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ULTRASOUND-ASSISTED TRANSESTERIFICATION OF SOYBEAN OIL USING *COMBI-LIPASE* BIOCATALYSTS

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Abstract - We applied ultrasonic technology for the transesterification of soybean oil catalyzed by a mixture of lipases (*combi-lipase*: 75 % Novozym 435; 10 % Lipozyme TL-IM; and 15 % Lipozyme RM-IM), verifying the effects of ultrasonic amplitude, pulse conditions, the ethanol:oil molar ratio, enzyme concentration, and the influence of solvent (tert-butanol on the reaction. We also compared this system against ultrasound combined with mechanical stirring, and the efficiency of the *combi-lipase*, compared with the individual use of each lipase. The optimum conditions for the transesterification reaction were determined as enzyme concentration of 15 % (in relation to oil mass); ethanol:oil molar ratio of 3:1; ultrasonic amplitude of 30 %, duty cycle of 50 % and time pulse of 15 sec. The yields of conversion of ethyl esters with and without solvent were similar, indicating that the use of solvents during enzymatic transesterification reactions is not necessary when ultrasonic technology is applied to the system. The combination of mechanical stirring and ultrasound did not improve the yields of conversion compared to ultrasonic technology alone. The proposed *combi-lipase* produced higher yields of ethyl esters (75 %) than the individual lipases (55 %) in 5 h under ultrasonic-assisted batch reactions.
Keywords: Transesterification; *Combi-lipase*; Ultrasound-assisted technology; Biodiesel.

INTRODUCTION

Biodiesel is a renewable and a biodegradable fuel, which is becoming commercially important because of environmental concerns over the use of fossil fuels (Santin et al., 2017) and because it might mitigate problems with fuel supply in the near future (Aarthy et al., 2014). Biodiesel is a mixture of alkyl esters with long-chain fatty acids, easily produced through transesterification of vegetable oils, animal fats, and microalgal oils with short chain alcohols. This biofuel can be used by itself or in blends with petro-diesel in existing diesel-engines without any mechanical modifications (Aarthy et al., 2014).

Vegetable oils (edible and non-edible oils) are promising raw materials for biodiesel synthesis because they can be produced on large scale and are renewable in nature (Leung et al., 2010). Edible oil resources, such as sunflower, rapeseed, palm, and soybean, are considered the first generation of biodiesel feedstock materials. The cultivation of these crops has been well established in many countries and 95 % of the world biodiesel production comes from these raw materials (Atabani et al., 2012). Soybean oil is one of the major feedstocks used to this end, and Brazil represents one of the bigest producers of this crop (Koc et al., 2011). Besides the availability of edible oils, another advantage of using these materials is the properties of the biodiesel produced, which is suitable to be used as a direct diesel fuel substitute (Leung et al., 2010).

The transesterification reaction occurs in the presence of catalysts, which may be chemical (acid or

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base) or enzymatic. Currently, the chemical catalysis, mainly using alkalis, is the most used route in the industrial production of biodiesel because it is fast and has a relatively low cost (Yu et al., 2010; Gupta et al., 2017). However, the chemical synthesis requires high energy inputs, is environmentally problematic, and undesirable side reactions, such as the saponification of free fatty acids occur, consuming part of the catalyst and reducing the reaction yields (Leung et al., 2010; Tan et al., 2010). To avoid this, the use of lipases as biocatalysts for biodiesel synthesis is a promising alternative (Aguieiras et al., 2015).

Lipases (glycerol ester hydrolases, EC 3.1.1.3) are enzymes able to catalyze the hydrolysis of oils and fats, producing free fatty acids, di- and monoglycerides, and glycerol. In non-aqueous media, these enzymes catalyze interesterification, esterification, and transesterification reactions (Aguieiras et al., 2015). The main advantage of this group of biocatalysts is their versatility, which is related to their properties of being substrate specific, presenting regio, chemical, and stereo-selectivities, allowing biodiesel production from low refined oil. Most lipases are classified into two groups: sn-1,3-specific, which hydrolyzes ester bonds at sn-1 and sn-3 positions, as examples Lipozyme TL-IM (TLL) and Lipozyme RM-IM (RML), and nonregiospecific (or random), which act on all three positions, such as Novozym 435 (CALB) (Tongboriboon et al., 2010). Notwithstanding these advantages over the chemical route, the enzyme-mediated reaction presents a slow rate and the costs of biocatalysts are high (Gog et al., 2012; Aguieiras et al., 2015).

Based on lipases with different substrate specificity and regio-selectivity, the concept of *combi-lipase* (a mixture of different lipases) has been reported to be an alternative to reduce reaction time and to increase the conversion rates of transesterification, because the *combi-lipase* will act synergistically, attacking different positions of triglycerides in oil composition (Alves et al., 2014). Furthermore, the use of a mixture of lipases can reduce the biocatalyst costs, since biocatalysts present varying market prices (Li et al., 2010).

