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Evaluation of Lipases from Wild Microbial Strains as Biocatalysts in Biodiesel Production

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Abstract: In this study, the evaluation of the catalytic behavior of several wild bacterial strains in the 1,3-selective ethanolysis of triglycerides with ethanol to produce a new type of biodiesel (Ecodiesel) that integrates glycerol as monoacylglycerols was carried out. The Ecodiesel production not only avoids the elimination of glycerol, which is largely generated as a by-product in the biodiesel industry, but also results in an increase in the biofuel yield. The wild microbial strain samples were obtained from several lipophilic organisms. In addition to evaluate the enzymatic extracts, the minimum grade of purification of the strains, necessary to obtain similar results to those attained with commercial lipases was studied. This purification treatment included a dialysis followed by a lyophilization process. Such extracts were directly used as biocatalysts in the transesterification reaction of sunflower oil with ethanol, attaining much better results (yield close to 100%) than those obtained with strains which were not submitted to the purification process (yields lower than 10%). Furthermore, the results here obtained are similar to those obtained with commercial lipases but were achieved under mild conditions and lower reaction time (2 h). In addition, the stability of the enzymatic extracts was corroborated by subsequent reactions, showing no loss of activity. Thus, this study brings to light that enzymatic extracts obtained by a very simple purification process can be economically competitive with the conventional biodiesel production methods.

Keywords: biodiesel; ethanolysis; wild microbial lipase; transesterification; Ecodiesel; sunflower oil

1. Introduction

The evaluation of alternative energies which can substitute the non-renewable ones (natural gas, coal, and fossil fuels) for electricity generation is a growing trend for reasons associated with environmental preservation [1,2]. However, there is no such equivalent in the transport sector. To date, electric motors as well as vehicles capable of using fuel cells are not able to compete with explosion or combustion engines, especially in the aviation field [3]. Thus, the search of renewable materials capable of replacing fossil fuels and that allow working with the current engines without any modifications seems to be essential [4].

At present, the only renewable materials considered for the substitution of diesel fuel are vegetable oils or animal fats used as biofuel, and the main existing technology to obtain biodiesel is based on the transesterification of triglycerides with methanol to obtain fatty acids methyl esters (FAME). Different alternative processes have been proposed, such as dilution of the vegetable oils, emulsification,

pyrolysis, and hydrotreating [5]. However, nowadays, the industrial production of biodiesel is carried out by homogeneous alkali-catalyzed transesterification of vegetable oils with methanol [1]. After the reaction, biodiesel is recovered by repeated washing with water to remove glycerol, soap, and excess methanol [5–7]. Nevertheless, the generation of glycerol as a byproduct is a major issue, because a high amount of water must be employed to remove it. In fact, this is one of the main difficulties associated with biodiesel production in many regions where water is not abundant, such as the Mediterranean countries [6].

In this sense, different methods to obtain glycerol derivatives during the transesterification process preventing waste generation have been considered. Furthermore, the glycerol derivatives would allow to increase the yield of the process (always higher than the nominal 10%), as some of this glycerol is integrated in the reaction products. These alternative methodologies to prepare esters from lipids using different acyl acceptors are still under development [8]. The transesterification reaction of triacylglycerols with dimethyl carbonate (DMC) [9], methyl acetate [10], or ethyl acetate [11] could generate a mixture of three molecules of FAME or FAEE (fatty acid ethyl ester) and one of glycerol carbonate or glycerol triacetate (triacetin). These mixtures that integrate glycerol derivative molecules exhibit relevant physicochemical properties for being employed as novel biofuels [9–12], because the glycerol derivatives possess rheological properties close or similar to those of FAME or FAEE, while containing a higher amount of oxygen and thus reducing the pollutants produced during the combustion.

In this regard, we have recently succeeded in the preparation of a new type of biodiesel that integrates glycerol into their composition via 1,3-regiospecific enzymatic transesterification of sunflower oil, using free [13,14] and immobilized porcine pancreatic lipase (PPL) [15]. Thus, the already patented Ecodiesel integrates glycerol as a derivative product in the diesel fuel, with the advantage that dimethyl carbonate or methyl acetate, more expensive than methanol or ethanol, are not employed (in contrast with other reported strategies). The procedure employed takes advantage of the 1,3-selective nature of lipases, which allows to "stop" the process in the second step of alcoholysis, obtaining a mixture of two moles of FAEE and one of monoacylglycerols (MG), as can be seen in Figure 1.

