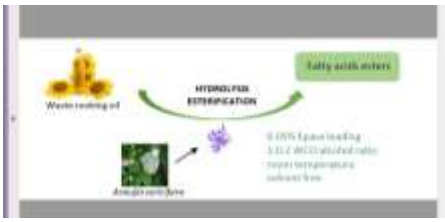
Graphical abstract



Highlights

- Plant lipase catalyzes hydrolysis and transesterification of waste cooking oil
- The hydrolysis of triglycerides goes to monoglycerides and FFAs
- Reaction takes place with water added and room temperature in short period of time
- Hydrolysis, esterification and transesterification achieved in tandem process
- Complete conversion of FFAs to esters is achieved with short chain alcohols

Abstract

The present contribution investigates the biocatalytic performance of the lipase obtained from the latex of the native plant known as *Araujia sericifera* (ASL) in the hydrolysis followed by the esterification of released free fatty acids and transesterification of glycerides of sunflower waste cooking oil WCO. A specific enzymatic activity of 719.05 µmol mg^{-1} h^{-1} (60 % conversion of triglycerides towards free fatty acids) was obtained in the hydrolysis of WCO with 0.05 % of biocatalyst (typically, 10.0 g WCO and 5.0 mg of enzyme) and 50 % w/w of water added in 30 min of reaction at 25 ^{o}C in homogeneous type of reaction system. The lipase has the capacity to catalyze the hydrolysis of the triglycerides mainly towards monoglycerides and diglycerides in a lesser extent. The released FFAs and the remaining glycerides reacted in tandem by addition of short chain alcohols immediately after the hydrolysis (without addition of more enzyme to the reaction media). Above 90 % conversion of the FFAs was obtained with methanol, ethanol, n-propanol and n-butanol at contents as low as 1:0.2 oil: alcohol molar ratio at 25 ^{o}C. The transesterification of the remaining monoglycerides (about 20 %) was also observed although no further reaction of the triglycerides was detected even under a great excess of alcohol.

Keywords: waste cooking oil; biocatalysis; lipase; esterification; transesterification

1. Introduction

The production of biodiesel has gained special attention in the last few years due to the decrease in the petroleum reserves. Nowadays, biodiesel is obtained through the transesterification of refined vegetable oil (the refinement involves 70-80 % of production costs) with methanol using potassium hydroxide as catalyst. However, this method has several drawbacks such as the formation of soap and the generation of aqueous wastes [1].

Other method for the generation of biodiesel is the transesterification catalyzed with acids even though high temperatures are required and secondary reactions occurs [2]. In the last years, several investigations on the enzymatic transesterification of oils of various sources catalyzed with lipases, demonstrated that this is an emerging technology that provides high yields towards biodiesel [3, 4]. In this context, the production of biodiesel catalyzed with lipase based biocatalysts provides certain advantages, such as a facile separation of product and glycerol, wastewater treatment requirement diminishes and the absence of side reactions [5].

In addition, raw materials with high content of free fatty acids such as waste cooking oils are suitable to be converted in biodiesel without deactivation of the biocatalyst. In contrast with the homogenous alkaline catalyzed transesterification that deactivates due to saponification reaction, the use of lipases allows both the esterification of the free fatty acids along with the transesterification of triglycerides towards fatty acids methyl and ethyl esters at mild reaction conditions. Nevertheless, the use of biocatalysts in the biodiesel production provides a slower reaction rate than the alkaline catalyst and the enzyme might inactivate in the presence of alcohols [5].

It is known that lipases (triacylglycerol hydrolases E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols and act at an oil-water interfase. X-ray studies have revealed that the active site of lipases is composed by a serine, an aspartate or glutamate, and a histidine. Lipases catalyze the hydrolysis of triacylglycerols, they are active at the oil-water interface. Several studies indicate that the lipase would present an interfacial activation. This is because lipases can be in two different conformations, open (active) and closed (inactive). The open form of lipases is described as more stable than the closed one. Interfacial activation would occur when the lipase meets a hydrophobic surface where its conformation would go from closed to open, increasing the enzyme activity [6, 7].

Lipases are one of the most widely used enzymes in biotechnology. The benefits offered by enzymes are high specificity, mild conditions and reduced waste. The plant using enzymatic reactions can be built and operated at much lower capital and energy cost. Enzyme-based processes tend to have lower waste treatment costs, with the addition that enzymes are biodegradable [8, 9]. In other hand, lipases catalyze many reactions, namely hydrolysis, esterification, transesterification, acidolysis, aminolysis, among others [10, 11].

According to their origin, lipases can be obtained from microbial, animal or plant sources. For industrial applications, microbial lipases are the most used; however, their cost is very high. Within this context, plant lipases could be considered as a cheaper alternative, since their production would be easier [12-14]. Nevertheless, there are some drawbacks that have to be taken into account. In general terms, lipases should be present in those tissues/organs rich in lipids, being the seed the most important one. In this regard, lipases play the physiological role of hydrolyzing the stored lipids in the seed that account to the energy needed for plant germination. Besides some exceptional cases such as the *Ricinus communis* seed [15], in which lipases are constitutively present, these enzymes are only expressed during that brief period

of germination before the seedling is able to do the photosynthesis in a very low content [16]. ~~To circumvent this problem, the presence of lipases in other plant tissues should be investigated.~~

It is well known that some plant belonging to certain families plant produce latex, this is a natural fluid, usually of milky aspect, that produce as defense against pathogens and herbivory [17]. Its composition may include terpenoids, polysaccharides, alkaloids and proteins such as enzymes. Among these enzymes proteases are frequently found [18]. Lipid hydrolases are less commonly present, or at least, less investigated [19]. In this sense, *Carica papaya* latex (CPL) represents the paradigmatic case. Lipase activity in this plant material is present in the insoluble fraction of the latex, consisting mainly of terpenoids (triterpenoids and polyisoprenoids), turning the isolation of the enzymes responsible of the hydrolytic activity extremely difficult. However, three lipolytic enzymes were identified by *Carica papaya*'s genome analysis and two of them were produced either as recombinant protein fully active (CpLip 1), or by transient expression (CpLip 2, the GDSL lipase previously identified and cloned) [20-23]. A phospholipase (CpPLD1), was partially purified and identified according to Abdelkafi et al. [20].

Since very few plant latex lipases were purified up to complete isolation (see the rationale for this below), their properties, application and mechanism of action were mainly determined by means of the biochemical properties of partial purified extracts. Plant latex lipases have in common optimal pH activities at neutral to alkaline pH values, preference for carboxylic acids of short and medium chain lengths, and sn-1,3 regioselectivity. These enzymes proved to be very interesting biocatalyst for diverse monoesters synthesis, hydrolysis of triglycerides, alcoholysis of sunflower oil, resolution of naproxen, lipids modification and asymmetric resolutions [21, 24-30]. Within this context, ASL has an optimal pH and temperature of 8.5 and 60 °C respectively, and showed a marked preference towards the hydrolysis of butyrate esters [31]. When the esterification of oleic acid with primary alcohols of different chain length was tested, ASL demonstrated a remarkable preference for those having between five and eight carbons. Interestingly, ASL demonstrated not to be inhibited by alcohols of shorter chains as methanol or ethanol, but the ester yield obtained was much lower [32].

Plant latex lipases, as well as almost all hydrolytic enzymes, have the canonical esterase mechanism that uses the core Ser-His-Asp catalytic machinery, an oxyanion hole and a lid, conforming together the active site [33]. This active site can accommodate different

substrates according to their size and thus, contributing to the great variety of preferences of each enzyme. For this purpose, in addition to study the affinity of the enzymes towards different substrates, investigation about the effect of inhibitors is a very useful tool to map the active site. In contrast with other hydrolases, specific lipase inhibitors are scarce. Tetrahydrolipstatine is a specific inhibitor of pancreatic lipase and other lipases of the kind belonging to different organisms, and it has shown good inhibitory activity against plant latex lipases of *Vasconcellea heilbornii* and *Carica papaya* esterase, (more than 90% of inhibition in both cases), whereas for ASL the rate of inhibition was around 70% (unpublished data) [21, 28, 34-37]. All these results would indicate that these lipases present in plant lattices could share some degree of structural resemblance.

