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Sustainable preparation of a novel glycerol-free biofuel by using pig pancreatic lipase: Partial 1,3-regiospecific alcoholysis of sunflower oil

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ABSTRACT

The preparation of a novel biofuel denoted as Ecodiesel-100 from the partial 1,3-regiospecific alcoholysis of sunflower oil is reported. Pig pancreatic lipase (PPL) was employed in the reaction as both free and immobilised enzyme on sepiolite. The resulting biofuel is composed of fatty acid ethyl esters and monoglycerides (FAEE/MG) blended in a molar relation 2/1. The novel biofuel has similar physico-chemical properties compared to those of conventional biodiesel and/or petrodiesel, avoiding the production of glycerine as by-product.

The biocatalyst was found to be strongly fixed to the inorganic support (87.5%). Nevertheless, the efficiency of the immobilised enzyme was reduced to less than half (42%) compared to that of the free PPL. Quantitative conversions of triglycerides and high yields to FAEE were obtained under mild reaction conditions (20–80 °C, oil/alcohol 2/1 v:v ratio and PPL 0.01–0.1% w/w of total substrate). The immobilised enzyme showed a remarkable stability as well as a great reusability (more than 11 successive reuses) without a significant loss of its initial catalytic activity. Both immobilised and free enzyme exhibited the same reaction mechanism, according to the coincidental results in the Arrhenius parameters ($\ln A$ and E_a). The immobilised PPL was found to be very suitable for the continuous production of biofuel due to its facile recyclability from the reaction mixture.

1. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases that operate on carboxylic ester bonds. They are widely spread in animals, plants, moulds and bacteria. The natural physiologic role of lipases is the hydrolysis of triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol but they can also catalyse esterifications, alcoholysis and transesterifications in non-aqueous media [1]. Such versatility makes them ideal candidates for various applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries [2,3]. The limitations of the industrial use of lipases have been mainly due to their high production costs, which may be overcome by molecular technologies to enable the production of the enzymes in big quantities as well as in a virtually purified form.

Triglycerides (TG) of vegetable oils and fats are becoming increasingly important as alternative fuels for diesel engines due to the diminishing petroleum reserves. However, their high viscosities and low volatilities do not allow their direct use or in oil/petrol blends in any diesel engine type [4–6]. Nowadays, the main process developed to overcome this drawback is the methanolysis reaction to produce biodiesel, a biodegradable, non-toxic diesel fuel substitute that can be used in unmodified diesel engines [7,8]. Biodiesel has a significant added value compared to petro-diesel due to its higher lubricity, which extends engine life and reduces maintenance costs as well as contributing to fuel economy [9]. The conventional methodology in the production of biodiesel primarily involves the use of NaOH and KOH as homogeneous catalysts. Three molecules of fatty acid methyl esters (FAME) and one molecule of glycerol are generated for every molecule of TG [10] (Scheme 1).

However, the process is far from being environmentally friendly as the final mixture needs to be separated, neutralised and thoroughly washed, generating a great amount of waste (e.g. salt residues, waste water). The catalyst cannot also be recycled. These several additional steps inevitably put the total overall biodiesel

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Scheme 1. Transesterification reaction of vegetable oils to produce FAME (biodiesel) and glycerol as by-product.

production costs up, reducing at the same time the quality of the glycerol obtained as by-product [11]. Ethanol could potentially be used instead of methanol, but the rates of reaction are comparatively slower.

Several reports can be recently found on the production of biodiesel involving other chemical [9,12] or enzymatic catalytic protocols as greener alternatives [13–18]. The increasing environmental concerns have led to a growing interest in the use of enzyme catalysis as it usually produces a cleaner biodiesel under milder conditions [19]. It also generates less waste than the conventional chemical process. Many reports on the preparation of biodiesel using free [20] or immobilised lipases can also be found [21–23].

Pig pancreatic lipase (PPL) has been widely employed in the last decades for the resolution of mixtures of chiral enantiomers, either by enantioselective hydrolysis [24,25] or by alcoholysis or transesterification [26]. The widespread use of the enzyme in fine chemicals is due to relative low price, accessibility (several suppliers in the international market) and high stability. The enzyme does not also require the use of cofactors. Despite various attempts in which the enzyme was also tested for the efficient production of biodiesel, the FAME conversions were lower than 60% [27–31]. Recently, Paula et al. obtained that the preparation of biodiesel via transesterification of babassu oil with alcohols (e.g. ethanol, propanol or butanol) using PPL as catalyst was feasible, regardless of the type of alcohols. Results revealed that the immobilised PPL could efficiently convert triglycerides to fatty acid alkyl esters obtaining yields that varied from 75% to 95% [32].

The major drawback of the process is the high cost due to the various steps involved that can limit somehow the widespread use of enzymes. Another limitation of the enzymatic method compared to the conventional base catalysed process deals with the alcoholysis of the 2-fatty acid esters of glycerol. Lipases have indeed a peculiar 1,3-regioselectivity which means that they selectively hydrolyse the more reactive 1 and 3 positions in the triglyceride [33]. In this regard, the production of biodiesel using lipases needs to take into account such regiospecific character [34,35]. In general, the challenging full alcoholysis of triglycerides involves long reaction times and gives conversions lower than 70 wt% in fatty acid methyl or ethyl esters [36,37].