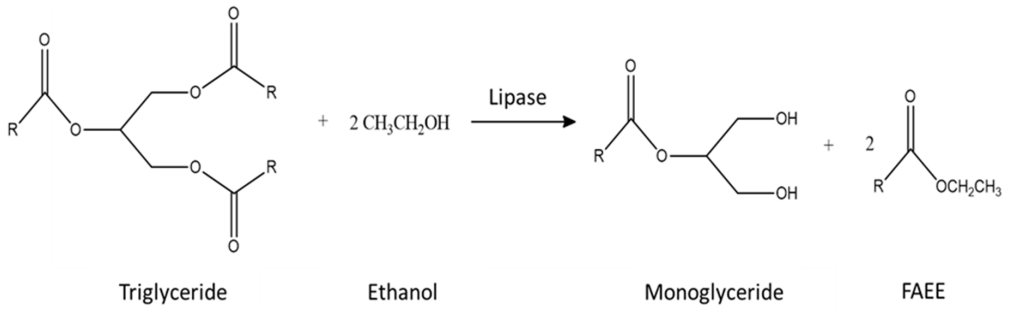

Figure 1. Reaction scheme of the 1,3-selective enzymatic catalysis to produce Ecodiesel, a biodiesel-like biofuel.

In comparison with the conventional method, the experimental conditions for Ecodiesel production are much smoother, the removal of impurities is unnecessary, and the biofuel produced exhibits similar physicochemical properties to the conventional biodiesel. Furthermore, MG enhance biodiesel lubricity, as it has been demonstrated in recent researches [16–18]. Besides, as ethanol is in excess, there is some amount of it in the product blend, allowing its direct employment as a fuel. Thus, the term Ecodiesel is ascribed to any blend of fatty acid alkyl esters with ethanol, alone or with any proportion of diesel fuel [19–23].

Initially, Ecodiesel was obtained by using PPL. Remarkable results were also attained with the microbial lipase Lipopan 50 BG (Novozymes AS, Denmark) [7,14], a low-cost purified lipase from the microorganism *Thermomyces lanuginosus*, usually employed as a bread emulsifier (bread improver) [24] and never previously described as a biocatalyst in chemical processes. Similarly, the enzymatic

ethanolysis of sunflower oil with ethanol, in free solvent media, has been studied in the presence of different lipases as well [25]. Likewise, *Rhizopus oryzae* lipase, from Biolipase-R, immobilized on Sepiolite, an inorganic support, was also studied to get the economic viability of the procedure [26]. In fact, the application of an available lipase on an industrial scale is a significant step towards the economic feasibility of biofuel production by an enzymatic method, because the high production costs of lipases is actually the main drawback in the application of this procedure.

In a recent work, some microbial lipases, from several wild microorganism strains selected in several lipophilic microorganisms such as olive oil press, animal fats, etc., have been also selected in order to improve the viability and competitiveness of the enzymatic process [27]. In that study, the selected strains belonged to the genera *Pseudomonas*, *Acinetobacter*, *Enterobacter*, *Bacillus*, and *Terribacillus*, and the best results were attained on a gram-positive bacterium belonging to the genus *Terribacillus* (OS-2) (yield > 80%), which had not been previously reported as a biofuel producer. Thus, these new microorganisms and their lipolytic enzymes can open a window of possibilities for synthetic processes, because enzymatic extracts can be easily obtained, reducing the cost of enzyme production in a notable way and, therefore, producing the biofuel in a cheaper way.

These previous researches demonstrated that it is possible to operate with non-excessively purified enzymatic extracts in order to achieve the partial transesterification process of the triglycerides with a high performance [27,28]. It is evident that the presence of other enzymes does not modify the lipases activity, because of the absence of their corresponding substrates. Therefore, we assumed a new paradigm, that is, to operate with very little purified enzymatic extracts of lipases. Thus, in the present study, we tried to determine the minimum purification degree of some wild microbial strains, previously described, necessary to get lipases which can be efficient in the 1,3-selective ethanolysis process. Thereby, we would be able to considerably reduce the production costs, making the production of alternative biofuels technically and economically viable.

2. Materials and Methods

2.1. Chemicals

Commercial sunflower oil was obtained from the local region. The ethyl esters of palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid were from Accustandard, and methyl heptadecanoate was from Sigma-Aldrich; all of them were chromatographically pure. Other chemicals, like absolute ethanol and sodium hydroxide (>99% of purity), were from Panreac Química SLU, Castellar del Vallès (Barcelona) Spain.

2.2. Collection, Isolation, and Screening of Lipolytic Microorganisms Able to Carry out the Transesterification Reaction

The wild-type microorganisms used in this study were obtained from a previous selection made among a thousand samples of vegetable oils (OS) supplied by an olive oil mill located in Seville, (Spain) and from animal fats (FS) from sawdust of fish (FS1) obtained in Cadiz (Spain) [27]. The samples were evaluated in a preliminary screening by a qualitative plate assay to select the microorganisms showing lipolytic activity. In a second step, the lipolytic bacteria were analyzed using a colorimetric assay to detect transesterification activity. This method consists in the transesterification of para-nitrophenyl palmitate (p-NPP) with ethanol in the absence of water to release the yellow-colored compound para-nitrophenol (p-NP), which can be subsequently detected by using a spectrophotometer. Thus, all positive hydrolytic microorganisms were grown in a typical liquid medium for bacteria with 2% tributyrin for 3 days at 30 °C and then centrifuged. The supernatants were freeze-dried for 24 h and mixed with 1 mL of 10 mM p-NPP (in n-hexane) and 60 µL of absolute ethanol. These mixtures were incubated at 37 °C with shaking for 16 h. A negative control was obtained by using a mixture of absolute ethanol and p-NPP. The mixture of p-NPP and freeze-dried supernatant (without ethanol) was used as a control of the hydrolytic activity of lipases. Thirty of the best lipolytic strains were selected

