Synthesis of biodiesel from high FFA alperujo oil catalysed by immobilised lipase

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ABSTRACT

Synthesis of biodiesel was carried out through enzymatic biocatalysis by the transesterification of natural waste olive oil (alperujo oil) using a recombinant 1, 3-positional selective *Rhizopus oryzae* lipase (rROL). The most relevant property of this uncommon substrate is the high content of free fatty acids (FFAs), making it impossible to produce biodiesel through basic catalysis. The synthesis reactions were carried out in a solvent-free media using a covalent immobilization on a glutharaldehyde-treated polymethacrylate amino-epoxide carrier. Methanol was added by stepwise addition to avoid lipase inactivation. FFAs effects on the kinetics, yields and stability of biocatalysts were studied. It was concluded that the high free fatty acids content allows an increasing on the initial reaction rate and it gives more stability to the immobilised enzyme, demonstrating the efficiency of this new substrate for the biodiesel production.

RESEARCH HIGHLIGHTS

KEYWORDS

***Rhizopus oryzae* lipase, free fatty acids effect, biodiesel synthesis, alperujo oil**

1. INTRODUCTION

Production of biodiesel (mono-alkyl esters of long chain fatty acids) is widely implanted and described nowadays, due to the fossil fuel reserves depletion and also to its environmental benefits. Biodiesel is able to be directly used as a fuel in current automobile motors [1], and also it is known to reduce the monoxide carbon and polycyclic aromatic hydrocarbon emissions [2].

The most common way to produce it is through chemical transesterification, this is, using a basic catalyst, mainly. There is a wide range of substrates able to produce biodiesel through this method. Most of them are vegetable oils such as corn, palm [3], cottonseed, sunflower or soybean [4]. However, the disposition of agricultural lands to biodiesel producing companies and also the utilization of these edible oils generates a constant ethic conflict.

Thus, the substrates source for biodiesel production has changed recently in order to keep away this problem. Nowadays, edible oils should be avoided for this application in preference to the non-edible, for instance waste oils from industry or restaurant sector. Another source is the derived lipid from microalgae or oleaginous yeast. Species with high lipid content and relatively small cultivation areas are *Chlorella* and *Dunaliella*, whose biodiesel productivity can be up to 800 times more than the productivity when using oils from crops [5]. Advantages when using oleaginous yeasts, such as *Candida sp.,* are the high amount of accumulated lipids in its biomass (>20% w/w), its short life cycle and it can be obtained independent of climatic factors [6].

The major problem that appears when using these substrates is the high content of FFAs. To carry out the base catalysed transesterification correctly, FFA values lower than 3% are needed [7]. However, it should be noted that the range of FFA values in non-edible oils or fats can be from almost 0% up to 63%, such as tallow [8]. In these cases, the reaction stops because of the soap formation due to the basic catalyst. So, basically, the substrates are previously pre-treated in order to reduce this FFA content and also to remove some impurities and other components [9]. It is known that this process may take some time and also may be particularly expensive. In this way, alternatives have been developed in order to

avoid the problem of saponification, and also to enhance productivity and the environmental benefits. Biodiesel synthesis through enzymatic biocatalysis has been applied by far as the most attractive solution to this problem. Lipases (tryacylglycerol acyl-hidrolase E.C.3.1.1.3) are the enzyme which catalyses biodiesel synthesis is also used for a high other applications [10].

It has been reported some alternative methods in order to carry out direct esterification of FFA with solid acid catalyst, which reduce the FFA levels during the biodiesel synthesis reaction [11].

In the present study, a*lperujo* oil was used as a substrate for biodiesel production. *Alperujo* is waste non-edible oil that comes from the olive extraction processes. It is a by-product easily available (only Spain generates approximately 4-5 tons per year) and a low-cost material [12]. Basically, *alperujo* oil is made of a high content of organic matter, corresponding in a range of 38.04-57.02% of lignin, 32.16-42.60% of hemicellulose, 16.51-25.51% of cellulose, 9.13-19.94% of fats and some soluble carbohydrates and small portions of active phenol compounds representing a range between 2.25-19.25% [13]. These high concentrated components lead to serious ecological problems once it is scattered on the soil, so it is non friendly to the environment [14] . Thus, biodiesel synthesis using this substrate could be an important solution to minimize the previous problems commented.