A series of improvements in conversion levels and/or the use of methanol as alcohol to mimic the results of the base catalysed transesterification reaction are currently ongoing as a consequence of the present legal regulations for biodiesel (EN 14214). Reasonably good results are sometimes reported due to the 1,2-acyl migration in the monoglycerides [38–40].

The current standard biodiesel production (under alkaline chemical conditions) is considered to be the most technically simple way to reduce the viscosity of vegetable oils from a range of 11–17 to about 2 times to that of petroleum diesel [41–43]. Various fuel properties of pure soybean oil, three B100 biodiesel types (soybean methyl esters, rapeseed methyl esters and rapeseed ethyl esters) and high-grade petrodiesel are summarised in Table 1.

The viscosity is the only significant parameter that may affect the performance of the diesel engine as the other parameters are

Table 1
Physico-chemical properties of soybean oil, biodiesel (B100) obtained from soybean oil and rapeseed oil and No. 2 diesel (D2) [43].

Properties	Soybean oil	FAME[a]	FAME[b]	FAEE[c]	D2
Specific gravity (g cm^{-3})	0.920	0.86	0.8802	0.876	0.8495
Viscosity (40 °C)	46.68	6.2	5.65	6.11	2.98
Cloud point (°C)	2	−2.2	0	−2	−12
Pour point (°C)	0	−9.4	−15	−10	−18
Flash point (°C)	274	110	179	170	74
Boiling point (°C)	357	366	347	273	191
Cetane number	48.0	54.8	61.8	59.7	49.2
Sulphur (wt%)	0.022	0.031	0.012	0.012	0.036
Heat of combustion (kJ/kg)	40.4	40.6	40.54	40.51	45.42

[a] FAME stands for fatty acid methyl esters from soybean oil.
[b] FAME stands for fatty acid methyl esters from rapeseed oil.
[c] FAEE stands for fatty acid ethyl esters from rapeseed oil.

very similar. Interestingly, the diglycerides (DG) and triglycerides (TG) are mainly responsible of the increase in viscosity of pure vegetable oils. Therefore, a novel biofuel containing a FAME/MG or FAEE/MG blend (in which we exclude the presence of significant quantities of DG and TG) can be expected to have similar physical properties to those of conventional biodiesel, eliminating the production of glycerol as by-product. The achievement of a glycerol-free biofuel is most convenient and advantageous in a market flooded by the production of glycerol as by-product in the preparation of biodiesel [44–48].

The biofuel obtained is also cleaner and the efficiency of the production can be increased more than 10% when the glycerine is somehow integrated into the biofuel. The last step of washing and cleaning the biodiesel in the conventional synthetic process [to mainly remove the traces of glycerol up to 0.02% glycerol (EN 14214)] can therefore be eliminated, reducing costs and avoiding the generation of waste water [49].

High levels of glycerol in the fuel causes various problems including coking, an increase in the viscosity of the fuel and a potential dehydration to acrolein which can be further polymerised. Coking can also generate deposits of carbonaceous compounds on the injector nozzles, pistons and valves in standard engines, reducing the efficiency of the engines [50,51].

Recent investigations have also shown that minor components of biodiesel, usually considered contaminants under the biodiesel standard EN 14214, including free fatty acids and monoacyl glycerols, are essentially responsible for the lubricity of low-levels blends of biodiesel and petrodiesel. Pure FAME exhibit a reduced lubricity compared to the biodiesel containing these compounds [52–57]. The presence of greater quantities of monoglycerides and/or free fatty acids enhances the lubricity of biodiesel, which is another key feature of this novel biofuel that incorporates high amounts of MG.

Here, we report the application of an enzymatic protocol using free and immobilised *pig pancreatic lipase* (*PPL*) as an economically viable biocatalyst for the production of a novel biofuel as potential petrol-fuel replacement.

2. Methods

2.1. Materials

A commercial crude PPL (Type II, L3126, Sigma–Aldrich), sunflower oil for food use and ethanol (Panreac, 99%; Alcoholes del Sur, 96%) were employed in the enzymatic ethanolysis reactions. Various short-chain alcohols including methanol, 1-propanol, 2-propanol, 1-pentanol (all reagents from Panreac, 99%) were also used.

The sepiolite (Tolsa S.A, Spain) is a natural silicate that presents a fibrous structure (Fig. 1). The theoretical formula of the unit cell is $Si_{12}O_{30}Mg_8(OH)_6(-H_2O)4.8H_2O$, where Si^{4+} and Mg^{2+} can be partially replaced by Al^{3+}, Fe^{2+} and alkaline ions. Each atom of Mg completes their coordination with two molecules of water.

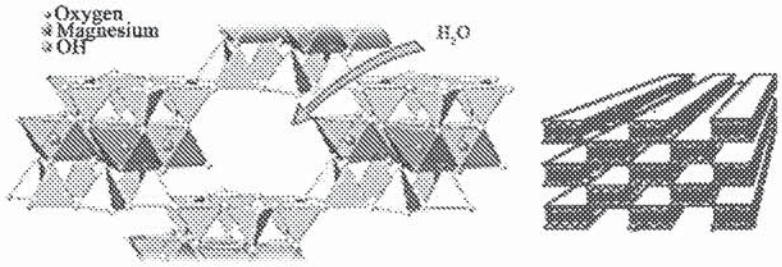

Fig. 1. Structure of the sepiolite.