for further characterization. Phylogenetic analysis was performed through a16S rRNA sequences of bacteria with neighbor-joining clustering, revealing that 23 of the bacterial isolates were Gram-negative and 7 were Gram-positive, belonging to different clades. The bacterial strains previously selected were grown for 3 days. The growth media were liquids in the form of broth. After the preparation, they were conveniently sterilized in autoclave at a temperature of 121 °C for 20 min. The broth media were prepared and stored in Pyrex containers for storage or in flasks (500 mL) for the growth of the strains (100 mL medium). Then, 25 mL of culture supernatants was concentrated by dialysis (12 KDa) and lyophilized for 48 h to obtain an extract (0.5 g) in the form of fine dry powder which could be used directly as a biocatalyst in the production of biofuels by sunflower oil ethanolysis [27].

2.3. Ethanolysis Reactions

These reactions were performed according to the procedure previously described [14–18,28–31]. A preceding study to determine the optimal experimental conditions of the reaction was carried out with a commercial low-cost lipase, Lipopan BG (Novozymes AS, Denmark), which then served as a reference for the expected variability in lipases extracts from wild microbial strains [14,15,26].

To determine the efficiency of the wild enzymatic systems, the assays were carried out applying the optimum conditions predetermined by the addition of 6 mL of commercial sunflower oil, 1.75 mL of absolute ethanol, and 12.5 µL of 10 N NaOH into a 20 mL tube containing the enzyme extract of the corresponding wild microorganism strain to be tested. The transesterification reactions were carried out directly in the same sterile plastic tube used for enzymatic extraction. So, after adding the reagents and a small magnetic stirrer, the tube was introduced for 24 h in a water bath at 30 °C, with stirring higher than 300 rpm. Less than 10% conversion of the starting material was found under these conditions without any lipase, so that a blank contribution can be considered as negligible under the current investigated conditions. For all experiments, a minimum of three replicates were carried out in order to see the reproducibility of the procedure. The standard deviation was always lower than 6%.

Reusability studies were performed with eight selected enzymatic extracts, operating under the same standard experimental conditions. The reaction products were extracted each time, then we left the previously used extract in the bottom of the tube and we added the reagents to generate a new reaction cycle.

2.4. Analytical Method

The reaction products were monitored by gas chromatography (GC), using a Varian 430-GC gas chromatograph (Europe, 4330 EA Middelburg, The Netherlands), equipped with a HT5 capillary column (25 m × 0.32 mm ID × 0.1 µm, SGE, Supelco, Sigma-Aldrich Quimica SL, Madrid, Spain) and a FID detector at 450 °C and splitless injection at 350 °C. The analysis program employed was the same as that used elsewhere, using n-hexadecane (cetane) as an internal standard to quantify the content of ethyl esters and glycerols (mono-, di-, and triacylglycerols) with the help of some commercial fatty acid esters standard [13–15,25,27].

Considering that sunflower oil is constituted by a mixture of fatty acids in variable proportion (mainly linoleic, oleic, palmitic, and stearic acids), the reactions results were expressed as the relative amounts of the corresponding ethyl esters (FAEE), MG, and diacylglycerols (DG) that were integrated in the chromatogram. By difference and with respect to the internal standard (cetane), the amount of triacylglycerols (TG) which did not react was calculated in mass percentage. The conversion included the total amount of triacylglycerols transformed (FAEE + MG + DG) in the ethanolysis process, and selectivity referred to the relative amount of FAEE + MG obtained, which presented lower retention times (RT) than those hydrocarbons that compose fossil diesel (RT < 26 min).

2.5. Viscosity Measurements

The transesterification reactions of oils or fats was carried out to obtain an important reduction in viscosity, as they share similar other physicochemical parameters to the fossil diesel, except for viscosity, which has values in the range of 30–45 mm^2/s (or cSt), while fossil diesel exhibits values in the range of 2.5–6 cSt. Because of the importance of viscosity for the correct running of diesel engines, this parameter becomes a critical factor that has to be modified before vegetable oils can be used as biofuel. Thus, accurate viscosity measurements are critical to assess the quality of biofuels produced. Viscosity was determined in a capillary viscometer Oswald Proton Cannon Fenske Routine Viscometer 33200, size 150 Sigma Aldrich, Spain [14–18,28–31].