Within the context of exploitation of agroindustrial wastes, plant lipases have been explored for sustainable biodiesel production. Lipases obtained from plant seeds or seedlings are usually the first choice, due to an historical reason, and therefore many of them are commercially available [38]. Bioprospection of other plant sources of lipases is continuously ongoing [39-41]. In this emerging topic of investigation, lipases from plant latex are also being studied, consisting CPL the standard of this kind of enzymes [42-44]. Other latex producing plant species having lipase activity belong to the Vascocellea (a close relative of the Caricaeae family) [30], Moraceae [45], Euphorbiaceae [46], and Apocynaceae [31, 47], all showing very similar catalytic characteristics to CPL. Among the Apocynaceae family, the South American native climbing milkweed *Araujia sericifera* demonstrated lipase activity in its latex, whereupon which has been proved to be a promising biocatalyst for its use in the biorefinery industry [32, 48].

The present contribution extends the use of the lipase of the *Araujia sericifera* in the biocatalytic hydrolysis and further conversion of waste cooking oil WCO into methyl, ethyl, n-propyl and n-butyl fatty acid esters. The influence of the addition of water in the reaction media, the amount of lipase and the temperature in the hydrolytic performance of the lipase was investigated. Additionally, insights in the effect of the amount of methanol and ethanol in the esterification and transesterification of previously hydrolyzed WCO towards FAME and FAEE are provided.

2. Experimental

2.1. Materials

Waste sunflower oil (commercial brand Caracas) was collected from the restaurant of the National University of La Plata (Buenos Aires, Argentina). This oil was used continuously for a week in a commercial deep fryer with an aqueous layer at the bottom (for cleaning purposes). The lipase (called ASL from now on) is the insoluble fraction of the latex of fruits of *Araujia sericifera* (Apocynaceae) obtained according to the method described previously in the literature [31]. Unripe fruits of *Araujia sericifera* (synonym: *Araujia hortorum*) were harvested from wild plants grown in the surroundings of La Plata, Buenos Aires Province, Argentina, in mid-summer. Fruits were washed with tap water, drained and dried with absorbent paper. Latex was collected by removing the petiole of each fruit and by gathering the latex drops in a solution of EDTA and Na_2SO_3 5 mM to avoid the activity of peroxidases and to maintain a reducing medium, respectively. The whole process was performed in an ice bath. The resulting homogenate was centrifuged 30 min at 4 °C and 9600 × g in order to separate a water soluble fraction and an insoluble pellet. This last fraction, which contained the lipase activity, fractioned and liophylized. The resulting dry powder was grounded and finally stored at -20 °C for further use.

Potassium hydroxide (Carlo Erba, 85%), methanol (Cicarelli 99.5%), ethanol (Cicarelli 99.5%), n-propanol (Sigma Aldrich, 99.5%), n-butanol (Sigma Aldrich, 99%) and toluene (Dorwil, 99.5%) were also used.

2.2. Hydrolysis and transesterification of waste cooking oil biocatalyzed with ASL

The influence of the addition of water (0 mL to 7.5 mL), the amount of ASL lipase (from 2.5 mg up to 7.5 mg), temperature (25 ^{o}C – 60 ^{o}C) and type of stirring (shaker shaking and magnetic stirring) in the hydrolysis of waste cooking oil (WCO) was investigated. Typically, the reactions were carried out with 10.0 g of WCO and a certain amount of water in closed flasks placed in a shaker bath at 200 rpm. When the mixture reached the desired temperature, a certain amount of ASL was added (time zero of reaction) and left reacting for up to 72 hours.

Once the optimal conditions of the hydrolysis were ascertained, the esterification using different alcohols at several reaction times was carried out. Typically, the alcohols were added after 30 min of hydrolysis at R.T. Additionally an excess of alcohols at 1:3 and 1:6 WCO: alcohol

molar ratio were investigated. Methanol, ethanol, n-propanol and n-butanol have been assayed for 1, 3, 6, 8 and 10 hours of reaction. The hydrolysis and the reaction with the alcohols have been performed at 25 °C in a shaker at 200 rpm.

2.3. Quantification of mono-, di- and triglycerides, glycerol and acidity index

The collected samples were analyzed employing a GC-2010 Plus Tracera, equipped with a BID detector and a capillary column MEGA-Biodiesel 105 (15 m × 0.32 mm × 0.10 µm), with helium as carrier gas. The temperature program started at 50 °C, then going to 180°C with a ramp rate of 15 °C min^{-1}, then to 230 °C at 7 °C min^{-1}, and, in a last step, to 350 °C, at 30°C min^{-1}. The injector and detector temperatures were maintained at 350 °C. The injection volume was 1 µL [49]. The results allowed the determination of the conversion of glycerides, yield towards the esters and the moles percentage of monoglycerides, diglycerides and triglycerides. For each analysis, 100 mg of sample were taken, adding (S)-(-)-1,2,4-butanetriol and tricaprin as stock solution (80 µl and 100 µL, respectively), and 100 µL of N-methyl-N-(trimethylsilyl)tri fluoroacetamide (MSTFA). This mixture was shaken and finally diluted with 8 mL of n-heptane. The analysis through this technique possesses 5-10 % error.

The acidity index of the oil phase after hydrolysis and esterification was determined using the European normative EN 14104 [50]. At a first step, the reaction mixture was centrifuged to separate the aqueous from the oil phase that contains the free fatty acids. Then, 1.00 g of the oil phase was diluted in 10.00 mL in a mixture of toluene and ethanol (1:1) previously neutralized. This was titrated with an ethanolic solution of KOH 0.100 N using phenolphthalein as indicator. This titration possesses 5% error as maximum.

The acidity index **AI** was calculated using the equation (1):

$$AI = \frac{M_{KOH}\, V_{KOH}\, C_{KOH}}{m_{sample}} \qquad (1)$$

Where, M_{KOH} is the molecular weight of potassium hydroxide KOH; V_{KOH} is the volume of the hydroxide used in the titration in L; C_{KOH} is the molar concentration of KOH in mol L^{-1} and m_{sample} is the mass of sample used in the analysis in g.

The quantification of glycerol was performed according to the volumetric method reported by Pisarello et al. for the assessment of total and free glycerol after of the synthesis of biodiesel [51]. In brief, the glycerin was extracted first with HCl 5 w/v %, and then with water. Later, glycerin was oxidized to formic acid with sodium periodate (0.28 M) and the acid formed was titrated with sodium hydroxide (0.100 N). This technique is suitable for samples free from sugars and other organic compounds with more than two adjacent hydroxyl groups. The percentage of free glycerol was calculated with the equation (2):

$$\text{glycerol wt \%} = \frac{0.0921\ V_{NaOH}\ N_{NaOH}}{m_{sample}}\ 100 \qquad (2)$$

where, glycerol wt % is the mass of glycerol (in g) per 100 g of sample; V_{NaOH} is the volume of sodium hydroxide in mL; N_{NaOH} is the normality of NaOH solution and m_{sample} is the mass of sample used in the analysis in g.

Additionally, the specific enzymatic activity of the biocatalyst was calculated as the ratio between the amount of free fatty acids FFA formed in the hydrolysis or the conversion of glycerides in the transesterification in µmol per weight (mg) of biocatalyst and reaction time (h).

