

IMPROVEMENT STRATEGIES FOR THE ENZYMATIC PRODUCTION OF BIODIESEL IN THE PRESENCE OF PRIMARY ALCOHOLS

ESTRATEGIAS DE MEJORA PARA LA PRODUCCIÓN ENZIMÁTICA DE BIODIESEL EN PRESENCIA DE ALCOHOLES PRIMARIOS

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Abstract

In order to improve the enzymatic production of biodiesel, the reuse of an immobilized preparation of *Candida antarctica* lipase B (CALB) was assayed under different conditions. The improvement included: (1) reuse of CALB in the presence of ethanol (EtOH), (2) reuse of CALB in the presence of methanol (MeOH), (3) blended alcohols at different molar ratios (0:100, 20:80, 40:60, 50:50, 60:40, 80:20 and 100:0, %EtOH:%MeOH), and employing second generation raw materials: waste cooking oil (WCO) and castor oil (CO). When the first strategy was carried out the FAEE (fatty acid ethyl ester) content achieved was 96% in a first cycle but after four cycles the percentage decreased to 32%. For the second strategy the FAME (fatty acid methyl ester) content attained was 63% in a first cycle but after four cycles the percentage was only 13.5%. When the blended alcohols were tested, the negative effect of MeOH was evident for higher concentrations regardless the type of oil used.

Keywords: CALB, ethanol, methanol, reuse, biodiesel.

Resumen

Con el propósito de mejorar la producción enzimática de biodiesel, se evaluó el reuso de un inmovilizado de *Candida antarctica* lipasa B (CALB) bajo diferentes condiciones de reacción. Las mejoras incluyen: (1) reusar CALB en presencia de etanol (EtOH), (2) reusar de CALB en presencia de metanol (MeOH), (3) mezclas de alcoholes a diferentes relaciones molares (0:100, 20:80, 40:60, 50:50, 60:40, 80:20 y 100:0, %EtOH:%MeOH) y (4) empleo de materias primas de segunda generación: aceite de fritura (WCO) y aceite de higuera (CO). Cuando la primera estrategia se llevo a cabo el contenido de FAEE (ésteres etílicos de ácidos grasos) alcanzado fue de 96% en el primer ciclo y después de cuatro ciclos disminuyó a 32%. Para la segunda estrategia el contenido de FAME (ésteres metílicos de ácidos grasos) alcanzado fue de 63% en el primer ciclo y después de cuatro ciclos disminuyó a 13.5%. Cuando se emplearon mezclas de alcoholes, el efecto negativo del MeOH fue evidente a medida que su concentración aumentaba independientemente del tipo de aceite empleado.

Palabras clave: CALB, etanol, metanol, reutilización, biodiesel.

1 Introduction

Our economy and life style are still based on the use of non-renewable resources to obtain energy and a wide range of chemicals. This high dependency results in several problems, namely, unstable international market, decline in fossil global reserves, fluctuating prices and environmental impact. Therefore, sustainable energy alternatives such biodiesel must be attained. Biodiesel is defined as mono alkyl esters of long chain fatty acids derived from renewable lipids

such as vegetable oils, which are used in compression ignition engines (diesel engines) or boilers (ASTM).

The fundamental reaction in biodiesel production is transesterification, which can be either catalysed either chemically (acid or alkali) or enzymatically. Catalysts can be homogeneous or heterogeneous. Normally, alkali catalysts include hydroxides and alkoxides of alkaline metals, such as KOH, NaOH, CH₃ONa, CH₃OK (Li *et al.*, 2013; Evangelista-Flores

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et al., 2014). These catalysts have been explored not only for methyl ester production (FAME), but also for ethyl ester (FAEE) preparation with very high yields under mild conditions. The reaction generally takes about one hour for completion (Meher *et al.*, 2006). This reaction system is also the preferred industrial method for the commercial production of biodiesel from vegetable oils/fats.

Taking into consideration the biodiesel yield and quality, the alkali process does not offer the best economic and environmental scenario for biodiesel production from low-cost feedstock such as waste oils and animal fats that have high contents of free fatty acids (FFA) and water; hence, for better results, acid-catalysed transesterification reaction has become a viable option. Acid catalysis converts both FFA and triacylglycerols (TAG) to biodiesel; however, it is corrosive and the transesterification rates are significantly lower (several thousand times) than those of the alkaline catalysis, with a requirement for higher reaction temperatures and a greater number of process equipment for separation and purification of biodiesel from glycerol.