3. Results and Discussion

3.1. Comparative Chromatograms of Standardized Reaction Products

Figure 2a shows the most characteristic biofuels components, obtained by enzymatic alcoholysis. Furthermore, a representative sample of monoglycerides of sunflower oil, which was easily obtained by the substitution of methanol or ethanol for glycerol in a conventional alcoholysis process in the same experimental conditions, is also shown.

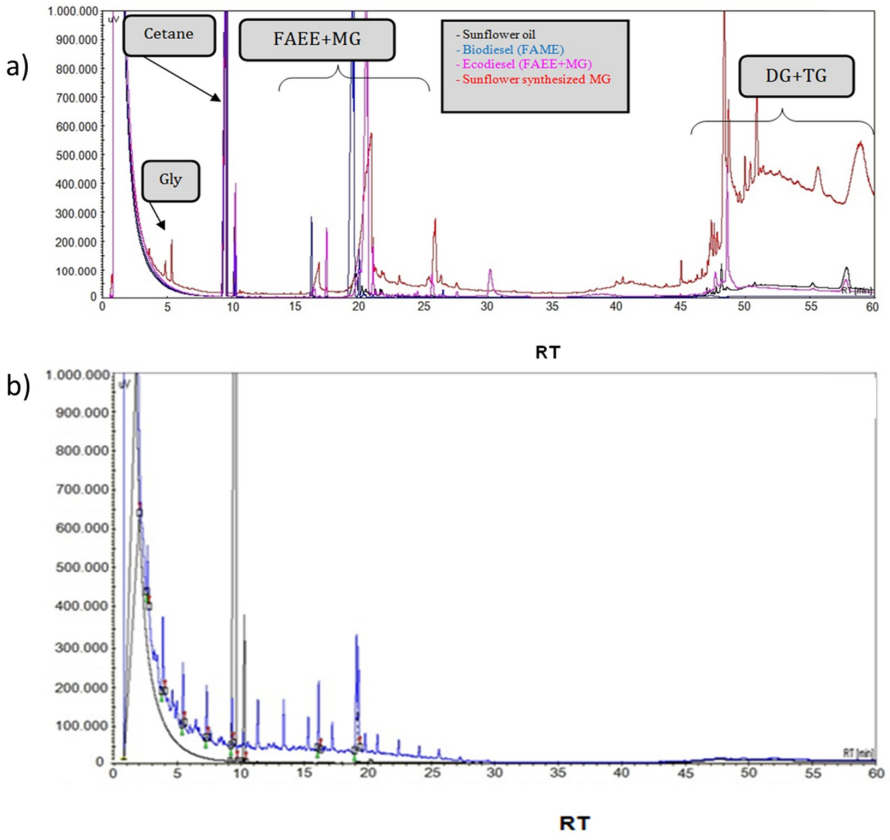

Figure 2. (**a**) Chromatograms related to the alcoholysis of sunflower oil with methanol over OS2; Fatty Acid Methyl Esters (FAME), ethanol, Fatty Acid Ethyl Esters (FAEE), glycerol (gly), monoacylglycerols (MG), diacylglycerols (DG), triacylglycerols (TG), and the initial sunflower oil are plotted in blue, pink, red, and black, respectively. (**b**) Commercial diesel fuel chromatogram.

The fatty acid esters (FAE), which constitute the lipid profile of the sunflower oil, displayed RT slightly higher (16–26 min) than cetane (10 min). The lipid profile included methyl, ethyl, and glycerol esters (the latter were MGs) of palmitic, stearic, linoleic, and oleic acids. Among them, palmitic acid derivatives (C16: 0) were grouped in a narrow RT range, 16–17 min, and oleic and linoleic acid

(C18: 1) derivatives were grouped between 19 and 21 min, except for oleic acid MG, which presented a different behavior, with RT = 26 min. Besides, glycerol RT was 5 min. The absence of this compound in the chromatograms brought to light the selectivity of the enzymatic transesterification reaction. Furthermore, the presence of DG, (RT of 40–60 min) can be also observed in Figure 2a. To determine the DG + TG amount, the use of an internal standard (cetane) was required because of the difficulty in peaks integration. On the other hand, the FAMEs, FAEEs, and MGs displayed slightly higher RT values than cetane, but within the molecular weight range, which suggested similar chemical-physical properties between the FAE and the hydrocarbons constituting the diesel, according to Figure 2b.

In fact, considering the chromatogram obtained for a diesel fuel (Figure 2b), we determined that it consisted of a complex mixture of hydrocarbons that appeared at RT in the range of that of the cetane. As it is well known, cetane is considered the "standard fuel" for internal combustion engines or diesel engines, so its "index" (in percentage form) is employed for checking the quality of fuels.