2.2. Experimental procedure

2.2.1. Support activation and PPL immobilisation

The acid demineralisation treatment of the natural sepiolite was carried out in a round bottom flask containing 400 mL 1 M HCl solution and 40 g sepiolite under vigorous stirring at room temperature. The presence of Mg was determined every 8 h using yellow titan as specific indicator. The acid treatment was repeated until absence of Mg in the filtrate. The final solid was preserved under incipient humidity to maintain the fibrous structure [58,59].

The entrapment of the PPL was carried out as follows: 1.7 g demineralised sepiolite, 0.04 g PPL and 6 mL ethanol were added to a reaction flask (50 mL) and kept in a refrigerator for 24 h, stirring occasionally, prior to its use. 6 mL ethanol were then added to the mixture and the solid with the entrapped PPL was separated by filtration and centrifugation from the solution containing the remaining non-immobilised lipase. The catalytic activity of this dissolution is proportional to the amount of PPL dissolved. Thus, we can easily determine the quantity of PPL which has not been immobilised, according to a previously reported methodology [58–61]. The comparison of this value with the activity of immobilised and free PPL enzymes will allow us to determine the amount of immobilised enzyme, and its efficiency [58–61].

2.2.2. Alcoholysis reactions

The alcoholysis reaction was performed in a 50 mL round bottom flask under continuous stirring at controlled temperature (20–80 °C) varying the pH values in the 7–12 range. The various pH environments were achieved by adding different quantities of aqueous solutions of NaOH 10N. In this regard, a blank reaction in the presence of the highest quantity of solution of NaOH was performed to rule out a potential contribution from the homogeneous NaOH catalysed reaction. Less than 15% conversion of the starting material was found under these conditions implying the production of the biofuel can be attributed to the activity of the enzyme added as catalyst.

The reaction mixture comprises of 9.4 g (12 mL, 0.01 mol) sunflower oil, a variable oil/alcohol volume ratio and 0.5 g of solid containing 0.01 g immobilised PPL. Free PPL (0.01 g) was also used as reference, to determine the efficiency and amount of immobilised enzyme.

2.2.3. Compositional analysis of reaction products by gas chromatography

Samples were periodically withdrawn at different times of reaction (6–48 h) and quantified using a gas chromatograph HP 5890 Series II Gas connected to a capillary column HT5, 0.1 UM (25 m × 0.32 mm, SGE). Dodecane was employed as internal standard. The results are expressed as relative quantities of the corresponding fatty acid ethyl esters (FAEE) and the sum of the quantities of MG and diglycerides (DG). The yield refers to the relative amount of FAEE produced (%). The conversion includes the total amount (%) of triglyceride transformed (FAEE + MG + DG). The reaction rates and turn over frequencies (TOF, mol h^{-1} $g_{PPL}{}^{-1}$) were calculated from the yield, considering the amount of FAEE generated per unit of time of reaction and weight of PPL employed.

2.2.4. Viscosity measurements

The viscosity was determined in a capillary viscometer Oswald Proton Cannon-Fenske Routine Viscometer 33200, size 200. This is based on determining the time needed for a given volume of fluid passing between two points marked on the instrument. It correlates to the speed reduction suffered by the flow of liquid, as a result of internal friction of its molecules, depending on their viscosity. From the flow time, t, in seconds, the kinematic viscosity (υ, centistokes, cSt) can be obtained from the equation: $\upsilon \times t = C$, where C is the constant calibration of the measuring system in cSt s, which is given by the manufacturer (0.10698 mm^2 s^{-1}, at 40 °C) and t the flow time in seconds. The kinematic viscosity also represents the ratio between the dynamic viscosity and the density (ρ, $\upsilon = \eta/\rho$).

3. Results and discussion

3.1. Effect of different parameters on the enzymatic activity

The ethanolysis of sunflower oil has been chosen as test reaction for the preparation of the novel biofuel in order to evaluate the behaviour of the PPL under different pH environments, temperatures and relative oil/ethanol ratio. Sunflower oil is composed of a mixture of fatty acids (mainly oleic, linoleic and stearic acids) in varying proportions. The effect of the different parameters in the preparation of the biofuel was investigated using free PPL in order to optimise the reaction conditions.

Demineralised sepiolite was used for the immobilisation of the PPL through entrapment into its channels, in a similar way to previously reported results [58–61]. The channels (11.5 Å × 5.3 Å) that move along the fibres confer the solid a microporous structure with a high surface area, similar to that of the AlPO-5 [62–64]. The extraction of the ions (i.e. Mg^{2+}, Al^{3+}) by acid treatment significantly increases the size of the pores, making them comparable to those of amorphous silica [65–68] or even to a mesoporous MCM-41-like structure [69,70]. These big pores are able to trap some macromolecules including various enzymes [58,59].