In this study the evaluation of the synthesis of biodiesel from an a high-FFA content non-edible substrate (*alperujo* oil) through enzymatic pathway using the recombinant 1,3-positional specific *Rhizopus oryzae* lipase (rROL), avoiding the generation of glycerol as a co-product, is proposed. Some recent works have demonstrated the efficiency of positional specific fungi lipases, such as ROL or

Rhizomucor miehei lipase (RML), by keeping glycerol as monoglycerides [15, 16] or enhancing yield due to the acyl-migration [17].

It is also evaluated the robustness of the enzyme comparing a covalent immobilization on commercial polymethacrylate epoxy-amino support (HFA-ReliZyme) with an ethylenediamine-glutharaldehyde-treated HFA-ReliZyme carrier (HFAGlut) immobilization.

2. MATERIALS AND METHODS

2.1. Materials

Heptane and methanol were purchased from Panreac (Barcelona, Spain). Olive waste oil (*alperujo*) was kindly donated from Sierra Mágina olive oil extraction mill (Mancha Real, Jaén, Spain). Oleic acid and standards of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate and methyl linolenate were obtained from Sigma-Aldrich (St Louis, USA). HFA403 ReliZyme carrier was purchased from Resindion (Binasco, Milano, Italy). Characterization and treatment of the substrate were carried out with ethanol and potassium and sodium hydroxide, materials also purchased from Panreac (Barcelona, Spain). Colorimetric kit for enzymatic assay (11821729) was obtained from Roche (Mannheim, Deuschland).

2.2. Lipases

Recombinant *Rhizopus oryzae* lipases were produced by the Bioprocess Engineering and Applied Biocatalysis group from Universitat Autònoma de Barcelona (UAB). Production methods are the same referenced in previous works [18]. Purification of the protein was carried out with an ultrafiltration Centrasette® Pall Filtron set (New York, USA) [19].

2.3. Treatment and immobilization on HFA-Relizyme

Two types of biocatalysts were used: rROL immobilized on non-treated commercial HFA-Relizyme (HFA) and rROL immobilized on the same HFA but previously treated with ethylenediamine and glutharaldehyde (HFAGlut).

The HFAGlut carrier was pre-treated by incubation in 80 mL of 1 M ethylenediamine solution at pH 10 under orbital incubator during 4 h at 60 °C, followed by solution removal by vacuum filtration. After that, the carrier were incubated in 80 mL of 2.5 w/v glutharaldehyde solution at pH 8 on a roller during 2 h at room temperature, also followed by solution removal by vacuum filtration.

In order to immobilize the rROL on both carriers, 100 mL of a 0.1 M phosphate buffer at pH 7.5 containing approximately rROL activity between 4000-6000 UA/mL was prepared, dissolving lyophilized lipase under magnetic stirrer for 1 h at 4 °C. After that, the solution was centrifuged during 20 min at 12000 rpm and then the supernatant was mixed with 1 g of treated carrier on a roller shaker for 42 h at 4 °C. After vacuum filtration, immobilized biocatalyst was dried on silica gel at room temperature until its weight reached a constant value. Finally, immobilized lipase was stored at -20 °C.

The activity and protein amount in the biocatalyst were 298 UA/mg of biocatalyst and 0.018 mg protein/mg biocatalyst for the HFAGlut, and 282 UA/mg of biocatalyst and 0.015 mg protein/mg of biocatalyst for the HFA. These values were calculated from the difference between final and initial activities and protein amounts of the supernatant, divided by the weight of the final biocatalyst.

2.4. Lipase Activity and Protein Concentration

Lipase activity was determined by Roche colorimetric kit assay, using a Cary Varian 300 spectrophotometer (Palo Alto, USA) at 30 °C in 200 mM Tris-HCl buffer

143 at pH 7.25 [20]. Protein concentration was determined by the widely used
144 Bradford method [21].