It is important to mention that, whereas the conversion values include of FAEE + MG obtained, which presented lower RT than those hydrocarbons forming part of fossil diesel the amount of triacylglycerols produced (FAEE + MG + DG), the selectivity refers to the amount (RT < 26 min). This implies that a high conversion, even 100%, could contain a high proportion of DG molecules, with high molecular weight and viscosity values. Consequently, a very high selectivity, i.e., a very high percentage of FAEEs and MGs, could promote a viscosity close to that of petroleum diesel; therefore, complete conversion is not a guaranty of low viscosity values.

3.2. Ethanolysis Reactions

In previous researches, the optimal experimental conditions of the ethanolysis reaction, using a purified lipase from *T. lanuginosus*, Lipopan 50 BG (Novozymes AS, Denmark), as a catalyst, were obtained [14,24]. Therefore, these results were employed as a reference when examining the expected variability for lipases from wild microbial strains. The wild-type microorganisms employed in this research were obtained from a previous selection among a thousand of samples, from both OS and FS [27], belonging to the genera *Pseudomonas*, *Acinetobacter*, *Enterobacter*, *Bacillus*, and *Terribacillus*, as indicated in the Introduction section. Once the bacterial strains were selected, they were grown for three days in their corresponding optimal broths cultures. Then, 25 mL of culture supernatants, containing extracellular lipases, was concentrated by dialysis (12 KDa) and lyophilized for 48 h to obtain an extract (0.5 g) in the form of fine dry powder which could be used directly as a biocatalyst in the production of biofuels by sunflower oil ethanolysis [27,28]. Thus, the catalytic behavior of the enzymatic extracts of the corresponding wild microorganism strains could be related to the amount and to the efficiency of the commercial lipases, taking into account that all the extracts (0.5 g fine dry powder) could contain a similar quantity of lipases, according to the identical procedure of extraction applied in each of them. Therefore, the different results obtained in the alcoholysis reaction could be ascribed to the variation in the efficiency of the lipase. As can be seen in Table 1, there was a wide range of lipase concentration in which the enzymatic activity was maintained. Thus, the results obtained with the wild microorganism strains extracts could be related to the quality of the lipases present in the enzymatic extracts.

Table 1. Influence of the amount of Lipopan BG (Novozymes AS, Denmark) in the ethanolysis reaction performance, developed under standard conditions; fatty acid esters (FAE).

Lipase Amount (g)	Conversion (wt %)	FAE (wt %)	DG (wt %)	TG (wt %)	Viscosity (cSt)
0.007	85.7	77.9	7.9	14.3	9.4
0.010	100	96.1	3.9	0.0	10.1
0.020	100	97.9	2.1	0.0	9.8
0.030	90.2	90.0	0.1	10.0	11.5
0.040	100	98.0	2.0	0.0	10.7

(a) Standard condition: 6 mL of sunflower oil, 1.75 mL of absolute ethanol, and 12.5 µL of 10 N NaOH, 300 rpm stirring, at 30 °C for 24 h and varying lipase amounts.

As aforementioned, the evaluation of the purification degree of the lipases extracts is mandatory for reducing the catalyst cost. In this sense, every lipase extract was directly used as a biocatalyst in the transesterification reaction of sunflower oil with absolute ethanol after a simple lyophilization process and an additional treatment, consisting in a dialysis process followed by filtration and pre-concentration of the extracts.

The efficiency of those extracts obtained by only a lyophilization treatment, without applying a previous dialysis, was negligible, since low yield were obtained (0–10%), Figures 3 and 4. However, after subjecting the same strain extracts to the additional treatment, the yield considerably increased, indicating the importance of the second step.

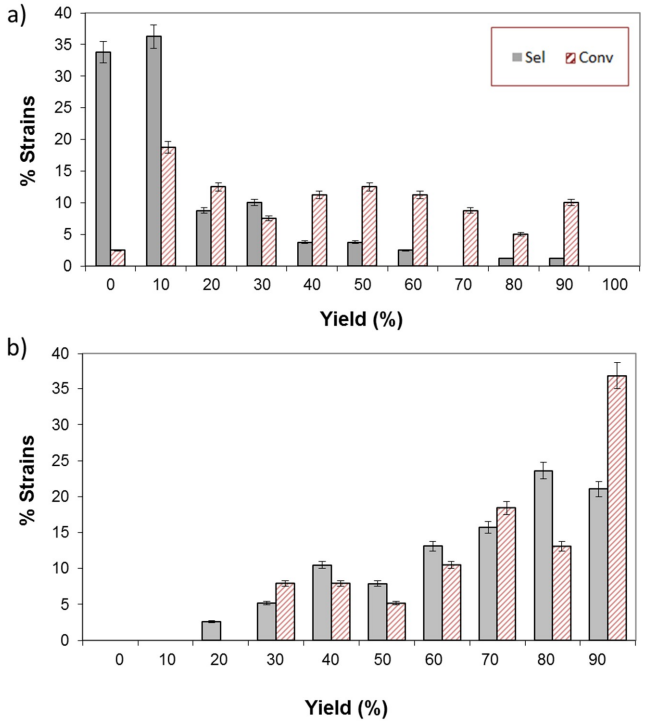

Figure 3. (**a**) Comparison of the catalytic performance of sawdust of fish (FS1) (percentage of studied strains that present a determined range of yield percentage) in the ethanolysis reaction of the totality of freeze-dried–lyophilized extracts without previous dialysis; (**b**) the same as (**a**) but after a simple previous dialysis treatment, under the same standard experimental conditions. Selectivity (Sel, %) is presented in grey, and conversion (Conv, %) in pink lines.