The drawbacks associated with the conventional chemical catalysis can be overcome with the introduction of an alternative, heterogeneous catalysts for biodiesel production. The chemistry of heterogeneous catalysis has been reported, and includes metal hydroxides (Dalai *et al.*, 2006), metal complexes (Abreu *et al.*, 2003), metal oxides such as CaO (Granados *et al.*, 2007), MgO (Wang & Yang, 2007), ZrO (Jitputti *et al.*, 2006), zeolites, hydrotalcites and supported catalysts (Xie & Huang, 2006). These types of catalysts have been investigated as solid catalysts, which overcome some of the drawbacks associated with the use of homogeneous catalysts. The order of activity among alkaline earth oxide catalysts was found to be BaO > SrO > CaO > MgO (Cantrell *et al.*, 2005).

With respect to heterogeneous catalysts, enzymes appear as a more versatile and as an environmentally friendly bio-based route for producing biodiesel. Enzymatic catalysis offers a number of environmental and economic advantages over the chemical method: (1) room-temperature reaction conditions, (2) reduced costs associated with the recovery of chemical catalysts, (3) enzyme re-use, (4) high substrate specificity, (5) single-step conversion of both FFA and TAG to biodiesel, (6) lower alcohol to oil ratio, (7) avoidance of side reactions and minimized impurities, (8) easier product separation and recovery; (9) biodegradability and environmental acceptance.

The ability of lipases to catalyse FAME synthesis from low-cost feedstock with high FFA (waste oil, grease, tallow, etc.) would lower the cost of enzymatic biodiesel (Caballero *et al.*, 2009; Dizge *et al.*, 2009; Zhao *et al.*, 2014). However, another challenge to undertake for the industrial implementation is enzyme inhibition by alcohols (Maceiras *et al.*, 2009).

With respect to the feedstock, in this study we employed waste cooking oil (WCO) and castor oil (CO), both oils classified as second generation raw materials. There are no protocols to control when WCO is illegally dumped in rivers and landfills in Mexico, causing environmental pollution. Thus, a proposal for biodiesel production from WCO may provide significant advantages. In this study, WCO was collected from the campus cafeteria.

Castor (*Ricinus communis L.*) is an oilseed plant with high adaptation; yield potential and high oil content (20-60 wt.%). This oil is highly soluble in ethanol (EtOH), dense and viscous. Mejía *et al.* (2013) cite multiple uses for this oil in several industries such as automotive, pharmaceutical, cosmetics, chemicals, fertilizers, pesticides, aircraft and currently the biofuels industry. In Mexico, the harvested area in 2011 was calculated in 500 ha's, with a production of 100 tons/ha. The producers are located in the states of Chiapas, Chihuahua, Coahuila, Colima, Mexico City, Guanajuato, Guerrero, Jalisco, State of Mexico, Michoacán, Morelos, Nayarit, Oaxaca, Sinaloa, Sonora, Tabasco, Tamaulipas, Tlaxcala, Veracruz and Yucatan (Benavides *et al.*, 2007). This oil seed has a potential for biodiesel production and according to our literature review we did not find any study conducted in Mexico related to the enzymatic production of biodiesel from this oil.

The present work deals with the enzymatic production of biodiesel. Compared to other studies previously reported, we prepared our own immobilized biocatalyst; the enzyme was reused in several cycles in the presence of primary alcohols and second generation raw materials were employed, in a solvent-free system. Such strategies are an attempt to demonstrate the ability of the enzymes for obtaining biodiesel.

2 Experimental

2.1 Materials

WCO was collected from the cafeteria located in the campus, while CO was extracted from the seeds of a

culture located in Zumpaguacan, State of Mexico. The biocatalyst, in a free form, was immobilized according to a previous reported protocol (García *et al.*, 2008). The fatty acid standard Supelco 37 Component FAME Mix was purchased from Supelco (Bellefonte, PA). All the other solvents employed were HPLC-grade from SIGMA-ALDRICH (St. Louis, MO).