145 ***2.5. Fatty Acid Methyl Ester and Oleic Acid Analysis***

146 FAMEs and oleic acid sample concentrations were analysed in a 7890A Agilent GC
147 (Santa Clara, USA) with a capillary column 1909BD-113 and an auto-sampler [22].
148 %RSD for FAMEs and oleic acid was about was about 3% and 7%, respectively.

149 ***2.6. Transesterification Reaction***

150 All reactions were carried out in 10-mL closed vials in an incubator (IKA KS 400,
151 Staufen, Deutschland) at 30 °C and under orbital stirring at 200 rpm. All reactions
152 were free-solvent reactions with 32,000 UA of biocatalyst (the total amount were
153 calculated according to each type of biocatalyst and to its inner activity), 8 g of
154 substrate and 0.16 mL of methanol (stoichiometric relation in order to achieve a
155 14% yield). Only one pulse of methanol was added instead the seven pulses that it
156 would need to achieve the maximum yield in order to cause the minor lipase
157 inactivation [22]. Reaction cycles were made by leaving the biocatalyst deposited
158 at the bottom of the closed vial, and then removing the entire reaction medium.
159 The vials containing the biocatalyst were stored at 4 °C until the next reaction.

160 ***2.7. Substrate preparation***

161 Three types of substrates were used. Centrifuged *alperujo* (initial) obtained by
162 centrifugation (5 min at 4500 rpm) of natural *alperujo* in order to dewax it, and
163 two treated substrates proceeding from initial: neutralized *alperujo* and
164 neutralized and then supplemented with oleic acid *alperujo*.
165 Neutralized *alperujo* was obtained by adding the necessary volume of a sodium
166 hydroxide solution to neutralize the whole FFAs amount present in the substrate.
167 This volume (V, in L) was calculated from:

$$V = \frac{m_o \cdot A}{M \cdot C}$$

where m_o is the total substrate amount (in g) to be neutralized; A, the acidity (values from 0-1) of the substrate; M, the oleic acid molar mass (in g·mol^{-1}); C, sodium hydroxide solution concentration (in mol·L^{-1}). Here was assumed the fact that all substrate acidity comes from the oleic acid presence. In order to assure the well neutralization of the substrate, a 10% more of solution volume is calculated. Then, the final volume is added to the substrate under magnetic stirrer for 20 min at room temperature. Next step is to heat up the solution at 60 °C for 20 min. Due to the soap formation during the process it is desirable to clean up the substrate with distilled water at 80 °C under magnetic stirrer, and then separated by decantation.

Supplemented *alperujo* was obtained by adding the necessary amount of oleic acid to match the original substrate acidity. The total amount of oleic acid needed is obtained here:

$$A = \frac{m_{oleic}}{m_{oleic} + m_o(1 - AN)}$$

where A is the substrate acidity (values from 0-1); m_{oleic}, the total amount of oleic acid (in g); m_o, the amount of substrate to be treated (in g); AN, the neutralized substrate acidity (values from 0-1).

2.8. Acidity Determination

In order to determine the total acidity of the substrates, acid-base titration was used. The method was carried out following European protocols: 702/2007 of 1991R2568.

3. RESULTS AND DISCUSSION

3.1. Substrates Acidity And Fatty Acid Content

Table 1 shows the substrates acidity characterization. Supplemented *alperujo* acidity fitted to the initial value, demonstrating the correct application of the oleic acid supplementation method. In order to understand better the reaction results, it was considered that the low percentage of neutralized *alperujo* acidity were negligible.

Table 2 shows the main fatty acid content of *alperujo*. *Alperujo* oil is a direct sub-product from the olive oil crop; therefore, oleic acid is the main fatty acid present in it, representing an 89% of the total fatty acid content, followed by palmitic and linoleic acid, representing a 5.53% and 3.32%, respectively.

