Title: Effect of acyl-acceptor stepwise addition strategy using *alperujo* oil as a substrate in enzymatic biodiesel synthesis.

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ABSTRACT

BACKGROUND: Using renewable feedstock sources for biodiesel production seem to be a promising strategy and even more when enzymatic catalysis with lipases is used. However, it is well known that these enzymes could be inactivated due to reaction conditions such as temperature or alcohol concentration. In this work, the effect of temperature and initial water activity (a_w) value on immobilised recombinant *Rhizopus oryzae* lipase (rROL) were studied. Methanolysis and ethanolysis reactions using *alperujo* oil with three different stepwise addition strategies were employed.

RESULTS: recombinant 1,3-positional selective rROL covalently immobilised on polymethacrylate amino-epoxy activated support showed maximum initial reaction rate at low a_w value (0,093). It was found that 30°C was the optimal temperature in terms of biocatalyst's stability during transesterification reactions. Adding alcohol at once, ethanol was clearly better acyl-acceptor in terms of stability than methanol. Productivity was found to be 2-fold higher when five pulses of ethanol were used instead of methanol.

CONCLUSIONS: *alperujo* oil has a great potential as a low cost feedstock for biodiesel production through enzymatic catalysis using a nearly semi-continuous alcohol addition strategy.

KEYWORDS

Rhizopus oryzae lipase , Biodiesel synthesis, Waste oil source, Water activity, Ethanolysis, Methanolysis

INTRODUCTION

Since the world fossil fuel reserves are nearly running out – the total depletion is forecasted for 2050-2060 [1,2] – production of biodiesel has been widely implanted in order to supply this deficiency, with a worldly production of 27,06 million tonnes in 2013 and an increase of a 68% since 2008 [3]. The use of biodiesel (mono-alkyl esters of long chain fatty acids) has its own benefits, such as its ability to be directly used in automobile engines without important treatments [4], it is considered safer than fuel oil because of its higher flash point and its ignition delay due to higher cetane number [5]. Environmentally, the key-point of using biodiesel is its potential for greenhouse gases emission reduction, concretely it was observed that on combustions of biodiesel-fossil fuel blends, the closed-carbon cycle, levels of both carbon monoxide and dioxide and smoke were reduced [6,7].

Biodiesel is produced worldly using alkali-catalysed transesterification, using feedstock oils such as corn, palm, soybean and rapeseed [4,8]. However, since the beginning of the 90s, several studies have been focused on biodiesel production using second generation oils because of the principal drawback of using edible oils: its high cost, which may represent around 75% of the

overall cost. Another problem associated with edible oils is its competition for food resources, available harvest land and deforestation [1,9].

In that way, second generation biodiesel, produced from such as non-edible oils, animal fats, waste cooking oil, etc., has been seen as an alternative to reduce environmental problems and utilisation of edible oils.

However, the main drawback of using the second generation substrates are its high concentration of free fatty acids (FFAs), making the standard alkali-catalysed transesterification impossible due to the saponification. In that way, FFA values lower than 1-3% are needed in order to carry it out correctly [4,10]. To overcome this problems, the first solution is the pre-treatment of the substrates to reduce FFA content and also to remove some impurities and other components [11], increasing the process steps that may lead to an increase of the process cost. Acid and heterogeneous solid base catalyst transesterification have been seen also as a solution, but these strategies exhibit slower reaction rates and they require high alcohol to oil molar ratios than the alkali [1,12–14].

One of the most attractive options due to its benefits compared with chemical transesterification is by far the enzymatic catalysis using lipases (triacylglycerol acyl-hydrolase E.C.3.1.1.3). This process requires less energy consumption, is more environmental-friendly because it does not generate as much as waste than the chemical one, and the immobilization of catalyst turns its recovery much easier [1,15]. Other advantage of using lipases is its perfect compatibility with FFAs. It has been widely reported not only the possibility of synthesise biodiesel by the direct esterification of FFAs [16–18], but also the reaction benefits when using substrates with high FFA content [19,20]. In this study it is assumed that the total amount of biodiesel produced (long chain alcohol esters) come from both reactions: transesterification of triacylgrlycerols and esterification of FFAs, as it is raised by other works [19,21,22]. The major problems present in enzymatic-catalysed transesterification, aside the high cost of the enzyme, is the inhibition of the lipase by the acyl acceptor, mainly methanol or ethanol, which

may cause the reduction of the enzyme lifetime. Methanol has been reported to be the principal cause of enzymatic deactivation during the transesterification reaction [15,23,24]. Although this major drawback, methanol is still the most used alcohol due to its availability and economic feasibility. Nevertheless, the problem of inactivation can be partially overcome by stepwise addition of the alcohol [4,20,25,26].

By the other side, ethanol – not as harmful for the lipase as methanol – has been also used widely for biodiesel production [27–30], because of some advantages such as major solubility in triacylglycerols (TAGs) [31] and its low toxicity in front methanol.