3.2. Experiments with free PPL. Optimisation of the reaction parameters

3.2.1. Effect of the pH

Table 2 and Fig. 2 summarise the main results of the alcoholysis reaction under standard reaction conditions (12 mL sunflower oil, 6 mL EtOH, 0.005 g free PPL, 40 °C) at different pH. The PPL activity increased on increasing the pH value, reaching a maximum activity at pH 12 (Fig. 2). The maximum FAEE yield found at quantitative conversion was around 55% (pH 12, 40 °C). The alkaline added (NaOH 10N solution, up to 0.1 mL) acts as an adjuvant in the alcoholysis process but never as catalyst as the reaction runs in the absence of the enzyme (even at pH 12 where the maximum quantity of NaOH solution-0.1 mL-was added) gave conversions of the starting material lower than 15%. The reaction rate (TOF) decreased gradually with the time of reaction regardless of the conversion in the systems. This behaviour can only be explained by the gradual denaturalisation of the enzyme that renders the enzyme inactive with time. This phenomenon became noticeable after 6–7 h of reaction.

3.2.2. Effect of the oil/alcohol ratio

The conversion and yields to FAEE were significantly affected by the oil/alcohol molar ratio (Fig. 3). An increase in the catalyst efficiency at longer times of reaction (>16 h) was found at lower oil/alcohol molar ratios (1/5 to 1/1) with an optimum at 1/2. In any case, relatively similar yields (at quantitative TG conversions) were

Table 2
Effect of the pH on the composition, yield, conversion (% by GC) and turn over frequency (TOF, mmol h^{-1} g_{PPL}^{-1}) of the Ecodiesel-100 obtained after the ethanolysis of sunflower oil[a].

pH	Time (h)	FAEE (%)	MG + DG (%)	TG (%)	Yield (%)	Conv. (%)	TOF (mmol h^{-1} g_{PPL}^{-1})
6	6	4.3	12.6	83.1	4.3	16.9	14.5
	24	4.6	13.5	82.0	4.6	18.0	3.8
6.4	4	3.1	9.4	87.5	3.1	12.4	15.7
	20	9.7	51.3	39.1	9.7	60.9	9.7
6.8	7	7.1	25.9	67.1	7.1	32.9	20.2
	24	8.7	44.8	46.5	8.7	53.5	7.2
7.2	6	7.7	23.4	68.9	7.7	31.1	25.7
	24	19.6	80.4	–	19.6	100.0	16.3
7.5	7	11.8	32.6	55.6	11.8	44.4	33.6
	24	12.6	35.8	51.6	12.6	48.4	10.5
8.0	5	10.6	84.6	4.8	10.6	95.2	42.5
	24	28.0	70.7	1.3	28.0	98.7	23.4
9.0	5	14.1	15.0	70.9	14.1	29.1	56.4
	24	28.5	71.5	–	28.5	100.0	23.8
10.0	4	16.3	18.6	65.1	16.3	34.9	81.6
	24	18.0	72.2	9.8	18.0	90.2	15.1
12.0	7	55.8	44.2	–	55.8	100.0	159.4
	24	56.3	43.7	–	56.3	100.0	46.9

[a] Reaction conditions: 12 mL sunflower oil (0.01 mol), 6 mL ethanol (0.11 mol), 0.005 g free PPL (0.005% w/w of total substrate), 40 °C, pH 12.

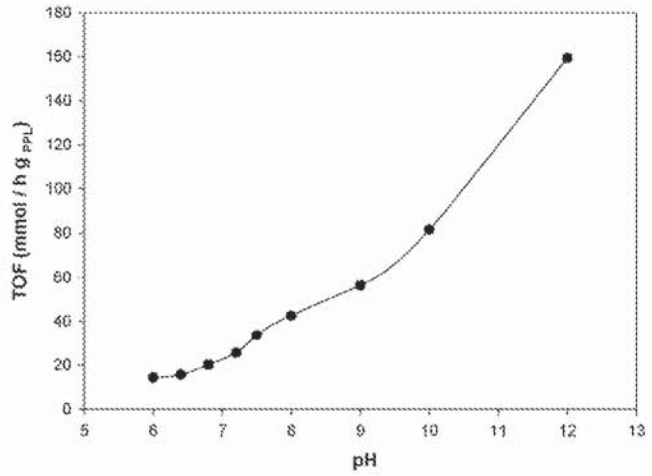

Fig. 2. Influence of the pH in the catalytic activity of the free PPL in the ethanolysis of sunflower oil at 40 °C.

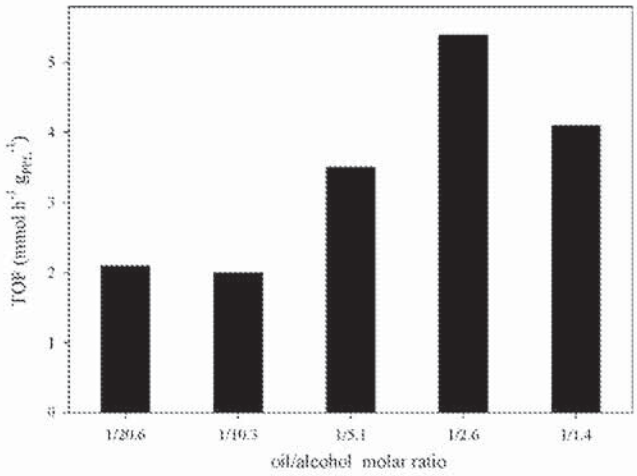

Fig. 3. Effect of the oil/alcohol molar ratio on the catalytic efficiency of the free PPL in the transesterification of sunflower oil with ethanol [reaction conditions: 45 °C, pH 12, 0.01 g free PPL (0.1% w/w of total substrate)].

found for all systems. The use of waste cooking oil implies a decrease in the conversion, as reported using other lipases [71–73].