2.4. Infrared analysis

WCO and the products of the reaction of hydrolysis and transesterification were followed through infrared spectroscopy with a liquid transmission cell with CaF_2 windows and a fixed path length that allows a quantitative comparison of the spectra. Solutions containing 1% v/v in carbon tetrachloride (Dorwil, 99.9%) were prepared for IR analysis. Additionally, the aqueous phase was analyzed by placing a drop of the liquid between two CaF_2 windows into a sealed cell. Spectra were collected in the 4000 to 400 cm^{-1} range (+/-2 cm^{-1} resolution) with a Bruker Vertex 70 equipment. The infrared analysis was recorded with 60 scans in the absorption mode. Deconvolution of the spectra obtained was performed by peak fitting of the signal by Lorentzian-shaped components on the non-deconvoluted spectra. The software used

with this purpose was a special peak fitting module of Origin 5.0. The positions and number of peaks were determined from the second derivative analysis of the spectra.

2.5. Analysis of the results using nonlinear fitting

The experimental data obtained in the hydrolysis of the WCO in terms of specific enzymatic activity as a function of the amount of ASL and water added to the reaction media was fitted using second order models according to the equation (3).

$$Z = z_0 + A(E-5)^2 + B(W-5)^2 \qquad (3)$$

The equation represents the quadratic model where E is the amount of enzyme ASL in mg, W the amount of water added in mL and Z is the enzymatic activity given in µmol.mg^{-1}.h^{-1}. The coefficients z_0, A and B correspond to: $z_0 = 7.38,\ A = -1.0672,\ \mathrm{B} = -1.0224$ with and adjustment coefficient $R^2 = adjR^2 = 0.99$.

On the other hand, for the case of specific enzymatic activity as a function of the amount of ASL and temperature, a second order model was also applied to approach the response surface of the experimental data according to the equation (4).

$$Z = z_0 + A(E-5)^2 + B(T-50)^2 \qquad (4)$$

Where, E is the amount of enzyme ASL in mg, T is the temperature in degrees Celsius and Z represents the enzymatic activity measured in µmol.mg^{-1}.h^{-1}. The coefficients z_0, A and B correspond to: $z_0 = -49.89577,\ A = 7.60808,\ \mathrm{B} = 1.11801$ with an adjustment coefficient $adjR^2 = 0.91$.

3. Results and Discussion

3.1. Screening of the esterification of WCO catalyzed with ASL lipase: a proof of concept

Currently, lipases are widely used in industry, such as ingredients in detergents and medicine formulations, in the biodiesel production by transesterification from edible and non-edible

oils, among others as discussed before. In fact, previous studies demonstrated that ASL lipase is active in the hydrolysis and esterification of cottonseed and soybean oils and the esterification of oleic acid [31, 32, 48]. Based on those studies, the catalytic activity of the ASL lipase on the direct transesterification of fresh and used sunflower oil was initially screened. In this context, the Table 1 shows the nature of the alcohol, WCO: alcohol molar ratio, temperature and time of reaction, specific enzymatic activity, yield towards the esters and conversion of free fatty acids (FFA) in the direct transesterification (see the data without pre-treatment in Table 1) of fresh and WCO at 45 ^{o}C for 24 h of reaction. Additionally, the results obtained in the transesterification after hydrolysis of the WCO are also presented for comparison. These results will be further discussed in this investigation.

The preliminary results of the esterification of sunflower oil before (fresh) and after being used in a cooking process without previous treatment demonstrated the capacity of the ASL to catalyze the transesterification of triglycerides towards the esters with an excess of methanol and ethanol at 45 ^{o}C [48]. Moreover, a certain amount of FFA was also present at 24 h of reaction evidencing that the lipase also catalyzes the hydrolysis of the sunflower oil (data not shown). It comes clear from the comparison of the specific activity, yield towards the fatty acid alkyl esters and the conversion of free fatty acids of the transesterification of the WCO with and without hydrolysis that, the enzyme ASL provides an improved catalytic performance if the oil is hydrolyzed in a first step. Previous investigations reported Vescovi et al. show the production of biodiesel from enzymatic catalyzed hydrolysis of waste cooking oil with *Thermomyces lanuginosus* lipase in a first step at 30 ^{o}C for 24 h [1]. Then, the hydrolyzed oil was esterified with ethanol using immobilized *Candida antarctica* lipase B at 40 ^{o}C . The combination of the hydrolytic and esterase activity of both lipases allowed 92% of fatty acid ethyl esters with low acid value. According to the preliminary results obtained in the present investigation, the ASL lipase combine both activities most probably due to the complex composition of the non-purified enzymatic extract of such vegetable lipase. These capabilities were further exploited in order to optimize the conversion of the WCO through a sequential two-step process of hydrolysis and esterification/trans-esterification.

3.2. Hydrolysis of the WCO: optimization of the biocatalytic performance of the plant lipase

Figure 1 shows the fitting surface graph that compares the influence of the amount of water added and biocatalyst in the specific enzymatic activity of ASL in the hydrolysis of WCO at 45 ^{o}C for 48 hs. The specific enzymatic activity was calculated as the amount of FFA formed in the hydrolysis (in micromoles) per amount of lipase (in milligrams) per time of reaction (in hours). The results clearly show that the highest enzymatic activity (7.4 µmol mg^{-1} h^{-1}) is reached with 5 mg of lipase and 5.0 mL of water added. An acidity index of 11 is obtained under this reaction conditions.

The decrease of the enzymatic activity upon increasing the amount of lipase was previously observed by some of us in the esterification of oleic acid with the ASL lipase [31, 48]. This observation is attributed to the agglomeration of the enzyme that occurs at high concentration of lipase, diminishing the exposure of the active sites to the substrate.

The observation that the catalytic performance of the lipase improves in an oil-water interface (due to the water added to the reaction system) is related with the interfacial activation observed in lipases with a lid covering the active site. In fact, the organic-aqueous media might provide the appropriate activation (by modifying the hydrogen bridged bonds and Van der Walls interactions) to modify the enzymatic conformation and "open" the lid for accessing of the substrates. The present findings are in accordance with Di Santo Meztler et al. that demonstrated the increase in the hydrolytic activity of the ASL upon addition of a surfactant (a nonionic detergent such as Triton X) to the reaction media [31].

Further studies combining the effect of various amounts of ASL lipase and the temperature, on the specific enzymatic activity are presented in Figure 2. Also in this case, the results indicate that the higher the amount of biocatalyst, the lower the activity of the lipase. Moreover, the biocatalyst possesses an optimum performance at 25 ^{o}C that is similar to the optimum reaction temperature in the hydrolysis of soybean oil reported by Sánchez et al. [32]. In this context, a specific enzymatic activity of 719.05 µmol mg^{-1} h^{-1} was reached when the hydrolysis was performed with 5 mg of ASL (0.05 % of biocatalyst considering the amount of oil and enzyme in the reaction media) and 5.0 mL of water added in 30 min of reaction. Additional experiments demonstrated that longer time of reaction (above 30 min) and magnetic stirring did not enhance the hydrolysis (data not shown for brevity). Furthermore, the addition of a co-solvent did not favor the catalytic performance. Table 2 compares the specific enzymatic activity in the hydrolysis of WCO with and without the addition of n-heptane under the optimized conditions of the present investigation, and also under those

previously published in the literature for the hydrolysis of soybean oil [32]. The addition of an organic co-solvent, extended periods of reaction (see the results for 5 h of reaction) and high amount of ASL lipase (5.0 mg vs. 91.7 mg) diminish two orders of magnitude the specific enzymatic activity.

Additionally, the reusability of the vegetable lipase ASL in the hydrolysis of the WCO was investigated. As a first attempt to establish the solubility of the enzyme in the aqueous and the oily phase, a system of 10,0 g of WCO with 5 mL of water and 5 mg of ASL was prepared. Then the aqueous phase was removed and replaced with a fresh aliquot, and the system was allowed to react for 30 min at 25 ^{o}C (the optimized reaction conditions). An acidity index equals to 1.49 was obtained resulting appreciably lower than the one obtained previously (10.96 activity index of the first use).