Other key parameter in enzymatic reactions is the water activity (a_w). Some studies have stated that this parameter is important in order to achieve higher yields because it is directly linked to the reaction's thermodynamics [32,33] and the hydrolytic activity of some lipases [34,35].

In this work, lipase dependence on initial water activity and temperature has been studied, as well as the utilisation of methanol and ethanol as an acyl acceptor via different stepwise-addition strategies. Covalently-immobilised recombinant 1,3-positional selective *Rhizopus oryzae* lipase (rROL) in a free solvent media was used in order to avoid the presence of glycerol, since acyl migration occurs in a long time reaction and favoured for the presence of solvent. Several studies have stated that glycerol may cause inactivation of lipases by adsorbing on the carrier forming a hydrophilic environment [15,36].

A part form biodiesel, 2-monoacylglycerol, which a product with an added-value mainly used as a emulsifier, lubricant and food surfactant [37,38], is produced. While non-specific lipases are the most used for biodiesel production, the use of positional-specific such as *Rhizomucor miehei* lipase (RML) have shown a great results and efficiency [39,40].

In the following study, raw *alperujo* oil was used as a substrate for biodiesel production. *Alperujo* is a high-FFA non-edible oil coming from the olive extraction processes. It is a low-cost material and is a by-product easily available [41]. As a waste oil, the content of FFA represents an important part of it, with a value of 24%wt; but also a high content of organic matter [20].

MATERIAL AND METHODS

Materials

Olive waste oil (*alperujo*) was kindly given from Sierra Mágina olive oil extraction mill (Mancha Real, Jaén, Spain). Heptane, ethanol and methanol were purchased from Panreac (Barcelona, Spain). Oleic acid and standards of methyl/ethyl palmitate, methyl/ethyl stearate, methyl/ethyl oleate, methyl/ethyl linoleate and methyl linolenate were obtained from Sigma–Aldrich (St Louis, USA). HFA403 ReliZyme carrier was purchased from Resindion (Binasco, Milano, Italy). Colorimetric kit for enzymatic assay (11821729) was obtained from Roche (Mannheim, Deuschland). Salts (LiBr, KOH, NaI, NaBr, NaCl and K_2SO_4) were purchased from Sigma Aldrich.

Lipase production and immobilisation on HFA-Relizyme

Recombinant *Rhizopus oryzae* lipase was produced by the Bioprocess Engineering and Applied Biocatalysis group from Universitat Autònoma de Barcelona (UAB). Production methods are the same referenced in previous works [42,43]. Purification of the protein was carried out with an ultrafiltration and diafiltration in Tris-HCl buffer 10mM ph=7 with a Centrasette Pall Filtron set (New York, USA) [44] and then lyophilised. Support pre-treatment was carried out as referenced in a previous work [20].

Lipase activity and protein determination

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 at pH=7.25 [45]. Protein concentration was determined by the widely used Bradford method [46].

FAMEs, FAEEs and oleic acid quantification

FAMEs, FAEEs and oleic acid sample concentrations were analysed in a 7890A Agilent GC (Santa Clara, USA) with a capillary column 19095N-123 and auto-sampler [25]. Values of %RSD for FAMEs and oleic acid was 3% and 7%, respectively.

Fatty acid methyl esters and oleic acid determination was carried out as referenced in previous works [19,20]. Fatty ethyl esters determination was carried out using the same method.

Transesterification reaction

All reactions were carried out in duplicate in 10-mL closed vials, using an incubator (IKA KS 400, Staufen, Deutschland) under orbital stirring at 350 rpm, at different temperatures depending on the experiment (30°C, 40°C, 50°C). Free-solvent reactions with 8 g of *alperujo* oil and the total amount of biocatalyst corresponding with a 32,000 UA were employed. The total stoichiometric amounts (2:1 alcohol to oil ratio) of methanol and ethanol were added in three different ways: one single pulse at the beginning of the reaction, five pulses with the same volume and ten pulses with decreasing volumes along the time. Stability-testing reactions were carried out by leaving the biocatalyst deposited at the vial's bottom and removing the medium at the end of the reaction. Then, vials containing the biocatalyst were stored at 4°C until the next reaction.

Water activity pre-equilibration

Saturated salts were employed in order to achieve desired initial water activities [47]. The salts used were: LiBr (a_w=0.066), KOH (a_w=0.093), NaI (a_w=0.397), NaBr (a_w=0.560), NaCl (a_w=0.755), K_2SO_4 (a_w=0.976).

All reaction components were pre-equilibrated overnight - minimum 16h- with each salt-hydrates in a jar with tight fitting lid [33].

RESULTS AND DISCUSSION

Effect of water activity and temperature

Some studies have stated that one of the most important reaction parameter, especially related with the kinetics, is the water activity [47]. Eventhough the reaction media contain mainly organic solvent and/or substrates, some water is needed to keep the enzyme active. Lipolytic activity of lipases is afected by a wide range of water activity values, depending on the specie and genus [33,48]. Evenmore, the optimal water activity value differs significally depending on the enzyme surround and the reaction system [15,48]. In this work, a recombinant *Rhizopus oryzae* lipase was used in free-solvent medium and it is worth noting that activity water effect in these kind of reaction media is yet understudied.