3.2.3. Effect of the different alcohols

Another important advantage of the enzymatic process is the possibility of using various alcohols different to methanol or ethanol. We have investigated the alcoholysis process of different short-chain alcohols, obtaining the corresponding fatty acid esters (FAE, Table 3). The biofuels could smoothly be produced using the various alcohols employed, obtaining quantitative triglyceride conversions and selectivities to FAE higher than 50% in most of the cases. The reaction took typically 8–12 h to complete and the selectivity to FAE increased with the time of reaction as expected.

The enzyme catalysed biofuel production (Tables 2 and 4) does not generate any glycerine as a result of the 1,3 selective hydrolysis of the triglycerides in the ethanolysis of sunflower oil. A potentially useful biofuel blend of FAEE, MG and traces of DG, in varying proportions (depending on the conversions) was obtained. The FAEE/MG ratio was around 2/1 molar at quantitative triglyceride conversion.

3.2.4. Effect of the temperature of reaction

The influence of the temperature of reaction on the catalytic activity of the free enzyme is summarised in Table 4 and Fig. 4. The reaction rate (TOF) gradually increases with temperature having a maximum activity (almost 60% FAEE at quantitative conversion) at 60 °C. A remarkable decrease in activity was found at temperatures higher than 60 °C due, most likely, to the denaturalisation of the protein structures of the enzyme. Similarly, the reaction rate values decreased with time, regardless of the level of conversion, maybe related to the deactivation of the PPL.

3.3. Experiments with immobilised PPL

Table 5 summarises the results obtained employing the immobilised PPL compared to the free enzyme. The same quantity

Table 3
Effect of the different short-chain alcohols, on composition, yield and conversion (% by GC) and TOF (mmol h^{-1} g_{PPL}^{-1}) of the Ecodiesel-100, obtained in the alcoholysis of pure and waste frying sunflower oil[a].

Alcohol	Time (h)	FAE (%)	MG + DG (%)	TG (%)	Yield (%)	Conv. (%)	TOF (mmol h^{-1} g_{PPL}^{-1})
MeOH	24	55.1	44.9	–	55.1	100.0	22.9
EtOH	10	58.7	41.3	–	58.7	100.0	58.7
	24	60.7	39.3	–	60.7	100.0	25.5
EtOH 96%	10	27.8	72.2	–	27.8	100.0	27.8
	24	35.3	64.7	–	35.3	100.0	14.7
1-PrOH	16	56.9	43.1	–	56.9	100.0	35.6
	24	58.9	41.1	–	58.9	100.0	24.5
2-PrOH	16	19.6	80.4	–	19.6	100.0	12.3
	24	56.4	43.6	–	56.4	100.0	23.5
1-BuOH	16	47.5	42.2	10.3	47.5	89.7	29.7
	24	49.3	42.1	8.6	49.3	91.4	20.5
2-BuOH	13	59.6	40.4	–	59.6	100.0	45.8
	24	65.7	34.3	–	65.7	100.0	27.3
t-BuOH	24	52.3	38.3	9.4	52.3	100.0	21.8
1-PeOH	24	58.9	41.2	–	58.9	100.0	24.5

[a] Reaction conditions: 12 mL oil (0.01 mol), 1/3 oil/alcohol molar ratio, 0.01 g free PPL (0.1% w/w of total substrate), 45 °C, pH 12.

supported biocatalyst was used in each reaction. The number of re-uses is an essential parameter to assess the efficiency of the physical entrapment of the PPL into the pores of the demineralised sepiolite.

In principle, the enzymatic activity is proportional to the amount of enzyme in solution. Thus, the quantity of immobilised enzyme can be determined from the differences in activity between the PPL in the supernatant (Table 5, entry PPL filtrate) and the standard quantity (0.01 g) of free PPL (Table 5, entry free PPL) [58–61]. The resulting solution (after the enzyme immobilisation) was filtered off, the reaction flask washed with 6 mL of ethanol and its catalytic activity was then tested in the ethanolysis process. The filtrate gave a 26.9% yield compared to the 57.7% yield obtained using the 0.01 g of free PPL. The calculations showed that only 12.5% of the enzyme (0.005 g of PPL) was in the filtrate. Therefore, an 87.5% of the enzyme was immobilised, in good agreement with previously reported results [58–61]. A good correlation was also obtained between the corresponding TOF values obtained with the filtrate (53.8), as compared to the free PPL solution (57.7).

The activities of the free and immobilised PPL (up to 6 reuses) under identical reaction conditions (Table 5, entries free PPL and 4) were then investigated. Two different series of reactions were carried out. Different temperatures, oil/alcohol ratios and oil/immobilised PPL ratios have been also investigated and included in Tables 5 and 6.

The efficiency of the PPL can be obtained comparing the TOF values of free and immobilised PPL (Table 5), both obtained under the same experimental conditions and temperature. The PPL reduced its efficiency to a 42.5% [(24.5/57.7) × 100 = 42.5] after

Table 4
Effect of the temperature on composition, yield, conversion (% by GC) and TOF (mmol h^{-1} g_{PPL}^{-1}) of the Ecodiesel-100 obtained after the ethanolysis of sunflower oil[a].