In a second assessment of reusability, the hydrolysis of the WCO was performed under optimized conditions with 0.05 % of biocatalyst (5 mg) and 50 % w/w of water added in 30 min of reaction at 25 ^{o}C as described before. Then, the aqueous phase was centrifuged and separated of the oil phase and reused to hydrolyze a fresh aliquot of 10.0 g of WCO under similar optimized conditions. Under this circumstances an acidity index equals to 0.94 was obtained that again is significantly lower than in the first use. This process was repeated for a third time and the hydrolysis was even lower (acidity index equals to 0.45) demonstrating that ASL is not suitable for reuse under the conditions of this investigation.

3.3. Insights on the mechanism of the biocatalyzed hydrolysis of WCO

A deeper analysis of the nature of the hydrolysis products through infrared spectroscopy and further quantification allowed obtaining insights in the mechanism of action of the ASL lipase. Figure 3 shows the infrared spectra of the starting WCO and the hydrolyzed oil phase obtained after centrifugation and removal of the aqueous phase. The inset graphs show the deconvolution of the signals at about 1700 cm^{-1} and 3600 cm^{-1}. Additionally, Figure 4 compares the evolution of the acidity index and the deconvoluted areas of the infrared signals at 1709 and 3533 cm^{-1} of the fatty acids; 1746 cm^{-1} due to the carbonyl C=O stretching of COOR of glycerides; 3619 and 3676 cm^{-1} attributed to O-H stretching vibration of monoglycerides [52-54].

Figure 4 shows that the release of FFA during the hydrolysis (indicated with the acidity index) is accompanied by the decrease of the area of the infrared signal at 1746 cm^{-1} corresponding to triglycerides (major component of the WCO), along with the increase of those belonging to monoglycerides (see the signals at 3619 and 3676 cm^{-1}) and free fatty acids (see the signal at 1709 cm^{-1}). Further quantification through GC provided additional evidences that the monoglycerides are the main product of the enzymatic hydrolysis of the WCO as can be observed in the Figure 5. In this context, 5 mg of ASL catalyzes the conversion of 60% of the triglycerides of 10.00 g of WCO (with 5.0 mL of water added) towards monoglycerides in 30 min of reaction at 25 ^{o}C.

This observation resembles the typical 1,3-regioespecific behavior of the microbial lipases that hydrolyze the ester bonds in positions R1 and R3. In fact, the 1,3-selective lipases have been successfully used to catalyze the transesterification of vegetable oil with ethanol in order to obtain fatty acid ethyl esters with monoglyceride, avoiding the production of glycerol [55-57]. Glycerol has necessarily to be removed but the monoglycerides are soluble in diesel, and can be integrated in the biofuel. In fact, this is the case of the ASL lipase as will be presented in the following sections.

3.4. Tandem esterification with short chain alcohols of the hydrolyzed WCO

The esterification of free fatty acids after hydrolysis of the WCO under optimized conditions (5 mg of ASL, 30 min at 25 ^{o}C) was performed by direct addition of short chain alcohols to the reaction media. In this experiment, an excess of 15% in the amount of alcohol required to convert the WCO (1:0.2 WCO: alcohol molar ratio) considering 100 % yield towards the esters was used.

Figures 6A to 6D show the conversion of glycerides and the free fatty acids generated during the hydrolysis, the moles percentage of triglycerides, monoglycerides, diglycerides and glycerol, and the yields towards the esters of methanol, ethanol, n-propanol and n-butanol at 25 ^{o}C upon time of reaction. Additionally, Figure 7 compares the specific enzymatic activity of ASL in the transesterification with the four alcohols.

The esterification of the FFA towards the esters raises 90 to 98 % conversion with the four alcohols assayed (in 10 h of reaction) indicating that enzyme remains active after the hydrolysis of the WCO. Additionally, the ASL catalyzes the transesterification of the monoglycerides and diglycerides leaving the triglycerides unaltered. The specific enzymatic

activity evidences that even though the activity of the lipase towards the transesterification is somehow lower for methanol at the first hour of reaction, it is not influenced by the nature of the alcohol afterwards (see Figure 7).

In this context, further experiments with the addition of a great excess of methanol and ethanol (1:3 and 1:6 WCO: alcohol molar ratio), were performed in order to establish if an excess of alcohol would improve the transesterification of the remaining glycerides after the hydrolysis. Experiments extracting the aqueous layer before the addition of the alcohols and further addition of ASL were also assayed.

Figures 8A and 8B compare the conversion of the glycerides and FFA, the yield towards the esters and the specific enzymatic activity of ASL upon addition of methanol and ethanol, respectively. It comes clear that the best bio-catalytic performance of ASL occurs under the presence of water and low alcohol contents [compares the data for 1:0.2w (with water) and 1:0.2 (after extraction of the aqueous layer) in Figures 8A and 8B]. Moreover, ASL catalyzes the esterification of FFA only in the presence of water (see grey columns in the Figure 8 that correspond to FFA conversion). Neither an excess of alcohol nor a high concentration of enzyme favors the catalytic activity. This observation is ascribed to the inhibition of the lipase due to the alcohol and the agglomeration of the protein at high lipase loadings [31, 48].

Back to the results obtained in the direct transesterification of the WCO (without hydrolysis) showed in Table 1, it comes clear that the specific enzymatic activity under such conditions are two orders of magnitude lower than the ones obtained when the WCO is previously hydrolyzed.

4. Conclusions

The present investigation demonstrates that the insoluble fraction of the latex recovered from the fruits of *Araujia sericifera* (an inexpensive native plant) has the capability to catalyze the hydrolysis, esterification and transesterification of sunflower waste cooking oil. The non-purified enzyme catalyzes the hydrolysis of the waste cooking oil in a proportion of 0.05% with respect to the amount of oil with the addition of water at 25 ^{o}C and homogeneous conditions. In fact, in just 30 min of reaction a 60% conversion towards monoglycerides and free fatty acids was achieved without the addition of co-solvents to the reaction media. The remaining FFA and monoglycerides further reacted towards fatty acids alkyl esters with the addition of

methanol, ethanol, n-propanol and n-butanol (1:0.2 WCO: alcohol molar ratio) at 25 ^{o}C without the need of adding more enzyme to the reaction media. The FFA were esterified up to 90 to 98% in 10 h of reaction and the glycerides (mono- and diglycerides) were transesterified in a 25%. Nevertheless, no transesterification of the triglycerides is observed indicating that ASL is able to catalyze exclusively the hydrolysis of these glycerides. This observation is in accordance with the low specific enzymatic activity observed in the direct transesterification of both fresh and used sunflower oil.

Even though the remarkable activity of the vegetable lipase, it is not suitable for reuse at least under the conditions of this investigation. This observation somehow indicates the need of immobilization of the enzyme in order to maintain its biocatalytic stability.

Authors statement

Paula S. Mateos: first author, investigation, formal analysis, writing original draft.