Thereby, a set of methanolysis reactions were carried out with six different initial water activity values trying to cover the entire range (from 0.033 to 0.976). Initial reaction rate (in $\mu mol \cdot mL^{-1} \cdot min^{-1}$) was calculated for each reaction adding one pulse of methanol representing a 14% of total stoichiometric volume, in order to avoid inactivation effect on biocatalyst. As it is seen in Figure 1, higher rates were reached when lower water activity values were used.

Some studies have reported the same case for the same lipase [31]. The maximum value was achieved when KOH salt was used in the pre-equilibrium at a_w = 0.093 when nearly no initial water was added to the system while at higher water activity values – using K_2SO_4, 0.976 - hydrolysis took place leading to longer reactions.

In the following experiments, pre-equilibrium of all reaction components at a_w=0.093 were carried out because of this transesterification rate boost.

As it is known, temperature is also a key parameter in enzyme-catalysed reactions. Higher temperatures induce higher reaction rates and may reduce mass transfer limitations. On the other hand, higher temperature values can inactivate enzymes. Even though recent studies had well characterised the optimal temperature for free lipase activity [44], three mild temperatures were tested in order to observe which promotes better transesterification rate of immobilised lipase. Stability studies were carried out at 30°C, 40°C and 50°C adding five pulses of methanol in order to avoid alcohol inhibition. As it can be seen in Figure 2, higher biocatalyst's stability was obtained when reactions were carried out at 30°C, with an activity loss of 62% in the fourth cycle (total time in contact with methanol of 20h). Though higher yields and faster reactions were achieved at 40°C and 50°C (data not shown), the activity loss of the biocatalyst - 90% in 17h and 95% in 10h, respectively - was detrimental. In that way, it is preferable to maintain the enzyme activity to reuse it in further reactions.

Effect of stepwise addition, comparing methanol and ethanol

Methanol and ethanol have been the most commonly used acyl acceptors in biodiesel synthesis since these compounds are easily available and not as expensive as could be alcohols with longer carbon chains such as iso-propyl alcohol [49] or butanol [50,51]. However, it has been reported that methanol is one of the most harmful alcohols and may cause lipase deactivation [4,24], so in order to avoid this enzymatic damage that impact on the activity of the subsequent reuses, some strategies have been proposed. Adding water to the system reduces high concentrations of methanol, but it may promote the undesired hydrolysis reaction[25,52]. Here is presented a comparison of one the most frequently used methods, the stepwise addition of the acyl acceptors.

As it is shown in Figure 3, adding large amounts of methanol at once were detrimental for the lipase's activity and only a yield of 2.84% was achieved. Another data confirming this low initial rate is the oleic acid behaviour, which is maintained constant along the reaction. Moreover, adding the same stoichiometric amount of ethanol resulted in a reaction with a 49.61% yield in 360 minutes (taking into account that the maximum yield is 66.67% due to the *sn*-1,3-positonal specificity of the lipase) with a significant decreasing of the oleic acid (demonstrating both widely known reactions: transesterification and esterification). These results confirm how harmful is methanol in free-solvent and free-water systems, resulting in a reaction environment with high methanol concentration capable to inactivate lipase.

In order to evaluate the lipase stability at these conditions, a cycle-reactions were carried out, reusing the final biocatalyst with fresh substrate. Five methanolysis and ethanolysis reactions – a total of 30 hours – were performed. As it can be seen in Figure 4, a 52.4% of the initial activity was retained in the case of ethanol (values for methanol were not shown due to the low yield obtained).

Figure 5 shows both methanolysis and ethanolysis reaction with the same acyl acceptor's stoichiometric amount added by 5 pulses of equal volumes. In the case of methanol, due to its lower initial rate, pulses were added every 60 minutes. The final yield achieved was 48.06% in 300 minutes, a 17-fold improvement of the previous result just doing it stepwisely. In the case of ethanol, pulses were added every 40 minutes. Here, an improvement of 17% was achieved in terms of yield, obtaining a 58.16% in just 200 minutes.

These results match with some previous studies reporting that stepwise addition of ethanol may increase both the final yield and immobilised lipase's performance in free-solvent [53,54] or in solvent system compared with the same strategy using methanol [55].

In terms of stability (Figure 6), during 5 cycles of methanolysis reaction, ROL lipase lost nearly the whole capacity of synthesising biodiesel, reducing the initial activity up to 97.3%. It is clear that performing a fifth cycle was detrimental for the lipase's activity, since a 40.26% of initial activity remained after the fourth cycle (20 hours). In the case of ethanol, along the same 20 hours (6 cycles), more than 90% of lipase activity was retained. Thus, adding the acyl acceptor stepwisely, not only induces the obtaining of higher yield but also reduces damage on the lipase, retaining more activity at the end of the cycles. Even so, harmful effects of methanol are present.