Temp. (°C)	Time (h)	FAEE (%)	MG + DG (%)	TG (%)	Yield (%)	Conv. (%)	TOF (mmol h^{-1} g_{PPL}^{-1})
20	6	15.6	10.0	74.4	15.6	25.6	26.1
	24	42.8	19.8	37.3	42.8	25.3	17.9
25	7	44.1	26.7	29.3	44.1	70.8	63.0
	24	48.4	51.6	–	48.4	100.0	20.2
30	8	38.6	27.2	34.2	38.6	65.8	48.2
	19	39.4	35.1	25.5	39.4	74.6	20.8
	24	40.3	35.3	24.4	40.3	75.6	16.8
40	6	45.7	44.5	9.8	45.7	90.2	76.1
	10	57.7	34.2	8.1	57.7	91.9	57.7
50	4	43.3	24.0	32.7	43.3	67.3	108.3
	19	48.0	29.8	22.2	48.0	77.8	25.3
	24	46.7	34.7	18.6	46.7	81.4	19.5
60	3	46.1	27.5	26.4	46.1	73.7	153.8
	6	52.6	47.4	–	52.6	100.0	87.7
	9	55.7	44.3	–	55.7	100.0	61.8
	19	57.2	42.8	–	57.2	100.0	30.1
70	5	25.0	16.5	58.5	25.0	41.5	50.0
	8	35.8	22.8	41.4	35.8	58.6	44.7
	20	52.1	33.1	14.8	52.1	85.2	26.0
	24	56.2	43.8	–	56.2	100.0	23.4
80	24	5.6	27.9	66.6	5.6	33.4	2.3

[a] Reaction conditions: 12 mL sunflower oil (0.01 mol), 6 mL ethanol (0.11 mol), 0.01 g free PPL (0.1% w/w of total substrate), 45 °C, pH 12.

Table 5
Comparison of activities of the free and immobilised PPL [composition, yield and conversion (% by GC) and TOF (mmol h^{-1} $g_{PPL}{}^{-1}$)] in the ethanolysis of sunflower oil[a].

Run[b]	T (°C)	t (h)	FAEE (%)	MG + DG (%)	TG (%)	Yield (%)	Conv. (%)	TOF (mmol h^{-1} $g_{PPL}{}^{-1}$)
Free PPL (0.01 g)	40	10	57.7	34.2	8.1	57.7	91.9	57.7
PPL filtrate (0.005 g)	40	10	26.9	38.2	34.9	26.9	65.1	53.8
1	25	72	61.3	38.7	–	61.3	100.0	8.4
2	30	24	58.7	41.3	–	58.7	100.0	21.7
3	39	24	55.2	32.6	12.2	55.2	74.5	23.1
4	40	24	58.8	41.2	–	58.8	100.0	24.5
5	45	20	61.1	38.9	–	61.1	100.0	25.6
6	50	27	60.8	39.2	–	60.8	100.0	30.5

[a] Reaction conditions (unless otherwise stated): 12 mL sunflower oil (0.01 mol), 6 mL ethanol (0.11 mol), pH 12, 0.5 g of demineralised sepiolite containing 0.01 g of immobilised PPL (0.1% w/w of total substrate).
[b] 1 to 6 in the first column stand for the number of reuses of the immobilised PPL.

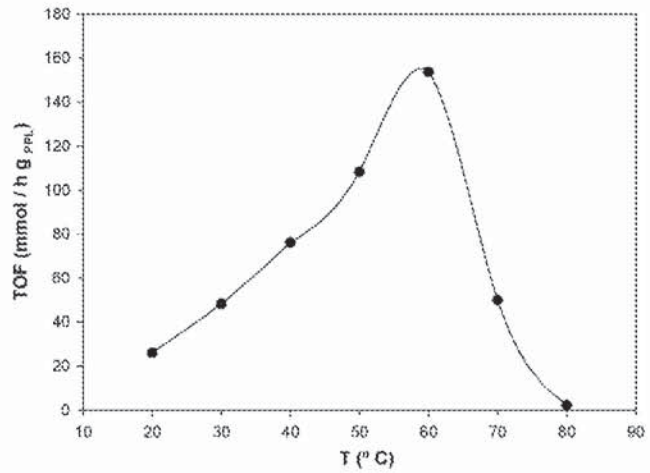

Fig. 4. Influence of the temperature in the catalytic activity of the free PPL in the ethanolysis of sunflower oil under standard experimental conditions.

immobilisation, that may be due to a potential steric effect of the immobilised enzyme in the reaction and/or to the deactivation of the active sites of the enzyme in the entrapment process.

TOF values showed that a decrease in the oil/alcohol molar ratio from 1/10 (Table 5) to 1/2 (Table 6) leads to an increase in the efficiency of the immobilised enzymes, in good agreement with results obtained for the free enzyme. Results also pointed out that in any case, even with an excess of ethanol, a maximum 66% yield could be obtained, corresponding to a 1,3 selective enzymatic process.

A comparison of the behaviour of the free and immobilised enzymes can also be established through their Arrhenius plots (Fig. 5). The activation energies (E_a) and Arrhenius constants (Ln A) can be obtained from the slopes and intercepts (Table 7) and let us quantify the influence of the immobilisation process and the reaction conditions. E_a provides an insight on the efficiency of the enzyme active sites whilst the Arrhenius constant (Ln A) gives information of the number of active sites in the process.