3.2. Transesterification Reactions

Transesterification reactions were carried out in a free-solvent media in order to avoid later stages for biodiesel recovery. In this study, four reactions were proposed according to each type of substrate (initial and neutralized) and biocatalyst (HFA and HFAGlut). The time evolution of oleic acid and the reaction yield are shown in Fig. 1A-D. It is important to note that 14% is the maximum yield to be achieved in the reactions, according to the methanol added volume. Initially, substrate contains a low amount of FAMEs that it had to be discounted from the final yield, so about 8-11% of yield was finally achieved. However, time reaction is quite different in some cases. In the case of using initial *alperujo* as a substrate (Fig. 1A-B), the maximum yield was achieved at 3.5 h when HFA was used as a biocatalyst and 2 h for HFAGlut. Nevertheless, the initial rate was a 2.6 fold higher in the second reaction (Table 3), just for the reason that rROL was immobilized in a glutharaldehyde-treated HFA support, instead of the commercial one. It is known that this method allows improving the immobilization rates by

orienting the enzyme in a different way [23] and extending its spacer arm between the support, giving more versatility [24].

However, when neutralized substrate was used (Fig. 1C-D), it took higher times to reach the maximum yield (9%) than when oil with FFAs was used. In the case of the reaction catalyzed by HFA biocatalyst, it took 8 h and the reaction catalyzed by HFAGlut biocatalyst took up to similar time, 9.5 h. Observing figures 1A and 1B, it would seem that this enhancement on the initial reaction rate would came from the immobilization method, but kinetic behaviours when neutralized *alperujo* was used led to think that there would be some kind of different reaction mechanisms. Indeed, it is clear that oleic acid played an important role in reaction kinetics because the absence of this component slowed the reaction up to 10 times. Moreover, as shown in Fig. 1A-B, the total amount of oleic acid remained stable during the whole reaction, suggesting that it did not react with acyl donator. However, it is known that the directly FAMEs synthesis reaction by oleic acid and methanol in free-solvent media is fully described [25]. Thus, it seems reasonable to expect that the minimal water resulting from the esterification reaction would lead in a continued oleic acid obtaining.

3.3. Stability-testing Reaction Cycles

Some studies have tested that lipases have an important decrease of their activity due to the methanol presence in the reaction media and specifically, when it is prolonged over long periods of time [26].

In order to know how the two chosen biocatalysts worked in the presence of methanol, a serial of biodiesel synthesis reaction cycles were carried out. The final yield of each reaction was compared to first one to determine the decreasing of the biocatalyst activity. The results are shown in Fig. 2A-D.

Lipase activity was quite preserved when *alperujo* oil was used as a substrate, regardless of the utilization of both biocatalysts as shown in Figure 2A-B. When HFA was used, a 36.5% of activity was lost during 10 batch cycles (a total of 35 hours of reaction). It is also true in regard to the case when HFAGlut was used: just the 21% of activity was lost during 9 batch cycles (a total of 18 hours of reaction). On the other hand, lipase activity lost more than 70% and 53% when neutralized *alperujo* and HFA, or HFAGlut were used, respectively (Fig. 2C-D). In both cases it occurred after 4 batch cycles. Taking in account that the biocatalyst spent up to 32 and 36 hours (HFA and HFAGlut respectively) in contact with the methanol, these results were only comparable to the results when initial *alperujo* and HFA were used, when the activity loss were 37% respect the first reaction after a similar time (35 hours). Therefore, these results led to think that the absence of FFAs in the substrate have adverse consequences for the biocatalyst stability.

Comparing the loss of lipase activity after 18 hours working with initial *alperujo* (5 and 9 cycles in the case of HFA and HFAGlut, respectively); it was the same in both cases, about the 20%. So, it is clear that the support used, and the immobilization method did not affect the enzyme in these reactions; also regarding no significant differences when neutralized *alperujo* was used.

To conclude, it demonstrates that this major loss in the lipase activity came from the presence of methanol in the reaction media, and the consequent lipase exposure to it during long terms of time. On the other hand, FFAs presence reduces negative effect of methanol in a same exposure time and in addition it allows faster reaction rates.