2.2 Pre-treatment of waste cooking oil (WCO)

WCO comes from commercial vegetable oil, which was reused several times. Thus, to guarantee the homogeneity of WCO and the removal of any suspended matter, the oil was submitted to filtration through filter paper. Subsequently, the oil was dried overnight at 60°C to remove water. After pre-treatment, WCO samples were prepared via selective derivatization in order to determine the fatty acid (FA) composition of the oil by gas chromatography (GC). Derivatization was conducted as follows: 200 μ L of WCO were mixed with 1 mL of 0.2N HCl-MeOH and then heated at 60°C during 4 h; then, 0.2 mL of distilled water and 2 mL hexane were added. After vortexing, the methyl esters were extracted in the hexane layer and then collected for GC analysis. FA composition of WCO is depicted in Table 1.

2.3 Castor oil (CO) extraction

For the extraction of oil, 150 g of seeds without endosperm were weighed, macerated and then submitted to solid-liquid extraction in a reactor under agitation, using hexane as solvent at 60°C for 24 h. The oil-solvent mixture was filtered through filter paper to separate the seeds and the liquid phase was placed in a rotary evaporator to remove the hexane. The recovered oil was further heated at 60°C, followed by the addition of distilled water (3% w/w) under stirring for 30 min. Next, the mixture was cooled and centrifuged at 13,000 rpm for 10 min. The second degumming step was then performed by the addition of 1% KCl in a ratio 98:2 (mixture:KCl), followed by heating (60-70°C for 15 min) and centrifugation at 3,500 rpm for 30 min. FA composition of CO is depicted in Table 1.

2.4 Enzyme-catalyzed transesterification reactions

In a parallel study, we found that 10% of CALB, a molar ratio of 6, 40°C and 200 rpm, were the best

Table 1. Fatty acid composition of waste cooking oil (WCO) and castor oil (CO)

Fatty acid	WCO* (wt.%)	CO* (wt.%)
Palmitic	6.18	11
Stearic	0.87	6.54
Oleic	61.54	24.70
Linoleic	23.84	
Ricinoleic		50.76
Linolenic	6.28	5.99

*The values depicted in table are the average of triplicate determinations from different assays.

conditions for enzymatic synthesis of biodiesel from WCO. Thus, in the present study we conducted the following strategies to prepare biodiesel.

The first strategy consisted of reusing CALB in the presence of EtOH. 5 g of the mixture of substrates (WCO and EtOH) were combined with CALB and allowed to react in 25 mL flasks. Several cycles were conducted to reuse CALB to evaluate its deactivation expressed as FAEE content. After each cycle, the residual reaction mixture was centrifuged to recover the enzyme, which was further washed with hexane and then dried overnight at 40°C to be used in a new cycle. For all the cycles performed, 200 μ L samples were withdrawn periodically to monitor the extent of the reactions by GC analysis. All trials were conducted in duplicate. Hexadecane was used as an internal standard. The second strategy was carried out following the protocol described for the former, except that in the second approach we employed MeOH, evaluating the deactivation of CALB as FAME content. The third strategy was performed with blended alcohols at different molar ratios (0:100, 20:80, 40:60, 50:50, 60:40, 80:20 and 100:0, %EtOH:%MeOH). Finally, the fourth strategy consisted of using CO as raw material under the best conditions found from the first three strategies, in order to compare its efficiency.

2.5 GC analysis

Samples from all the transesterification reactions were filtered through a 0.45 μ m nylon microfilter to remove the catalyst. After that, the samples were mixed with 2 mL hexane. One μ L of the extract was injected into a Varian 3800 GC (Palo Alto, CA) fitted with an Agilent HP-Innowax polar capillary column (30 m X 0.32 mm X 0.25 μ m). Injector (CP-8410) and FID temperatures were both set at 250°C. The oven temperature was kept at 50°C for 2 min, then raised to 220°C at rate

of 30°C/min and held at this temperature for 25 min. After that, temperature was taken to 255°C and held at this temperature for 7 min. FA were identified by comparing their retention times with those of the Supelco 37 FAME Mix standard.

3 Results and discussion

Compared to other reports in the technical literature, in the present study we used primary alcohols as acyl acceptors to evaluate the ability of CALB when it was reused for the production of biodiesel from WCO and CO, in a solvent-free system.

3.1 Effect of alcohol type on biodiesel production

Most commonly used alcohols for biodiesel production are MeOH, EtOH, propanol and butanol. In a study conducted by Hama *et al.* (2006), they observed a lower activity for Novozym 435 compared to whole-cell *R. oryzae* regardless of alcohol type. They also found that MeOH was the most active alcohol for production of biodiesel from jatropha oil.