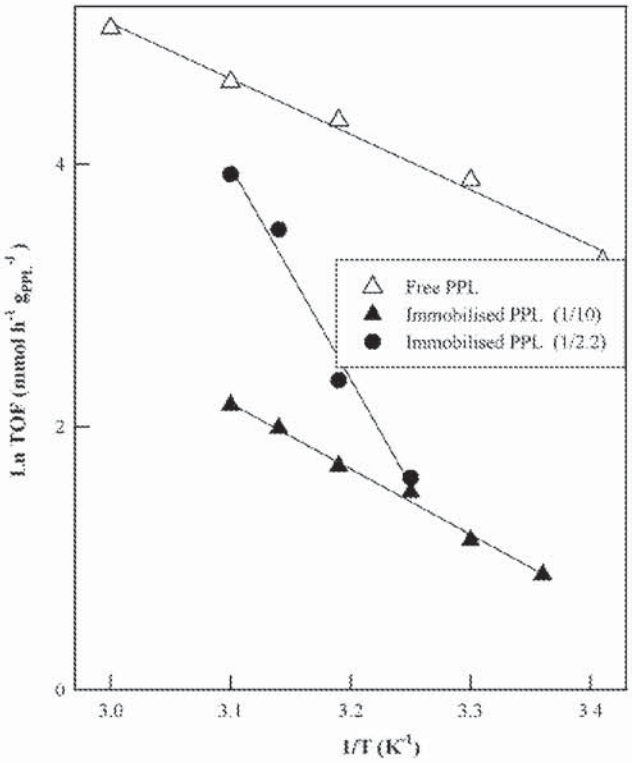

Fig. 5. Arrhenius plots (Ln TOF vs 1/T) comparing the enzymatic activity of the PPL in the ethanolysis of sunflower oil (pH 12) under various conditions. (△) Free PPL (1/10.3 oil/ethanol molar ratio): 12 mL sunflower oil, 6 mL ethanol, 0.01 g free PPL; (▲) immobilised PPL (1/10.3 oil/ethanol molar ratio): 12 mL sunflower oil, 6 mL ethanol, 0.5 g sepiolite containing 0.01 g immobilised PPL; (●) immobilised PPL (1/2.2 oil/ethanol molar ratio): 48 mL sunflower oil, 4.8 mL ethanol, 0.5 g sepiolite containing 0.01 g immobilised PPL.

Interestingly, the numerical values of E_a and Ln A are very similar for both free and immobilised PPL (under identical conditions; pH 12 and 2/1 oil/alcohol v:v ratio). Thus, the free and immobilised PPL may operate under the same reaction mechanism. A variation

Table 6
Composition, yield and conversion (% by GC) and TOF (mmol h^{-1} $g_{PPL}{}^{-1}$) of the Ecodiesel-100 obtained after the ethanolysis of sunflower oil[a]. Data corresponds to the number of runs of the biocatalyst, as a continuation of this table, under different reaction conditions.

Run	T (°C)	t (h)	FAEE (%)	MG + DG (%)	TG (%)	Yield (%)	Conv. (%)	TOF (mmol h^{-1} $g_{PPL}{}^{-1}$)
7	25	27	–	–	100.0	–	–	–
8	35	15	5.2	56.1	38.7	5.2	62.2	17.5
9	40	6	13.8	17.8	68.4	13.8	25.8	36.8
10	45	12	63.5	36.5	–	63.5	100.0	169.4
11	50	15	26.5	53.3	20.1	26.5	76.6	176.8

[a] Reaction conditions: 48 mL sunflower oil (0.04 mol), 4.8 mL ethanol (0.09 mol), pH 12, 0.5 g of demineralised sepiolite containing 0.01 g of immobilised PPL (0.1% w/w of total substrate).

Table 7
Activation energies (E_a, kcal mol^{-1}) and Arrhenius constant (Ln A, h^{-1}), obtained in the ethanolysis of sunflower oil[a].

Lipase PPL	Oil/ethanol (mL/mL)	E_a (kcal mol^{-1})	Ln A (h^{-1})	R^2
Free	12/6	8.4 ± 0.2	17.8 ± 0.8	0.99
Immobilised	12/6	9.9 ± 0.2	17.6 ± 0.6	0.99
Immobilised	48/4.8	32.1 ± 1.6	54.1 ± 5.1	0.98

[a] Reaction conditions: pH 12, 0.01 g free PPL or 0.5 g of demineralised sepiolite containing 0.01 g of immobilised PPL (0.1% w/w of total substrate).

of the oil/alcohol molar ratio (dotted line, 10/1 ratio) deeply changed the values of E_a and Ln A for the immobilised PPL (Table 7, Fig. 5). Smaller quantities of alcohol provided a greater number of active sites participants in the enzymatic process (greater Ln A) and improved the efficiency (greater E_a), therefore promoting the alcoholysis.

Of note was also the enzyme stability and recyclability. Although the efficiency was reduced compared to the free form, the immobilisation through physical entrapment of the PPL guaranteed the lifespan of the lipases. Free PPL was found to be completely deactivated in 48 h, whereas the immobilised enzyme was active for several weeks, even after successive reuses (Tables 5 and 6), preserving over 90% of the initial activity.