3.4. Supplemented Oil Reactions

It is known that *alperujo* oil contains a lot of components that make this substrate different from the others. The majority of these components are lignin, hemicellulose, cellulose and fats [13]. In order to assure that the great differences in the initial reaction rate, came from the presence of FFAs according to the substrate used, a new reaction was carried out. Thus, a new substrate was prepared (see 2.7 in Materials and Methods). Basically, a 19% of oleic acid was added to the neutralized oil in order to simulate the initial one acidity. HFAGlut-lipase immobilization was used in this reaction as a biocatalyst, because results in the previous reactions demonstrated that the initial reaction rate and stability was much better than the HFA.

As it is shown in Fig. 3A, the kinetics of the HFAGlut working in FFA-supplemented substrate is almost identical when compared to the HFAGlut kinetics when initial oil is used as a substrate. About a yield of 11% was achieved in both cases in 2 hours of reaction. Furthermore, initial reaction rate was maintained, as it is shown in Table 4. The slightly higher value in the supplemented oil reaction would come from the consideration that neutralized oil was supplemented entirely with oleic acid, whereas the initial oil acidity may come from different components.

In order to determine and compare the stability of the biocatalyst when it is used in supplemented substrate reactions, a serial of reaction cycles were carried out following previous steps (Fig. 3B). The biodiesel production capacity loss was only an 8.2% during 5 batch cycles. Comparing this result with the biocatalyst stability when used in initial oil (Fig. 2B) it was slightly similar, since only a 10% activity was lost during 10 hours in both cases. Thus, it is clear that the presence of FFAs in the substrate is the cause of a favourable increment of the initial reaction rate and also in the enzyme stability.

3.5. Methanol Stepwise Addition Reaction

The fact that rROL gave best results when used initial alperujo oil rather than neutralized one, suggested that indeed the enzyme would use FFAs to carry on the reaction. An important fact, previously mentioned, is that the oleic acid amount in the reaction media remained stable during the short reaction, shown in Fig. 1B. It is reminded that only a yield of 14% could be achieved in the reactions with only one methanol addition. In order to know the FFAs evolution profile achieving a higher yield, a methanol stepwise addition reaction was carried out. Alperujo oil and HFAGlut biocatalyst were used. Methanol was added every 2 hours, in a total of 3 additions.

As it is shown in Fig. 4A, a final yield of 28.62% was achieved after the 3 methanol additions and 6 hours of reaction and the oleic acid present during the whole stepwise addition reaction was quite constant.

Moreover, the lineal yield evolution seems to indicate that kinetics were proportional to the constant FFAs amount, suggesting a continuous oleic acid formation cycle.

In order to determine the enzyme stability in this methanol stepwise addition reaction, a total of 9 reaction cycles were carried out (Fig. 4B). The total activity loss at the end of the 9th cycle (54 h) was about the 43%. Nevertheless, it is important to note that the enzyme remained up to 3 times more in contact with methanol, demonstrating the robustness and stability of the immobilization used.

CONCLUSIONS

Alperujo oil could be perfectly used as a biodiesel substrate, allowing a new sustainable and optimized obtaining process. The FFAs presence on it has

provided a higher initial reaction rate and enhances the enzyme stability. Reactions spent up to 4 times more when neutralized oil was used. Ethylenediamine activation and glutharaldehyde treatment on the carrier have enhanced the initial reaction rate more than when non-treated one was used. Finally, the initial amount of oleic acid present in the substrate has not decreased during the reactions, suggesting that a continuous cycle of oleic acid formation could take place.

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422

FIGURE CAPTIONS

Fig. 1. Time evolution of FAMEs yield (●) and oleic acid total amount (○) in the transesterification reaction using initial *alperujo* oil as a substrate and HFA **(A)** or HFAGlut **(B)** as a biocatalyst, or using neutralized *alperujo* oil as substrate and HFA **(C)** or HFAGlut **(D)** as a biocatalyst.

Fig. 2. Relative yield (considering first reaction achieved yield as the 100 %) using the HFA **(A)** or HFAGlut **(B)** as a biocatalyst and the initial *alperujo* as a substrate, or using the neutralized *alperujo* and the HFA **(C)** or HFAGlut **(D)** as a biocatalyst, after cycle reactions.