Next experiments were carried out adding ten pulses of acyl acceptor with decreasing volumes and increasing the addition frequency along the time. This strategy was chosen in order to emulate the yield evolution in the 5-pulse reactions, trying to add alcohol as the reaction needed it. Thus, methanol or ethanol accumulation in the system was reduced and yield and stability should be enhanced.

For the case of methanolysis reaction, shown in Figure 7, a final yield of 57.16% was achieved (an increasing of up to 19%) in 360 minutes. In the case of ethanolysis, a 60.25% of yield was achieved (which represents 91.28% of the theorical maximum yield) in 260 minutes.

In contrast to 5-pulse reactions, where the decrease of total amount of free oleic acid was similar for both alcohols, when ten pulses were added - in the case of methanolysis - the disappearing of free oleic acid total amount was faster than in ethanolysis. It seems than methanol may be a better substrate for FFA esterification than ethanol, but inactivation caused by its high concentration in the 5-pulse reactions, may produce damage on the enzyme leading it to reduce the esterification rate.

When a mass balance of total FAMEs and FFAs was made in the cases of 5-pulse and 10-pulse addition, it could be seen that the sum of both the free FFAs still present in the reaction medium and produced FAMEs resulted in a value close to the maximum theoretical FAME yield, stating that nearly all the triglycerides present in the substrate were converted to biodiesel while the rest where in form of FFAs.

In terms of stability, shown in Figure 8, the differences seen in previous experiments get narrower. After 30 hours of reaction (5 cycles), the activity of the ROL lipase in presence of methanol was decreased only in a 12.31%. It was a notable improvement compared with the 5-pulse methanolysis, which lost a 60% of the initial activity just in 20 hours. On the other side, an 88.11% of activity was retained in 7 cycles when ethanol was used, which corresponds to a 30.3 hours of reaction.

Table 1 shows the obtained productivity for each reaction. Methanolysis reactions' productivity were 1.83-fold lower than ethanolysis when 5-pulse reactions were employed, and 1.45-fold lower than ethanolysis when 10-pulse were carried out. Comparing both methanolysis reactions, the final productivity did not increase although methanol was added in lower volumes in order to avoid the lipase inactivation. By the other hand, a decreasing of a 22.6% of the final productivity were obtained when ethanol was added using the 10-pulse stepwise addition, due to the fact that times between pulses in this case were overestimated, reducing productivity.

A fact that can be drawn from this is that, as the total amount of acyl acceptors is divided the differences of the harmful effect between them are minor, due to the capability of the lipase to handle the volume added. This automatically ensures in applying a semi-continuous or fed-batch system in order to add the chosen acyl acceptor. For the case of ethanol, this statement is not as clear as in the case of methanol, due to the higher times employed in 10-pulse reactions which reduce productivity achieved since no substantial yield enhancement is observed.

CONCLUSIONS

Recombiant *Rhizopus oryzae* lipase can be used as a biocatalyst in the biodiesel synthesis reaction using *alperujo* oil and methanol carried out at 30°C, giving better results in terms of enzymatic stability than higher temperatures. Previous pre-equilibration steps of enzyme were performed in order to obtain a fixed initial water activity, determining that a_w of 0.093 is the optimal to set the faster initial rate.

Methanol and ethanol as acyl acceptors were compared. Ethanolysis initial reaction rate was higher than when methanol was used as acyl-acceptor. Adding all alcohol at once, ethanol gave better results in terms of final yield and enzymatic stability, while as long as the stepwise additions were incremented, the difference between the two acyl acceptors became closer. When ten pulses were added, the ethanolysis reaction gave faster initial rate than methanolysis one, but in contrast, the lipase activity remained nearly the same in both reactions.

The time of stepwise addition should be optimized for each acyl-acceptor in a semi-continuous or fed-batch alcohol addition strategy in order to get the minimum inactivation of the biocatalyst.

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FIGURE CAPTIONS

Figure 1. Initial reaction rate profile of R. oryzae as a function of water activity at 30°C using alperujo as a substrate.

Figure 2. Relative yield (considering first reaction yield as 100%) of 5-pulse methanolysis reactions at three different temperatures (A, 30°C; B, 40°C; C, 50°C) on the biocatalyst's activity.

Figure 3. Time evolution of FAMEs, FAEEs yield and oleic acid of 1-pulse transesterification reaction using methanol and ethanol (reaction conditions: 8g of alperujo oil, 1:2 alcohol to oil molar, 30°C and 350 rpm).