Marisa B. Navas: investigation, formal analysis.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURE 1

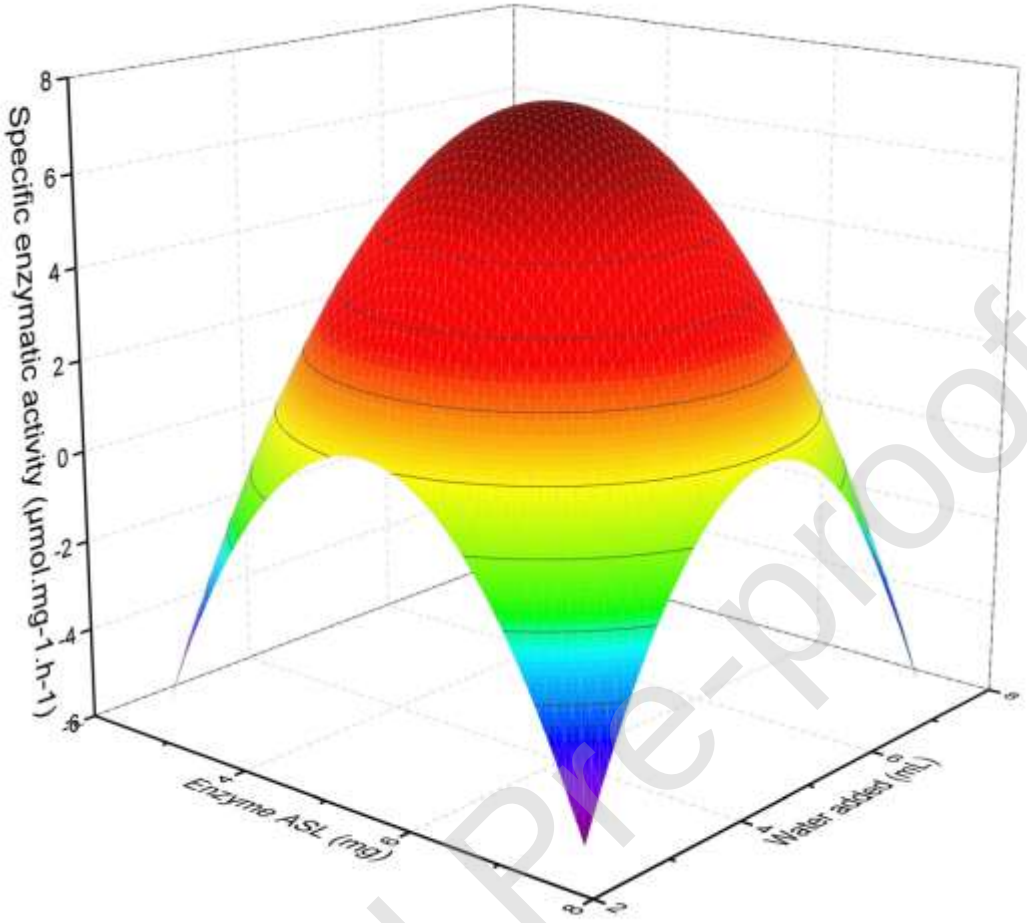

Figure 1 Surface graph of the specific enzymatic activity as a function of the amount of ASL lipase and water added in the hydrolysis of WCO at 45 ^{o}C for 48 h. The surface plot is the graph of the function: $\mathbf{z} = z_o + A(\mathbf{E} - 5)^2 + B(W - 5)^2$ where $z_o = 7.38$; A = -1.0672 and B = -1.0224

FIGURE 2

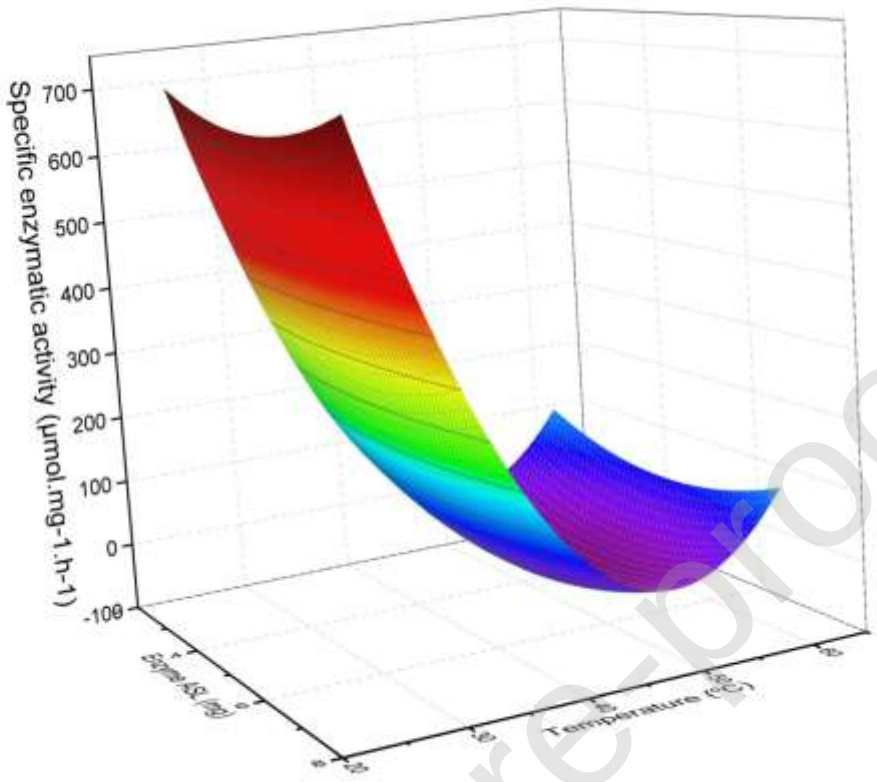

Figure 2 Graph of the surface response methodology of the specific enzymatic activity as a function of the amount of ASL lipase and temperature in the hydrolysis of WCO with 5.0 mL of water added, 30 min of reaction and 200 rpm. The SRM responds to the equation: $\mathbf{z} = z_o + A(\mathbf{E} - 5)^2 + B(\mathbf{T} - 50)^2$ where $z_o = -49.89577$; A = 7,60808 and B = 1.11801.

FIGURE 3

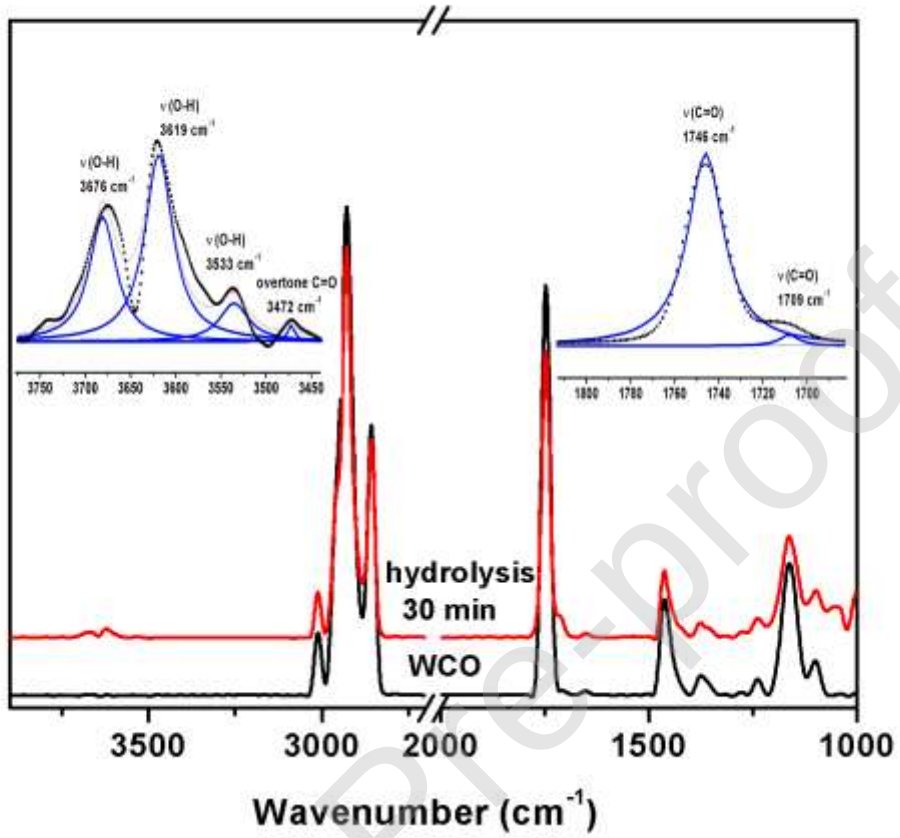

Figure 3 Infrared spectra of the starting WCO and after hydrolysis with 5.0 mg of ASL, 5.0 mL of water added, 25 ^{o}C and 30 min of reaction. Inset graphs showing the deconvolution of the fingerprint signals of FFA and glycerides.