Fig. 3. A: Time evolution of FAMEs yield when using initial (○) or supplemented (●) oil, and oleic acid total amount present in media when using initial (◊) or supplemented oil (♦), both reactions using HFAGlut as a biocatalyst. **B:** Relative yield (considering the first reaction yield as 100%) when using supplemented oil as a substrate after cycle reactions.

Fig. 4. A: Time evolution of FAMEs yield (●) and oleic acid total amount present in media (○) when *alperujol* oil and HFAGlut biocatalyst was used during the methanol stepwise addition reaction, with 3 methanol additions every 2 hours (marked with arrows). **B:** Relative yield (considering the first reaction yield as 100%) after cycle reactions.

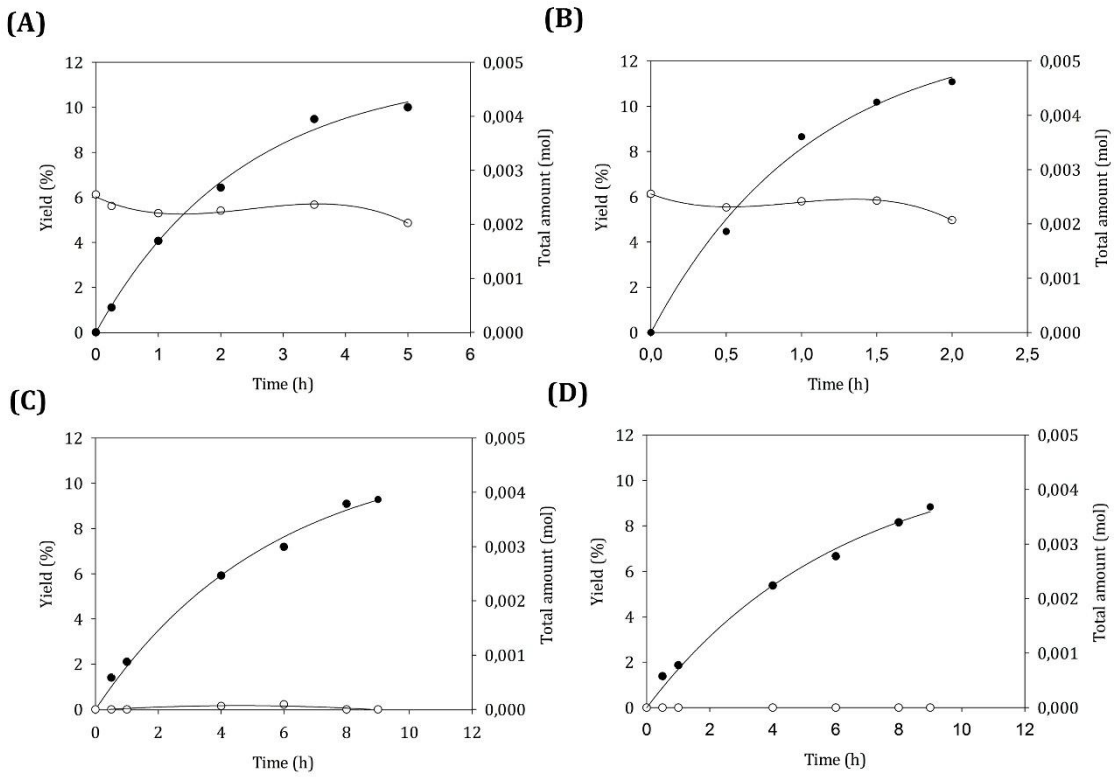

Fig. 1. Time evolution of FAMEs yield (●) and oleic acid total amount (○) in the transesterification reaction using initial *alperujo* oil as a substrate and HFA **(A)** or HFAGlut **(B)** as a biocatalyst, or using neutralized *alperujo* oil as substrate and HFA **(C)** or HFAGlut **(D)** as a biocatalyst.