In the present work, we employed both MeOH and EtOH as acyl acceptors for biodiesel production but instead of using the commercial available Novozym 435, we prepared our own immobilized biocatalyst to be evaluated in the presence of both alcohols and its blends. Immobilized lipase is distinguished from free lipase because it is easily recovered from the reaction mixture, facilitating its repeated use and also enhances enzyme resistance against inactivation by polar alcohols such as the ones we employed.

The first set of transesterification reactions was carried out using EtOH and WCO. Reaction conditions were 10% of CALB, a molar ratio of 6, 40°C and 200 rpm. As shown in Figure 1, four cycles were performed in order to evaluate the deactivation of CALB, expressed in terms of FAEE content. For fresh enzyme, the findings of these trials show that the highest FAEE content was 96% after 6 h of reaction. During subsequent cycles, FAEE contents decreased as follows: 43.55, 33.14 and 32.06%, suggesting that the residual activity of the enzyme was also decreased: 45.59, 34.69 and 33.56%, respectively. These results can be explained by the continuous exposure to EtOH and the thermal treatment to which the enzyme was subjected between cycles.

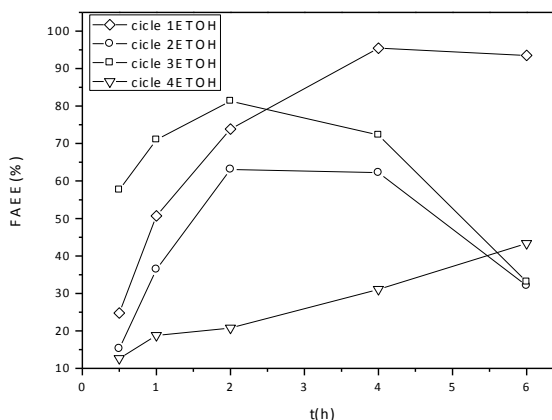


Fig. 1. Reuse of the immobilized preparation of CALB in the presence of ethanol and waste cooking oil. All trials were conducted for a molar ratio of 1:6 (oil:alcohol), 40°C, 10% of enzyme loading, at 200 rpm of mechanical stirring. The values shown in the plots are the average of triplicate determinations from different experimental trials.

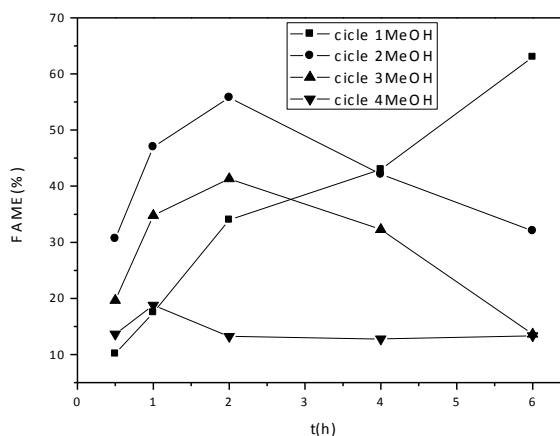


Fig. 2. Reuse of immobilized preparation of CALB in the presence of methanol and waste cooking oil. All trials were conducted for a molar ratio of 1:6 (oil:alcohol), 40°C, 10% of enzyme loading, at 200 rpm mechanically stirring. The values shown in the plots are the average of triplicate determinations from different experimental trials.

Our second set of transesterification reactions was carried out under the same conditions as described above, but using MeOH. As shown in Figure 2, four cycles were also performed in order to evaluate the deactivation of CALB, expressed in terms of FAME content. For fresh enzyme, the data show that the highest FAME content was only 63% after 6 h of reaction. This is a smaller content compared to that reached for the same enzyme in the presence of

EtOH. During the subsequent cycles, FAME contents decreased as follows: 32.06, 13.66 and 13.55%, indicating that the residual activity of the enzyme was also decreasing: 50.89, 21.68 and 21.19%, respectively.