FIGURE 4

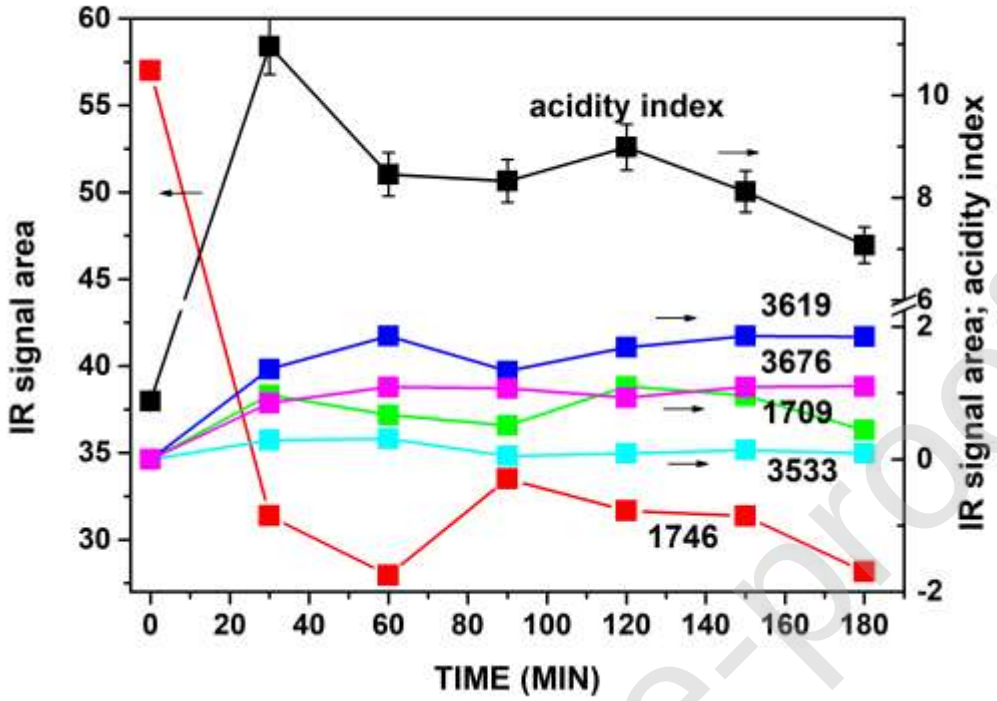

Figure 4 Evolution of the acidity index and the areas of the infrared signals of FFA, and glycerides of the starting WCO and along the hydrolysis with 5.0 mg of ASL, 5.0 mL of water added, 25 ^{o}C up to 180 min of reaction.

Figure 4 Evolution of the acidity index and the areas of the infrared signals of FFA, and glycerides of the starting WCO and along the hydrolysis with 5.0 mg of ASL, 5.0 mL of water added, 25 ^{o}C up to 180 min of reaction.

FIGURE 5

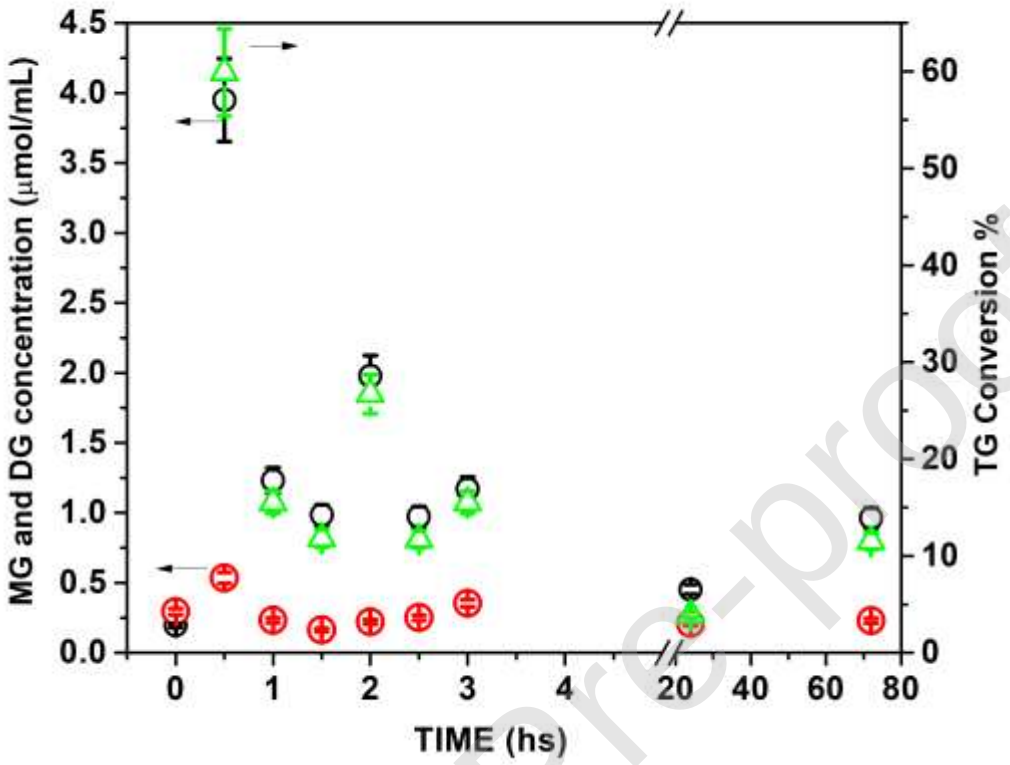

Figure 5 Conversion of triglycerides (△) and concentration of monoglycerides (O) and diglycerides (O) during the hydrolysis of WCO with 5.0 mg of ASL, 5.0 mL of water added, 25 °C up to 72 h of reaction. The values of mono- and diglycerides at time zero corresponds to the starting WCO.

Figure 5 Conversion of triglycerides (△) and concentration of monoglycerides (O) and diglycerides (O) during the hydrolysis of WCO with 5.0 mg of ASL, 5.0 mL of water added, 25 °C up to 72 h of reaction. The values of mono- and diglycerides at time zero corresponds to the starting WCO.