Another point in question is the need to increase the enzymatic-based reaction rates, allowing the use of these biocatalysts on an industrial scale (Yu et al., 2010). In this way, several studies have been devising new techniques to achieve this cost reduction. Applying ultrasound-assisted technologies in order to eliminate the low miscibility of reactants in a transesterification reaction, improving mass transfer, and increasing reaction rates has demonstrated to be a promising approach (Subhedar et al., 2015). Ultrasound generates cavitation, the phenomenon of formation, growth, and collapse of micro bubbles in the reaction medium (Ho et al., 2016). This phenomenon produces extreme conditions, such as acoustic microstreaming, turbulence, high pressure and temperature, as well as high shear forces, promoting the formation of fine emulsions of the immiscible reactants (Bhangu et al., 2017). Ultrasound-generated energy not only provides the mechanical energy for mixing but also the activation energy (Ea) required for the transesterification reaction. Furthermore, this technology contributes to chemical effects, since free radicals are produced during the short cavitation bubble collapse (Ho et al., 2016; Tan et al., 2019).

Ultrasound irradiation has been shown to reduce the reaction time, and the amount of required catalyst and alcohol necessary for the reaction, at the same time eliminating the need of solvents. It also produces glycerol, the main by-product of the reaction, with higher purity (Kumar et al., 2011). The performance of ultrasound-assisted transesterification reactions depends on several sonication parameters, such as the amplitude applied, the power output rate, the use of pulse mode, direct/indirect sonication, multiple frequencies, as well as the specific enzyme preparation (Kwiatkowska et al., 2011; Lerin et al., 2014; Manickam et al., 2014). Thus, it is crucial to evaluate and optimize these variables for each process in order to obtain good results and save energy consumption.

The ultrasound amplitude represents the intensity/strength of the sound wave at any point in time. The power provided to the reaction mixture and the intensity of cavitation are directly related to the amplitude. It is important to study this variable because it influences the degree of intensification of any chemical/biochemical process, directly affecting the yields of conversion (Subhedar and Gogate, 2016; Bhangu et al., 2017). For an enzymatic process, moderate amplitudes promote positive effects on reaction, whereas high amplitudes can induce the deactivation of the enzyme due to intense shockwaves released from cavitation bubbles (Subhedar and Gogate, 2016; Bhangu et al., 2017; Gupta et al., 2017).

Pulse sonication is a mode of ultrasonic operation in which the applied ultrasound is intermittently switched on-and-off, aiming to reduce the energy consumption in the system, at the same time allowing for sufficient cooling of the reaction mixture and the transducers, to avoid enzyme deactivation and tip erosion. The duty cycle, generally expressed as a percentage, represents the proportion of the pulse duration period relative to the total cycle time (Martinez-Guerra and Gude, 2016). This variable determine the dissipated energy, allowing its optimization to achieve maximum reaction yields and to obtain more economical processes (Martinez-Guerra and Gude, 2015; 2016). In particular, duty cycles are recommended for enzymatic reactions in order to decrease possible enzyme deactivation due to

continuous irradiation (Subhedar and Gogate, 2016). Finally, the pulse time is the variable that represents the time of one cycle of pulse, in which the ON and OFF time will be determined by duty cycle.

The use of ultrasound combined with enzymes has been showing great potential in terms of innovation; however, many studies still need to be developed in order to improve the use of this combined technology (Chatel, 2018). In this sense, the aim of this work was to optimize ultrasonic (amplitude, duty cycle and pulse time), and reaction (ethanol:oil molar ratio and enzyme concentration) parameters, as well as the influence of solvent (tert-butanol) on the transesterification of soybean oil, catalyzed by *combi-lipase*. We also compared the application of mechanical stirring combined with ultrasound on this reaction.

MATERIAL AND METHODS

Materials

The biocatalysts used in this work were Novozym 435, immobilized on macroporous resin (CALB), Lipozyme TL-IM, immobilized on acrylic resin (TLL), and Lipozyme RM-IM, immobilized on anion-exchange resin (RML), which were kindly donated by Novozymes (Novozymes, Spain). Refined soybean oil was purchased at a local market and was used without any treatment. Ethanol and tert-butanol were of analytical grade. Methyl heptadecanoate was purchased from Sigma Aldrich Co. (St. Louis, USA).

Transesterification reaction

The transesterification reactions were carried out in an enzymatic reactor designed and constructed in our group, which consists of a batch reactor, measuring 45 mm in length, 34 mm of inner diameter, and having the working volume of 30 mL. An ultrasonic device that operates at 700 W and 20 kHz frequency (Qsonica Q700, USA), equipped with a sonotrode probe of 1.27 cm diameter was coupled on the top of the reactor. We tested the homogeneity of the mixture under ultrasound energy application, finding the immersion of the tip of the probe up to 70 % of the liquid column as creating maximum turbulence of the reaction mixture. The reaction variables were: temperature (40 ^{o}C), time (1 h), ethanol:oil molar ratio (6:1) and *combi-lipase* (75 % Novozym 435 + 10 % Lipozyme TL-IM and 15 % Lipozyme RM-IM), biocatalysts concentration (15 % as mass fraction of oil). These reaction conditions were optimized according to the experimental design (described below in item 2.3).