Nelson *et al.* (2006), reported that MeOH was less efficient than ethanol in a solvent-free system and the low methyl ester yield of 25.7% was attributed to inhibition by MeOH of *C. antarctica* lipase activity. This was also observed by Abigor *et al.* (2000), who reported that the conversions of palm kernel oil using MeOH and EtOH were 15% and 72%, respectively. On the other hand, Hajar *et al.* (2009), described a repeated batch study in packed-bed reactor. According to their results, FAME yield was high (97%) even after six methanolysis cycles (i.e., 432 h) using the same packing materials (loofa plus the enzyme). Continuous circulation of the reaction mixture in the packed-bed reactor provided adequate mixing and this is considered another positive point for setting an efficient system for biodiesel production.

These important differences between FAEE and FAME contents can be explained by a stronger denaturing activity of MeOH compared to EtOH. On the other hand, mass transfer can be a limiting step for the transesterification reaction. The low miscibility of WCO and alcohols used, as well as glycerol formation, can interfere with the ability of CALB to produce the corresponding esters. Thus, our immobilized preparation was more stable and efficient in the presence of EtOH.

To overcome such operational difficulties several efforts are reported in the technical literature. Li *et al.* (2013) cite these works: (1) Watanabe *et al.* (1999) studied stepwise addition of EtOH and the conversion could reach up to 95%, (2) Rodrigues *et al.* (2010) also found that two-step ethanolysis was effective to avoid the negative effect caused by EtOH and a conversion rate of ca 100% could be obtained, (3) Du *et al.* (2004, 2007) proposed introducing a hydrophilic solvent tert-butanol as the reaction medium for FAME production and the operational stability of the lipase was improved significantly and (4) it was also found that using tert-butanol as the reaction medium was favorable for maintaining the high catalytic activity of the lipase in enzyme-mediated ethanolysis for FAEE production (Raita *et al.*, 2010).

Even when such efforts clearly improve the enzymatic synthesis of biodiesel, it is worth mentioning the advantages of our research: (1) we used WCO instead of vegetable oil, (2) we prepared our own immobilized biocatalyst, (3) we did not

introduce any organic solvent into our reaction mixture and (4) we reused the enzyme.

3.2 Effect of MeOH and EtOH contents in blended alcohol

Using blends of MeOH and EtOH as acyl acceptors for lipase-catalyzed transesterification could be an innovative strategy for overcoming the drawbacks of each alcohol. For the third set of transesterification reactions carried out in the present work, MeOH and EtOH were mixed at different molar ratios (0:100, 20:80, 40:60, 50:50, 60:40, 80:20 and 100:0, %EtOH:%MeOH) and allowed to react with WCO and 10% of CALB, at 40°C and 200 rpm.

Reactions carried out in the presence of different mixtures of alcohol (Figure 3) show that the yield of the enzyme activity decreased with increasing amounts of MeOH. For example, when the blended alcohol ratio was 80:20 (EtOH:MeOH), the amount of esters formed was 71.03%, whereas for a 20:80 (EtOH: MeOH) ratio, the ester content was 42%. We observed that when EtOH increased in blended alcohol, biodiesel yield also further increased: 27.09 (40:60, %EtOH:%MeOH) to 95% (100:0, %EtOH:%MeOH)

In a similar study, Zhao *et al.* (2014) using soybean oil reported that the yield of biodiesel increased significantly when the molar proportion of MeOH in blended alcohol decreased from 100 to 60 mol% during the first 8 h of reaction.

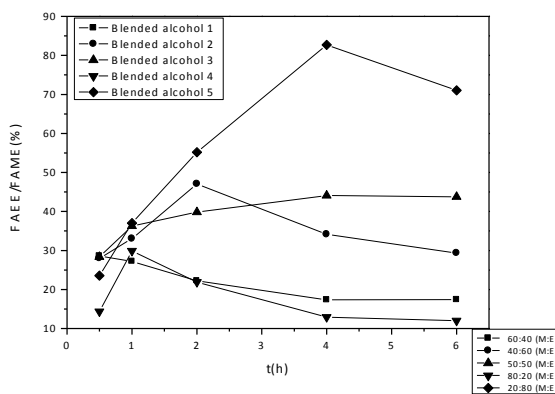


Fig. 3. Effect of blended alcohols at different molar ratios (0:100, 20:80, 40:60, 50:50, 60:40, 80:20 and 100:0, %EtOH:%MeOH) at 40°C, 10% of enzyme loading and 200 rpm of mechanical stirring. The values shown in the plots are the average of triplicate determinations from different experimental trials.