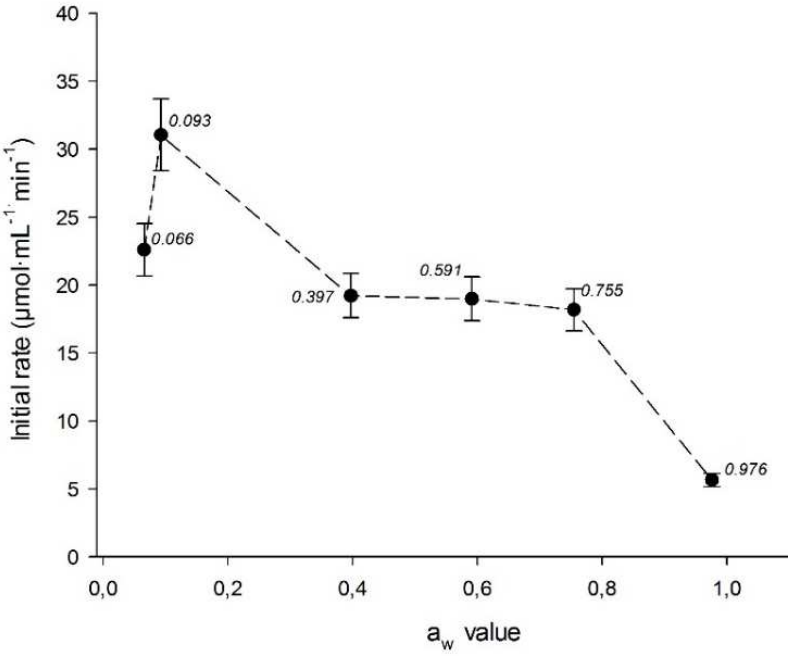
Figure 4. Relative yield (considering first reaction yield as 100%) of 1-pulse ethanolysis reactions. Methanolysis reaction is not shown due to the low yield achieved.

Figure 5. Time evolution of FAMEs, FAEEs yield and oleic acid of 5-pulse transesterification reaction using methanol and ethanol. First five points correspond to five pulses (reaction conditions: 8g of alperujo oil, 1:2 alcohol to oil molar, 30°C and 350 rpm).

Figure 6. Relative yield (considering first reaction yield as 100%) of 5-pulse methanolysis and ethanolysis reactions.

Figure 7. Time evolution of FAMEs, FAEEs yield and oleic acid of 10-pulse transesterification reaction using methanol and ethanol. The first 10 points correspond to the 10 pulses (reaction conditions: 8g of alperujo oil, 1:2 alcohol to oil molar, 30°C and 350 rpm).

Figure 8. Relative yield (considering first reaction yield as 100%) of 10-pulse methanolysis and ethanolysis reactions.



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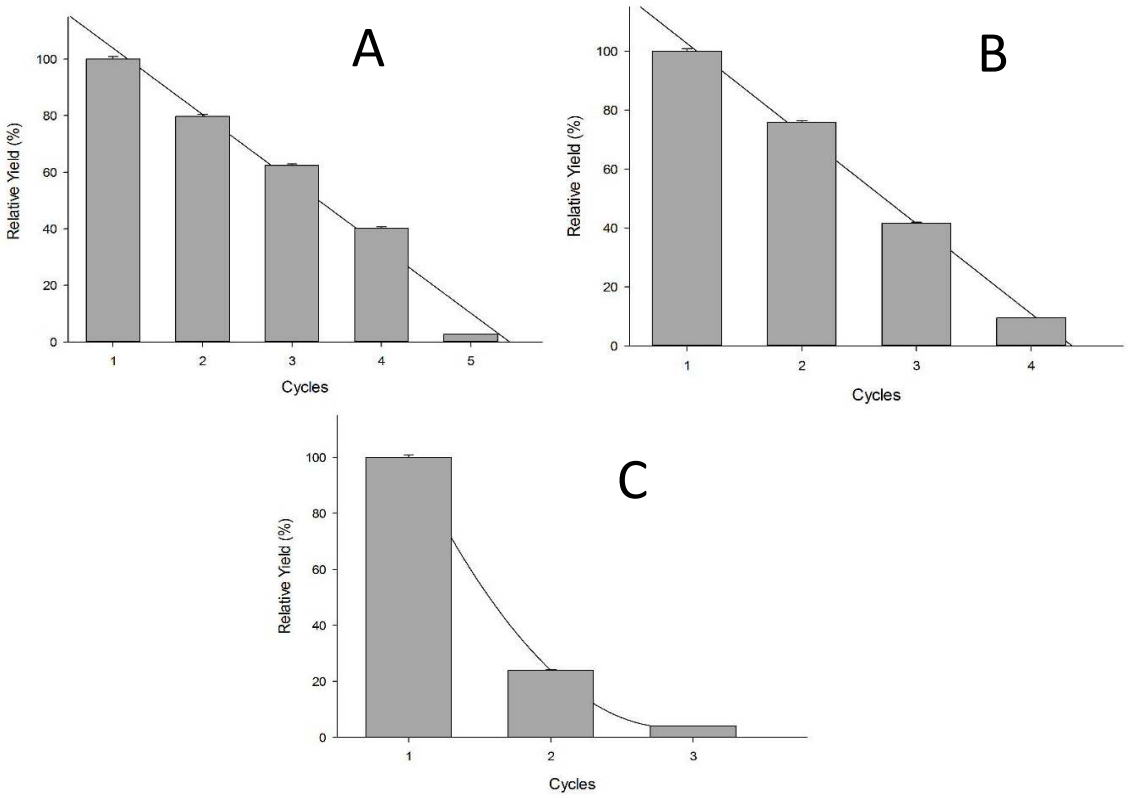