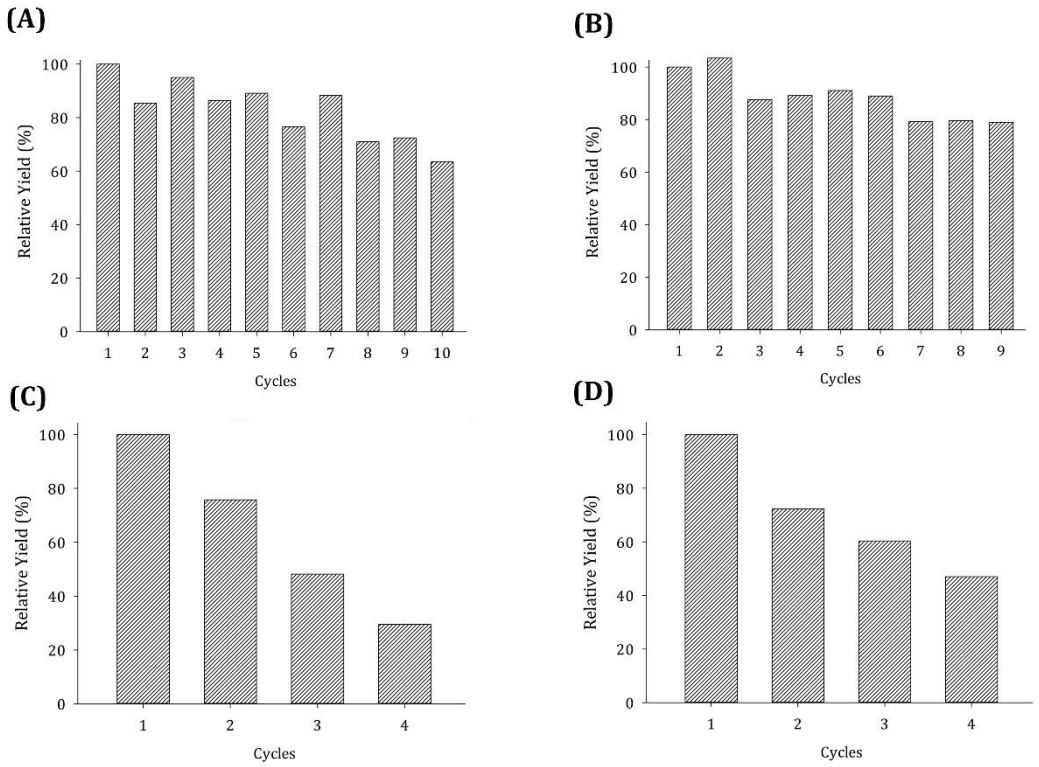

Fig. 2. Relative yield (considering first reaction achieved yield as the 100 %) using the HFA **(A)** or HFAGlut **(B)** as a biocatalyst and the initial *alperujo* as a substrate, or using the neutralized *alperujo* and the HFA **(C)** or HFAGlut **(D)** as a biocatalyst, after cycle reactions.