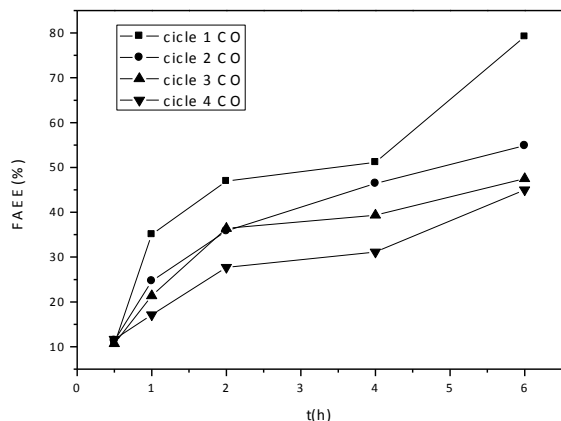


Fig. 4. Reuse of immobilized preparation of CALB in the presence of ethanol and castor oil. All these trials were conducted for a molar ratio of 1:6 (oil:alcohol), 40°C, 10% of enzyme loading, at 200 rpm of mechanical stirring. The values shown in the plots are the average of triplicate determinations from different experimental trials.

However, when the molar proportion of MeOH in blended alcohol was further decreased from 60 to 40 mol%, the incremental yield in biodiesel was small.

Based on studies reported in the literature, mixed MeOH-EtOH systems have proven to be a promising approach for the production of biodiesel using a homogeneous catalyst base. MeOH has the advantage of breaking the emulsion faster so that biodiesel and glycerol can be easily formed, while EtOH offers the advantage of improving oil solubility and therefore increase the rate of mass transfer in the system.

3.3 Using CO as an alternative feedstock for biodiesel

Different feedstocks have been employed for the synthesis of biodiesel. Most of them are obtained from vegetable oils (first generation raw materials) but it implies a partial substitution of such oils in food applications and consumption. Consequently, to avoid a competition between food and energetic applications, second generation raw materials represent the new and novel sources for biodiesel production.

Finally, we carried out the transesterification reaction with CO and based on the best conditions obtained from the strategies described above, we employed EtOH as acyl acceptor for a 10% enzyme load, 40°C and 200 rpm. As shown in Figure 4, four cycles were performed in order to evaluate the deactivation of CALB, expressed in terms of FAEE

content. For fresh enzyme, the findings of these trials show that the highest FAEE content was 79% after 6 h of reaction. During subsequent cycles, FAEE contents decreased as follows: 54.84, 47.5 and 45.01%, indicating that the residual activity of the enzyme also decreased: 69.26, 60 and 56.84%, respectively.

Compared to other studies using fresh lipases, Delgado & Pashova (2010), reported the transesterification of CO with EtOH using n-hexane as a solvent and Lipozyme TL IM. The maximum percentage of biodiesel was obtained at 35°C, a 5:1 (EtOH:CO) molar ratio, 15% of the enzyme and an initial water concentration of 2% w/w, reaching a yield of 80%. Palomino *et al.* (2010) mentioned the transesterification of CO with MeOH using a solvent additive and an immobilized lipase preparation from *Muchor miehi*. The authors reached 45% FAME content after 7 h.

Homogeneous catalysts have also been used for transesterification of CO. Ramezani *et al.* (2010) tested different basic catalysts, and found that KOCH₃ allowed them to achieved 76.2% FAME content at 65°C, catalyst concentration: 0.35% oil, 2 h and 250 rpm. Madankar *et al.* (2013) reported a yield of 96.1% with 1% KOH, a methanol/oil molar ratio of 250:1 at 65°C and a stirring rate of 600 rpm after 3 h.

Conclusions

To achieve high yields (> 90 %) in biodiesel conversion, we found best to use immobilized enzymes. In order to be reused in subsequent cycles of reaction it is necessary to recover the enzyme and wash it with non-polar solvent and submitted to mild drying temperature. For all the transesterification reactions where CALB was reused, we observed that the amount of FAEE or FAME decreased, however, the effect of MeOH was more severe for the activity of the enzyme. A relevant aspect of the present study is the use of second-generation raw materials, WCO and CO, which allowed us to achieve 96 and 79% FAEE content, respectively. According to our results, the next step is the continuous enzymatic preparation of biodiesel.

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