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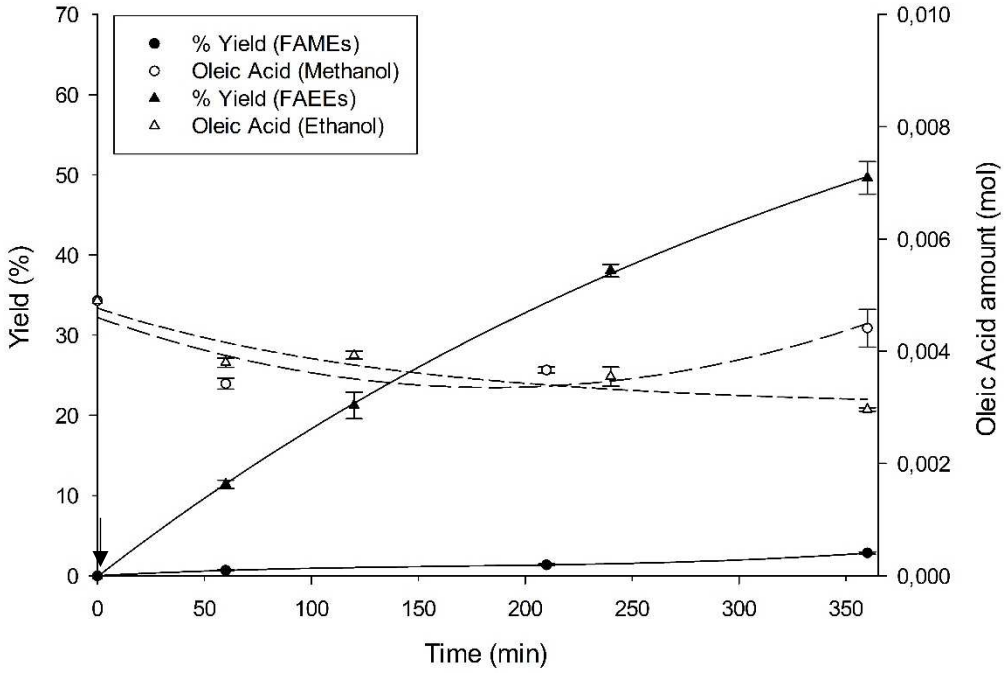

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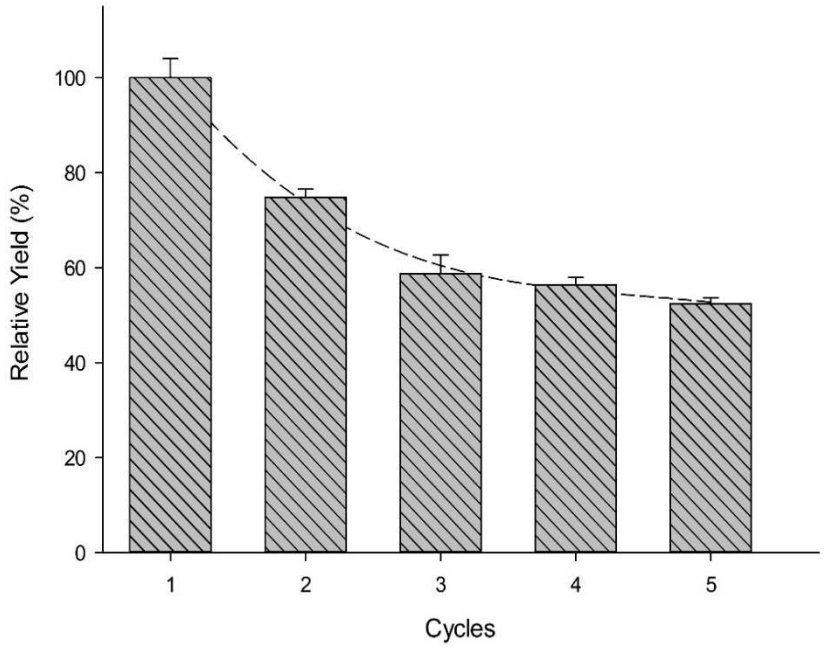