FIGURE 6A

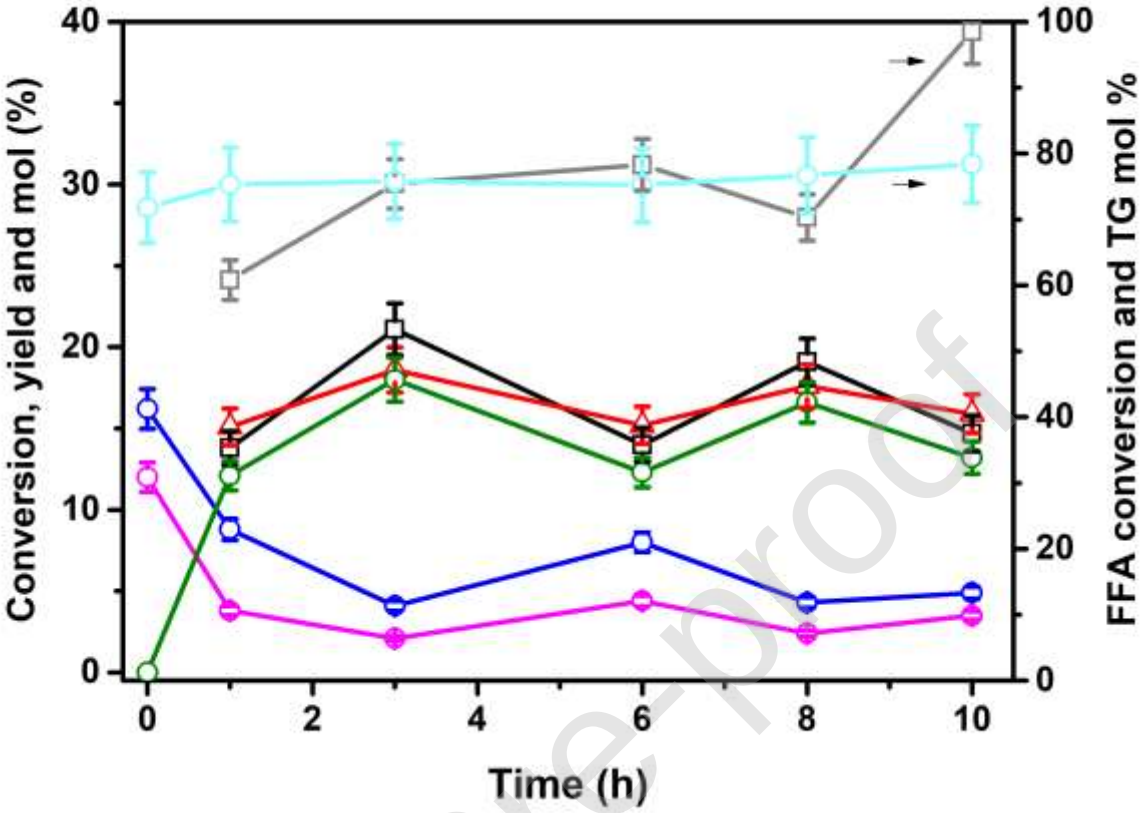

FIGURE 6B

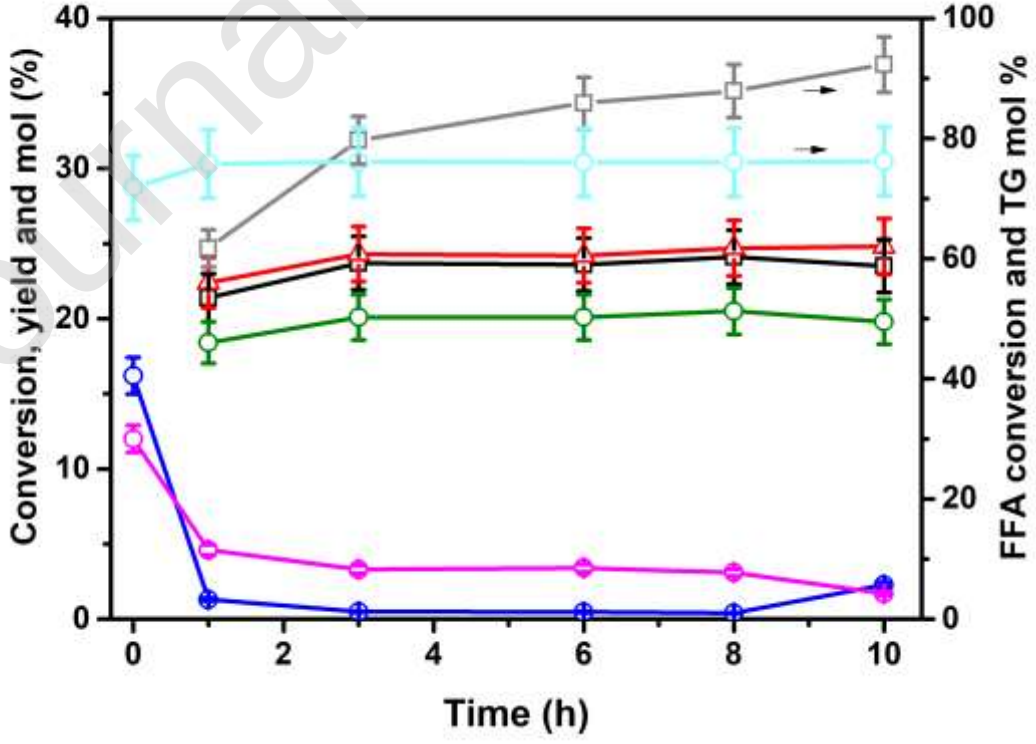

Figure 6 Conversion of glycerides (■) and FFA (□), mol percentage of monoglycerides (○), diglycerides (○), triglycerides (○) and glycerol (○), and the yield towards the esters (△) using: (**A**) methanol, (**B**) ethanol, (**C**) n-propanol and (**D**) n-butanol in a 1: 0.2 molar ratio of hydrolyzed WCO: alcohol at 25 °C.

FIGURE 7

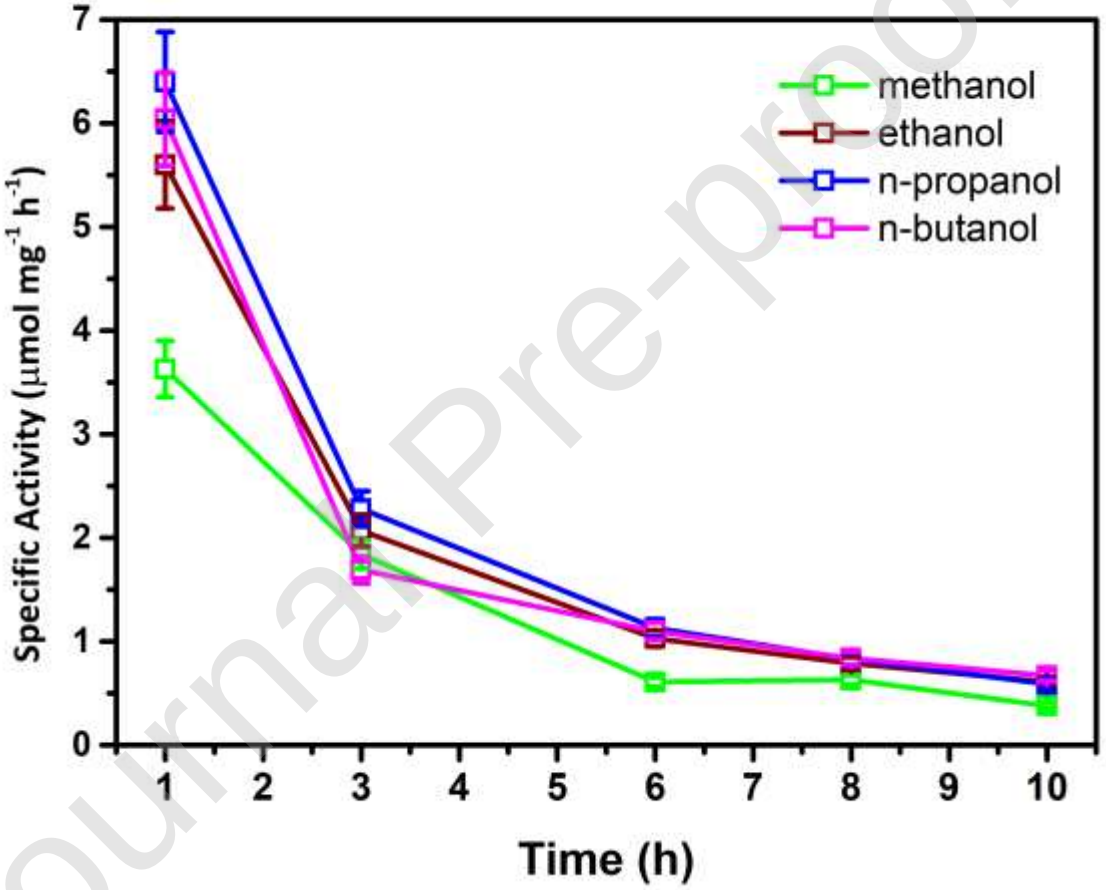

Figure 7 Specific enzymatic activity of ASL in the conversion of glycerides of WCO towards the esters methanol, ethanol, n-propanol and n-butanol as a function of time reaction for 1: 0.2 molar ratio of hydrolyz WCO: alcohol at 25 °C.