This simple methodology allowed us to access to the enzymatic material, which met the minimum requirements for performing the enzymatic alcoholysis process, in a simple and inexpensive way, in comparison to the commercial purified lipases, e.g., Lipopan 50 BG (Novozymes AS, Denmark).

The results obtained in the enzymatic ethanolysis of sunflower oil are collected in Table 2. In general, these results obtained with the enzymatic materials are quite similar to those obtained on commercial lipases, Table 1. The samples obtained from OS, i.e., OS-3, OS-4, OS-5, OS-6, OS-8, OS-10, OS-15, OS-19, and those obtained from FS, e.g., FS-4, exhibited conversion and selectivity values in the percentage ranges of 60–100%, respectively, which allowed to obtain viscosity values lower than 11.5 cSt. Taking into account that around 0.5 g of these materials was able to perform an enzymatic activity similar to that of 0.01–0.04 g of an expensive commercial lipase, the economic viability of the process must be highlighted. On the other hand, comparing the efficacy of the isolated extracts from vegetal oils with those from animal fats of all the studied strains, it could be affirmed that the extracts from animal fats had an acceptable overall performance, although inferior to that of extracts obtained

from vegetable oils. Among the animal fat samples, strains from FS1 presented much better yields than strains from ham fat (FS2).

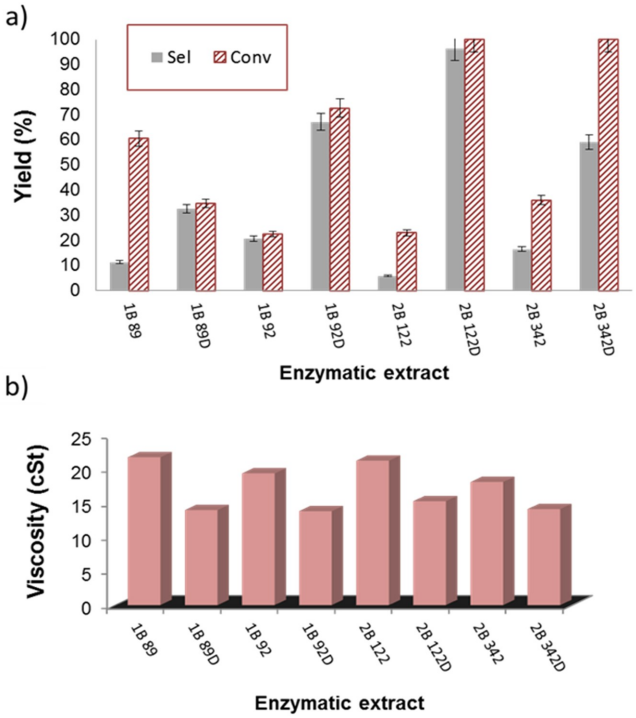

Figure 4. Comparison of the catalytic performance (**a**) and viscosity in cSt (**b**) obtained in the ethanolysis reaction over some cultured microbial wild strains either submitted to the dialysis treatment or not.

Table 2. Conversion, selectivity, and viscosity obtained in the enzymatic ethanolysis of sunflower oil under different lyophilized bacterial extracts, with previous dialysis, from vegetable oil (OS) and animal fat (FS) samples. Reaction conditions: 37 °C, 24 h, 300 rpm, operating with 6 mL of oil, 1.75 mL of absolute ethanol, and 0.0125 mL of 10N NaOH solution. Conversion = FAE + DG, being FAE = (FAEE + MG) the selectivity of the process. A minimum of three replicates for each experiment were carried out (less than 6% of error was obtained).