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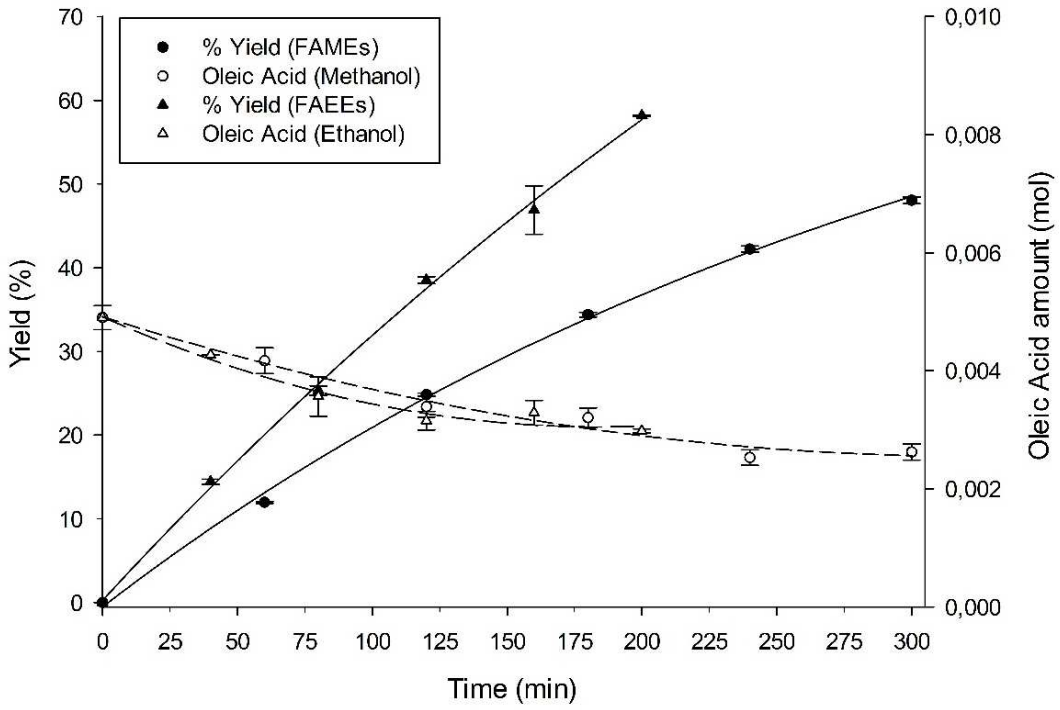
4



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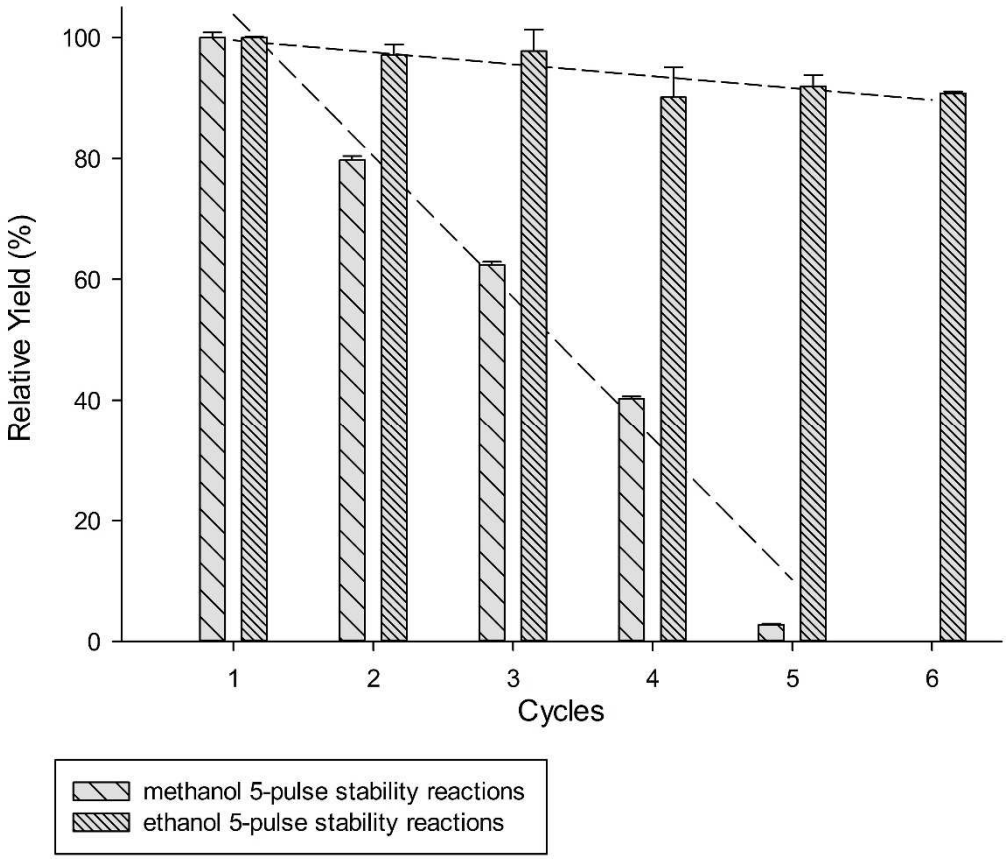