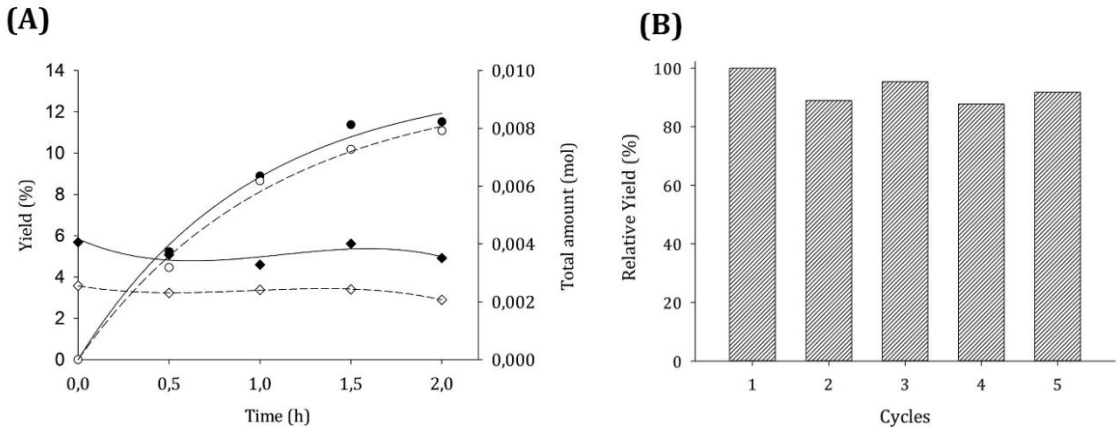

Fig. 3. A: Time evolution of FAMEs yield when using initial (○) or supplemented (●) oil, and oleic acid total amount present in media when using initial (◊) or supplemented oil (♦), both reactions using HFAGlut as a biocatalyst. **B:** Relative yield (considering the first reaction yield as 100%) when using supplemented oil as a substrate after cycle reactions.

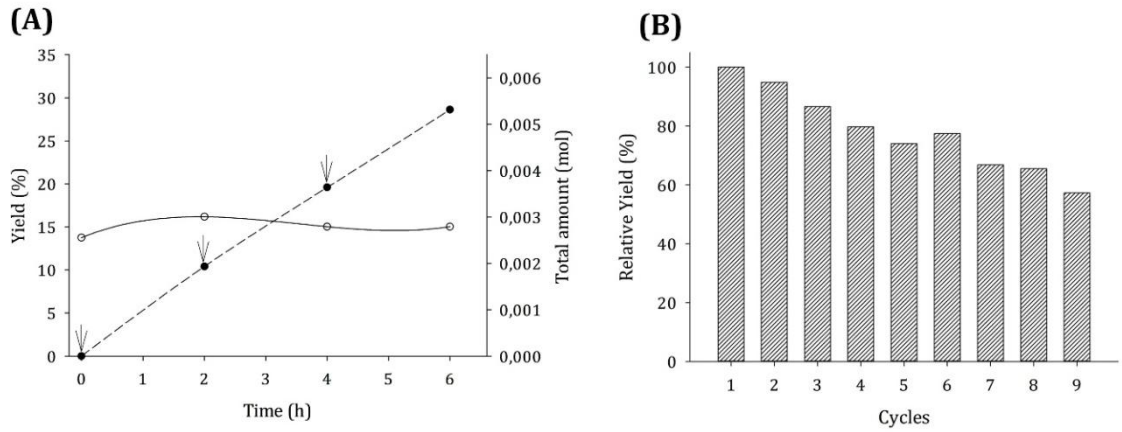

Fig. 4. A: Time evolution of FAMEs yield (●) and oleic acid total amount present in media (○) when *alperujol* oil and HFAGlut biocatalyst was used during the methanol stepwise addition reaction, with 3 methanol additions every 2 hours (marked with arrows). **B:** Relative yield (considering the first reaction yield as 100%) after cycle reactions.

Table 1

Acidity values of substrates

Substrate	**Acidity (%)**
Initial alperujo	18.93 ± 0.93
Neutralized alperujo	1.705 ± 0.03
Supplemented alperujo	17.99 ± 0.33

Table 2

Fatty acid composition (%wt) of *alperujo*

Fatty Acid	**Composition (%)**
Palmitic Acid (16:0)	5,53±0,20
Steraric Acid (18:0)	1,25±0,15
Oleic Acid (18:1)	89,73±0,64
Linoleic Acid (18:2)	3,32±0,24
Linolenic Acid (18:3)	0,17±0,09

Table 3
Initial rate values for each reaction

Substrate	Support	Initial rate (μmol FAME·mL^{-1}·min^{-1})
Initial *alperujo*	HFA	2.493
	HFAGlut	6.482
Neutralized *alperujo*	HFA	0.748
	HFAGlut	0.640

Table 4
Initial reaction rate values when different substrates were used

Support	Substrate	Initial rate (μmol FAME·mL^{-1}·min^{-1})
HFAGlut	Initial *alperujo*	6.482
	Supplemented *alperujo*	7.280