SAMPLE	Conversion (wt %)	FAE (wt %)	DG (wt %)	TG (wt %)	Viscosity (cSt)
OS 1	69.8	63.1	6.7	30.2	11.7
OS 2	100.0	93.7	6.3	0.0	11.8
OS 3	68.4	65.9	2.5	31.7	10.9
OS 4	92.9	80.1	12.9	7.1	10.8
OS 5	61.8	58.7	3.1	38.2	11.4
OS 6	69.8	67.4	2.5	30.2	11.3
OS 7	83.8	72.2	11.6	16.2	15.0
OS 8	87.6	80.1	7.5	12.4	11.7
OS 9	83.8	76.0	7.8	16.2	12.4
OS 10	71.9	66.8	5.1	28.1	10.8
OS 11	89.3	87.6	1.8	10.7	13.2
OS 12	79.9	71.8	8.1	20.1	13.7
OS 13	87.1	77.5	3.2	12.9	11.7
OS 14	48.8	48.1	0.7	51.2	11.9
OS 15	90.4	82.6	7.8	9.6	11.2
OS 16	59.8	49.3	10.5	40.2	12.2
OS 17	54.7	54.0	0.7	45.4	12.0
OS 18	100	59.1	41.0	0	13.1
OS 19	65.0	61.0	4.0	35.0	11.0
FS 1	100	93.9	6.1	0.0	12.6
FS 2	71.0	70.8	0.1	29.0	12.5
FS 3	100	91.3	8.8	0	12.8
FS 4	100	89.0	11.0	0	10.2
FS 5	90.0	90.0	0	10.0	12.6

An important limiting aspect of enzymatic catalysis, as it is usually described [29], is the very high reaction times needed, in comparison with conventional chemical methods. In this sense, the results collected in Figure 5 showed that, in only two hours, the selective process was completely performed. Thus, in the first hour, the yields displayed a broad peak (RT = 53) corresponding to DG and/or TG, that remained without undergoing the ethanolysis reaction. In the second reaction hour, this peak practically disappeared. The peaks corresponding to FAEE and MG (RT = 17–25 min) increased a lot in the second hour. These results were very similar in all the cases studied, employing eight different enzymatic extracts. Furthermore, the results obtained after two hours were pretty similar to those achieved after 24 h of reaction. This fact reinforced the possible application and profitability of this method from an industrial point of view.

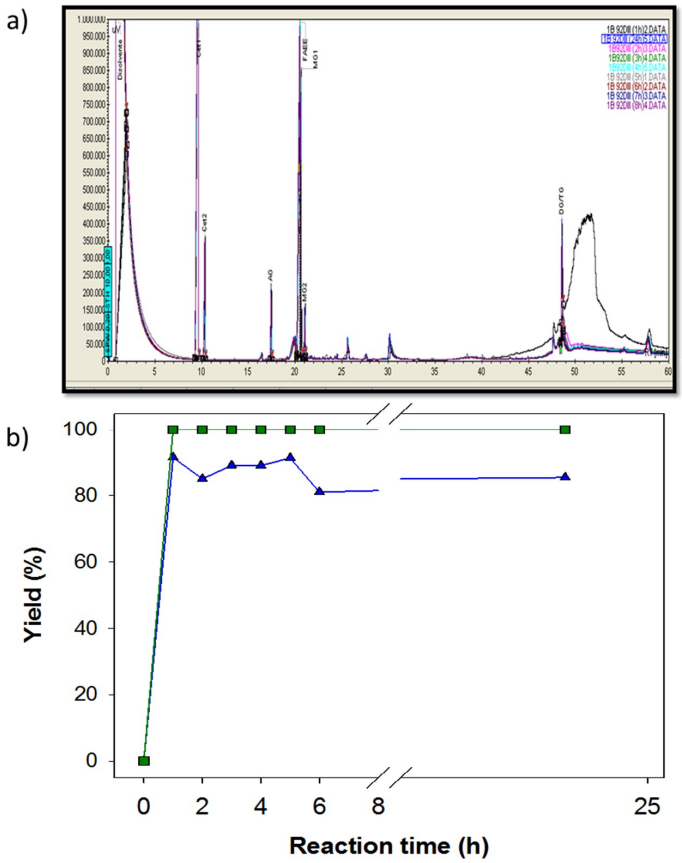

Figure 5. (**a**) Superimposed chromatograms corresponding to the ethanolysis reaction of sunflower oil, under standard conditions over the OS-8 wild microbial strain extract, at different reaction times. (**b**) Conversion (green squares) and selectivity (blue triangles) values obtained from these chromatograms.

In this respect, lipases exhibited several advantages in comparison with homogeneous alkali catalysts, such as high catalytic activity, moderate reaction conditions, ability to process vegetable oils containing a high degree of free fatty acid and water, diminishing the formation of soaps and emulsions, etc. [30]. However, sometimes it was too difficult to obtain complete conversion, which needed 48 h or more, although the yields near to 70% were obtained in relatively low reaction times, i.e., one or two hours. Obviously, this fact was due to the 1,3-selective nature of lipases which produce about a 30% of MG because of the impossibility of getting the alcoholysis of MG (Figure 1). We concluded, therefore, that the enzymatic catalysis is particularly suitable for obtaining Ecodiesel but is not applicable for producing conventional biodiesel that complies with EN 14214.