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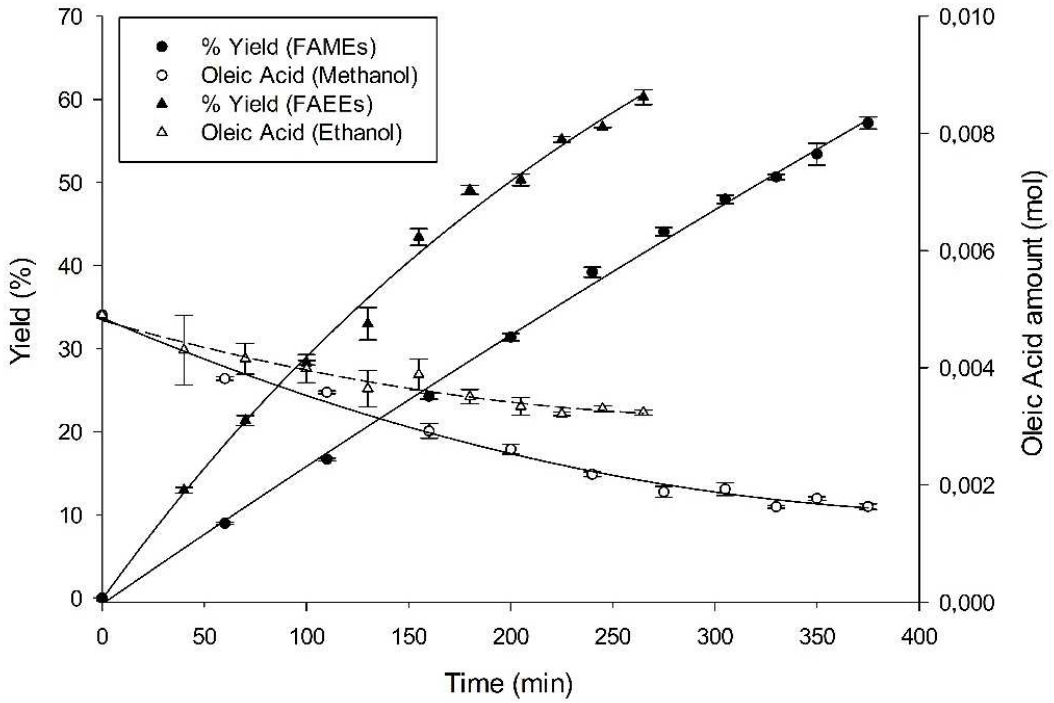

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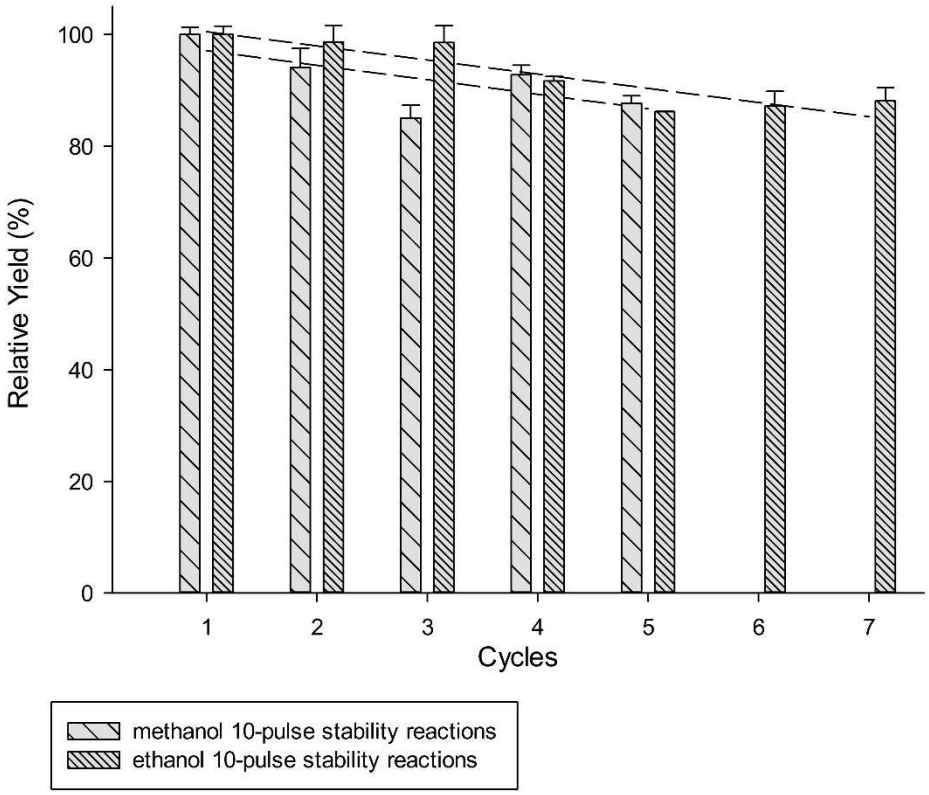

Figure 8. Relative yield (considering first reaction yield as 100%) of 10-pulse methanolysis and ethanolysis reactions.

Table 1. Productivity values of biodiesel synthesis reactions by stepwise addition along the stability tests

Reaction	**Productivity (µmol biodiesel/min)**
5-pulse methanolysis	3.91
5-pulse ethanolysis	7.17
10-pulse methanolysis	3.82
10-pulse ethanolysis	5.55