Figure 7 Specific enzymatic activity of ASL in the conversion of glycerides of WCO towards the esters of methanol, ethanol, n-propanol and n-butanol as a function of time reaction for 1: 0.2 molar ratio of hydrolyzed WCO: alcohol at 25 ^{o}C.

FIGURE 8A

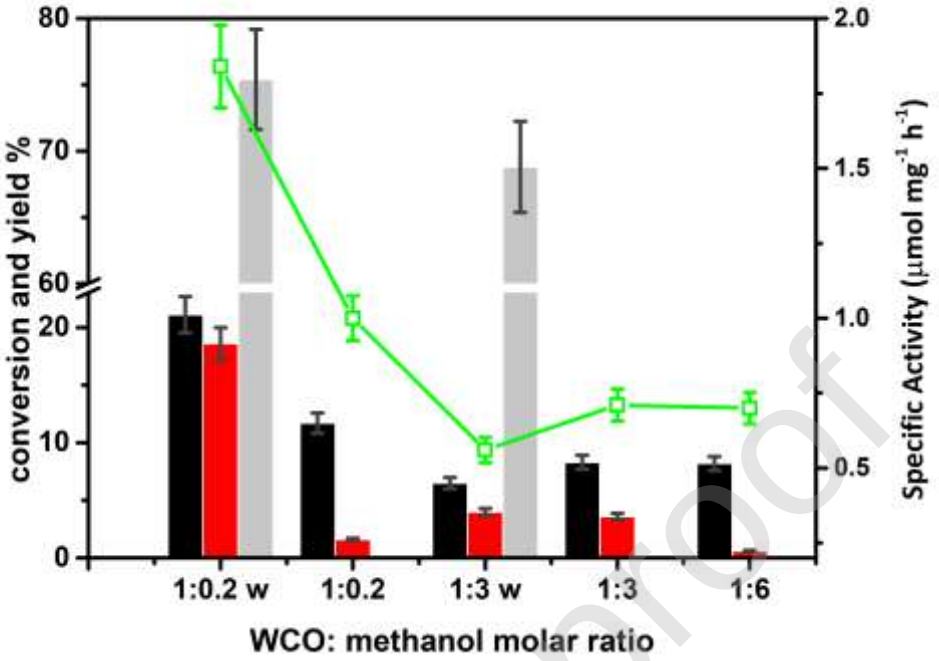

FIGURE 8B

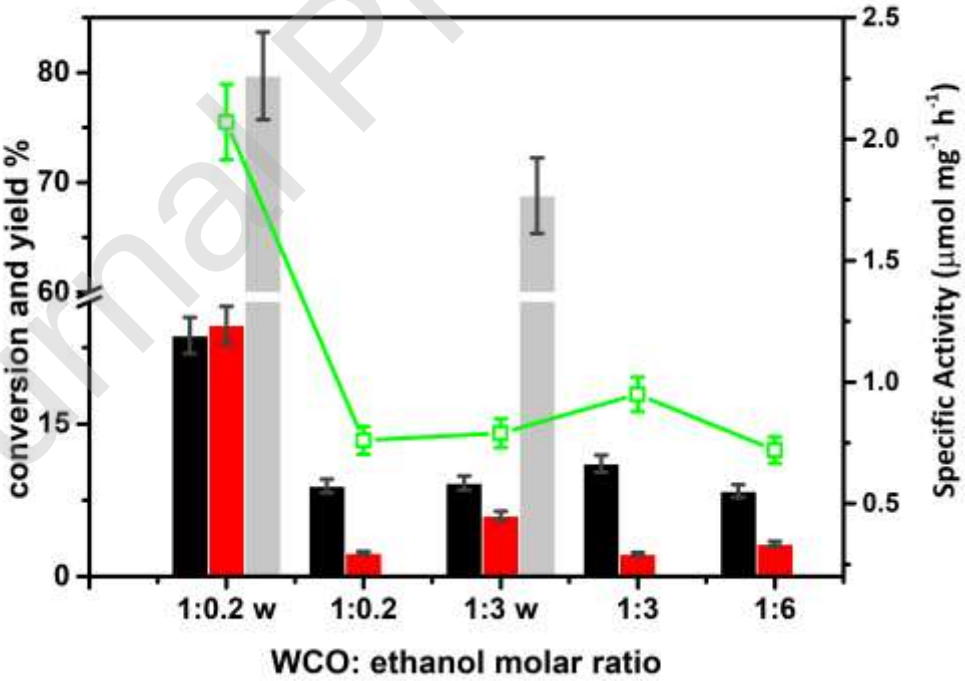

Figure 8 Specific enzymatic activity of ASL (□, right *y* axis), conversion of glycerides (black column, left *y* axis) yield towards the esters (red column, left *y* axis) and conversion of FFA (grey column, left *y* axis) for 1: 0.2 1:3 and 1:6 WCO: alcohol molar ratios for (**A**) methanol and (**B**) ethanol. The reactions were carried at 25 °C without water added and under the presence of water (indicated by "w" following the molar ratio) for three hours.

Figure 8 Specific enzymatic activity of ASL (□, right *y* axis), conversion of glycerides (black column, left *y* axis), yield towards the esters (red column, left *y* axis) and conversion of FFA (grey column, left *y* axis) for 1: 0.2, 1:3 and 1:6 WCO: alcohol molar ratios for (**A**) methanol and (**B**) ethanol. The reactions were carried at 25 ^{o}C, without water added and under the presence of water (indicated by "w" following the molar ratio) for three hours.

TABLE 1 Specific enzymatic activity, yield towards the esters and FFA conversion in the trans-esterification of fresh sunflower oil and WCO with short chain alcohols catalyzed with the ASL lipase with and without previous hydrolysis.

Oil	Previous treatment	Alcohol	WCO: alcohol molar ratio, temperature, time	Specific activity (μmol mg^{-1} h^{-1})	Yield %	FFA Conversion %
Fresh	none	Methanol	1:4.5, 45 ºC, 24 h	0.04	1.7	----
	none	Ethanol	1:3, 45 ºC, 24 h	0.03	----	----
WCO	none	Methanol	1:4.5, 45 ºC, 24 h	0.07	2.2	10.3
	hydrolysis	Methanol	1:0.2, 25 ºC, 3 h	1.84	17.1	75.4
	none	Ethanol	1:3, 45 ºC, 24 h	0.11	1.6	----

hydrolysis	Ethanol	1:0.2, 25 ºC, 3 h	2.07	22.7	79.7
hydrolysis	1-propanol	1:0.2, 25 ºC, 3 h	2.28	19.8	87.9
hydrolysis	1-butanol	1:0.2, 25 ºC, 3 h	1.69	19.0	88.8

TABLE 2 Specific enzymatic activity EA of ASL lipase in the hydrolysis of WCO with and without heptane (co-solvent added) as a function of the mass of biocatalyst and WCO, oil: water molar ratio and time of reaction at 25 ºC in a batch reactor under stirring at 200 rpm.

Mass ASL [mg]	**WCO [g]**	**Molar ratio Oil: water**	**Heptane [mL]**	**Time [h]**	**EA [μmol mg^{-1} h^{-1}]**
5.0	10.0	1:25	---	0.5	718.8
5.0	10.0	1:25	1.0	0.5	5.6
91.7	0.9	1:9	1.0	5.0	2.4
87.2 [a]	0.9 [b]	1:9	1.0	5.0	4.7

[a] Data from reference [32].

[b] fresh soybean oil