3.4. Comparison of the novel biofuel with reported methodologies

New areas of research for methodologies to prepare esters from lipids which directly afford alternative co-products are currently under development [74]. The transesterification reaction of triglycerides with dimethyl carbonate (DMC) [75–79], methyl acetate [80–84] or ethyl acetate [85] produced a mixture of three molecules of FAME or FAEE and one of glycerol carbonate (GC) or glycerol triacetate (triacetin). Such mixture (FAME + GC) has relevant physical properties to be employed as fuel, constituting a novel biofuel denoted as DMC-BioD [76,78].

Gliperol is another patented novel biofuel [83]. It is composed of a mixture of three molecules of FAMEs and one molecule of triacetin and it can be obtained after the transesterification of one mol of TG with three moles of methyl acetate employing lipases as catalysts [80–84].

Similarly, the already patented Ecodiesel-100 obtained through the 1,3-selective partial ethanolysis of the triglycerides with PPL is a mixture of two parts of FAEE and one part of MG, with minor quantities of DG [86].

DMC-BioD, Gliperol and Ecodiesel-100 incorporate glycerol in their monophasic homogeneous mixtures, avoiding the generation of residues or by-products in their preparation processes. The main difference with respect to the conventional biodiesel (FAME) production is that no additional separation steps are needed. GC and/or triacetin can perfectly be burnt together with the FAME in the blend. In terms of green chemistry, the incorporation of glycerol into the biofuel improves the efficiency of the process (from current 90% to 100%), without a substantial modifications of the physico-chemical properties of the biofuel (Table 8). The atom efficiency is also improved as the total number of atoms involved in the reaction is part of the final mixture.

Recent studies have also demonstrated that the presence of MG adds value to the biofuel by improving the lubricity on the engine [52–57].

The viscosity is indeed highly dependent on the proportion of the TG in the sunflower oil (given its high viscosity value, 31.9), and, to a lesser extent, on the mixture MG + DG (only traces of DG can be found at FAEE conversions above 50%).

Similar commercial biodiesel samples with values close to the 2/1 FAEE/(MG + DG) ratio (e.g. yield ~60% and conversion = 100%, Table 8, entries 6, 7 and 15) exhibited low viscosities compared to the EN 14214 standard (3.5–5 viscosity range). This may be due to the influence of the ethanol present in the FAEE (plus MG + DG), which is greater than the methanol present in FAME (plus MG + DG), due its higher solubility. The presence of MG was also expected to have a little influence on the viscosity of the biofuel.

Table 8
Kinematic viscosity values, υ (cSt or mm^2/s) at 40 °C of various representative biodiesel blends as well as commercial diesel and biodiesel.

No.	Oil/alcohol	FAE	MG + DG	TG	Yield	Conv.	υ
1	Sunflower oil	–	–	100	–	–	31.9
2	Commercial diesel	–	–	–	–	–	3.1
3	Commercial biodiesel	–	–	–	–	–	2.9
4	Used/MeOH[a]	95.7	4.3	–	95.7	100.0	3.9
5	Sunflower/EtOH[b]	94.8	5.2	–	94.8	100.0	6.6
6	Sunflower/EtOH[c]	55.7	44.2	–	55.7	100.0	6.9
7	Sunflower/EtOH	61.3	38.7	–	61.3	100.0	4.1
8	Sunflower/1-PrOH	62.0	35.8	–	62.0	100.0	9.2
9	Sunflower/2-prOH	33.9	55.6	10.8	33.9	89.5	12.9
10	Sunflower/EtOH	44.3	33.6	22.1	45.3	77.9	19.6
11	Used/EtOH	54.3	41.2	4.5	54.3	95.5	23.4
12	Used/EtOH	51.4	40.9	7.7	51.4	92.3	24.5
13	Used/EtOH	66.0	31.0	3.0	66.0	100.0	19.7
14	Sunflower/EtOH	58.4	41.6	–	58.4	100.0	15.0
15	Sunflower/EtOH	60.8	39.2	–	60.8	100.0	5.4
16	Sunflower/EtOH	26.5	53.4	20.1	26.5	76.6	20.7
17	Sunflower/EtOH[d]	13.4	84.6	2.0	13.4	98.0	24.5
18	Used/MetOH	71.9	28.1	–	71.9	100.0	13.1
19	Diesel/biodiesel (1:1)[e] B50	–	–	–	–	–	6.4
20	Diesel/biodiesel (8:2)[e] B20	–	–	–	–	–	4.2

[a] Homogeneous catalyst NaOH.
[b] Homogeneous catalyst KOH.
[c] Free PPL.
[d] Synthetic biodiesel blend.
[e] Blend of commercial diesel and biodiesel with viscosity, υ = 13.1 cSt.

4. Conclusions

The alcoholysis of TG with short-chain alcohols using 1,3-regiospecific PPL lipases can play an advantageous role, compared to the conventional base catalysed processes, to prepare new biofuels incorporating glycerine minimising the production of waste as well as improving the reaction conversion under greener conditions. Milder reaction conditions were employed and a cleaner biofuel (Ecodiesel-100) could be obtained. The efficiency of PPL was remarkably increased at higher pH, in contrast with reported results describing a poor activity of the enzymes at those pHs. The immobilised PPL was highly stable although the efficiency was reduced (42%) compared to the free enzyme. The catalyst can easily be recycled almost preserving the initial catalytic activity after several cycles.

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