On the other hand, the presence of MG in the reaction medium had some effect on the flow properties, e.g., viscosity, in comparison with the conventional biodiesel (pure FAME). Therefore,

its use as pure B100 in the ID engines is not completely advisable. However, the use of Ecodiesel as an additive for fossil diesel (e.g., at the typical B20, 20/80 biofuel/diesel ratio) seems to be adequate for diesel engines.

Furthermore, considering the use of lipases as possible enzymatic catalysts, another important factor to take into account must be the robustness of such enzyme, i.e., these enzymes must be reused in several successive processes, either free or immobilized [25–27]. In this sense, eight selected enzymatic extracts were subjected to successive ethanolysis reactions. In Figure 6, the catalytic performance of one of these enzymatic extracts (other results are not shown here) is plotted. As can be seen, the FS-5 enzymatic extract exhibited similar conversion and selectivity values even after five reuses, indicating a very positive aspect for its industrial application.

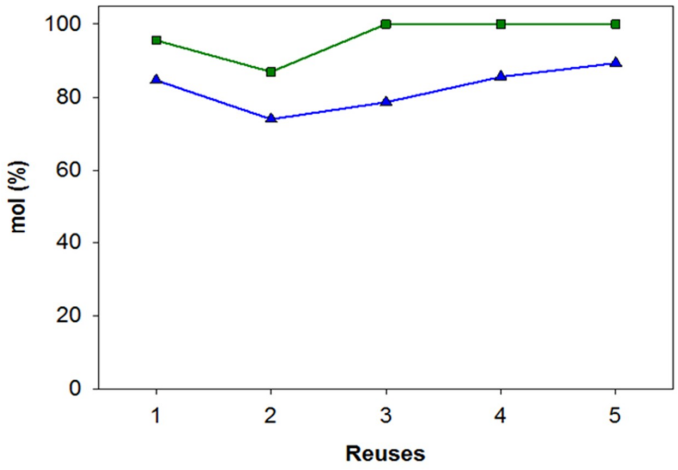

Figure 6. Catalytic performance obtained in successive reactions on FS-5 wild microbial strain extract in the sunflower oil ethanolysis reaction, under the same standard experimental conditions. Conversion (green squares) and selectivity (blue triangles).

According to recently new strains genera identified, some of the most efficient checked lipases from these wild microbial strains from vegetal oil samples were identified through a 16 RNRr phylogenetic analysis. Among these microbial strains, the one belonging to the genus *Terribacillus* (OS-2), seems a promising option for biofuel production. However, in spite of getting high yields in the partial ethanolysis of the sunflower oil, the kinetic viscosity values of the Ecodiesel obtained were not better than those obtained with some samples here studied, e.g., OS-2 and OS-11 (Table 2), with which higher yield values were also obtained. Thus, the results here obtained are promising for making this option economically competitive in comparison to the conventional biodiesel production methods.

4. Conclusions

To improve a new methodology that integrates glycerol as monoacylglycerols, several microbial lipases from different wild microorganism strains, sampled in some lipophilic organisms, were evaluated as useful and inexpensive 1,3-selective biocatalysts in sunflower oil ethanolysis. These strains, which were previously selected and described [27], after isolation and cultivation, were applied to select those microbial strains which showed better enzymatic activity. Thus, an enzymatic material capable of performing Ecodiesel synthesis was obtained by a very simple procedure, consisting in the concentration of enzymatic extracts through dialysis followed by a lyophilization process from the corresponding strains. This Ecodiesel was obtained after very short reaction times (1–2 h) and under soft reaction conditions. Besides, a higher atomic yield was achieved (no glycerol was generated as a by-product), and the purification step of residual glycerol was not needed, indicating that Ecodiesel can be employed directly after its production.

Furthermore, the freeze-dried bacterial cultures enzyme preparations can also be repeatedly re-used without losing efficiency, giving economic viability to the process. Thus, the results here obtained show the viability of a new paradigm, that is, the use of scarcely purified enzymatic extracts containing mixtures of several enzymes, instead of purified enzymes, which is currently the general procedure. In this sense, the presence of other enzymes should not affect the reaction, since these additional enzymes do not find in the reaction medium substrate molecules that allow their activity. However, only highly purified enzymes are commercially available, because these types of enzymes are required in biochemical research, although the high prices make them prohibitive for their application in fine chemical processes.

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Abbreviations

FAME	fatty acids methyl esters, components of conventional biodiesel
DMC	dimethyl carbonate
FAEE	Fatty Acid Ethyl Ester
PPL	porcine pancreatic lipase
FAE	esters of fatty acids
MG	monoglycerides or monoacylglycerols
DG	diacylglycerols
TG	triacylglycerols or triglycerides
GC	gas chromatograph
RT	retention times
p-NPP	para-nitrophenyl palmitate
p-NP	para-nitrophenol
OS	Vegetable Oil Samples/Strains
FS	Animal Fat Samples/Strains
B100	100% Biofuel

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