Central composite design

At first, a central composite design (CCD) of three variables was performed in order to optimize ultrasound conditions for the transesterification reaction. The variables (amplitude, duty cycle and pulse time) and their coded and uncoded values are shown in Table 1, whereas in Table 2 are presented the 16 treatments for the three variables, each at five levels and two replications at the central point. In each case, the reaction rate for the transesterification reaction was determined after 1 h of reaction.

The second-order polynomial equation for the variables is presented in equation 1.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

where Y is the response variable, β_0 is the constant, β_i, β_{ii} and β_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and Xi and X_j are the coded level of variables x_i and x_j, also respectively.

The software Statistica 10.0 was employed for the experimental design and to analyze the results. Statistical analysis of the model was performed by analysis of variance (ANOVA). The explained variance was given by the multiple determination coefficient, R^2.

Table 1. Process variables of application of ultrasound in the transesterification of soybean oil and ethanol and their levels used in the CCD.

Variables	Name	Coded levels				
		-1.68	-1	0	1	1.68
X_1	Amplitude (%)	30	38	50	62	70
X_2	Duty cycle (%)	30	38	50	62	70
X_3	Pulse Time (s)	5	7	10	13	15

Optimization of reaction parameters

After optimizing ultrasound variables, the influence of reaction conditions on biodiesel yields was analyzed. The parameters evaluated for the transesterification reaction were: enzyme concentration: 5, 15, and 25 % (in relation to the oil mass); ethanol:oil molar ratio: 3:1, 6:1, and 9:1; the influence of the solvent *tert*-butanol and the use of a mechanical stirring system combined with ultrasound. Also analyzed was the efficiency of the *combi-lipase* compared to individual lipases. Each variable was evaluated individually, maintaining the others constant, as previously described in section 2.2. The reaction was carried out in the same reactor and using the same ultrasonic device described in 2.2, under the ultrasound optimized conditions.

Gas chromatography analysis

First, 1 mL of distilled water was added to 1 mL of sample, followed by centrifugation (2,500 x *g*, 10 min, 4 °C), to separate ethyl esters from the glycerol formed during the reaction. From the upper phase containing esters, a 50 mg sample was mixed with 1 mL of internal standard of methyl heptadecanoate

(10 mg/mL in heptane) for further analyses by gas chromatography (Shimadzu, model GC-17A, Japan) equipped with a flame ionization detector (FID) and DB5 capillary column (30 m × 0.25 mm id × 0.25 mM; J&W Scientific, USA), with split ratio of 1:30, injector and FID detector temperature of 300 °C and 310 °C, respectively. Nitrogen was used as carrier gas, at a flow of 1 mL min^{-1}. Column temperature was programmed from 50 °C to 310 °C, with a heating rate of 10 °C min^{-1}. The amount of injected sample was 1 µL, and total time of the analysis was 32 minutes.

A standard FAEE (Fatty Acid Ethyl Esters) mix (C4-C24) from Supelco was used to identify the peaks at different retention times and to correct the peak area using the response factors of the compound. The FAEE content was quantified upon analysis following the European Standard DIN EN 14103, and then calculated based on the amount of ethyl esters in the analyzed sample.

RESULTS AND DISCUSSION

Optimization of Ultrasound Conditions

In order to optimize the ultrasonic system conditions for the enzymatic transesterification reaction, a central composite design was used to evaluate the influence of the variables: amplitude (X_1), duty cycle (X_2) and pulse time (X_3), on the initial reaction rate (mmol L^{-1} h^{-1}), and the results are presented in Table 2.

The highest reaction rates obtained in this study were 264.82 and 253.81 mmol L^{-1} h^{-1}, both with 50 % of amplitude and duty cycle, and 5 s (treatment 13), and 15 s (treatment 14) of pulse time, respectively. Analyzing the behavior of the amplitude from Table 2, it is possible to verify that increasing this variable and maintaining others constant, the initial reaction rate decreases. However, for the variables duty cycle and pulse time, this behavior is not clearly observed, being better evaluated through the statistical analysis, shown in Table 3.

Table 2. Experimental design variables of application of ultrasound in the transesterification of soybean oil and ethanol and results of the CCD.

Treatment	X1	X2	X3	Initial reaction rate ($mmol.L^{-1}.h^{-1}$)
1	38 (-1)	38 (-1)	7 (-1)	206.22
2	62 (1)	38 (-1)	7 (-1)	187.37
3	38 (-1)	62 (1)	7 (-1)	217.41
4	62 (1)	62 (1)	7 (-1)	182.48
5	38 (-1)	38 (-1)	13 (1)	225.28
6	62 (1)	38 (-1)	13 (1)	231.70
7	38 (-1)	62 (1)	13 (1)	221.33
8	62 (1)	62 (1)	13 (1)	188.22
9	30 (-1,68)	50 (0)	10 (0)	236.22
10	70 (1,68)	50 (0)	10 (0)	174.88
11	50 (0)	30 (-1,68)	10 (0)	141.55
12	50 (0)	70 (1,68)	10 (0)	187.23
13	50 (0)	50 (0)	5 (-1,68)	264.82
14	50 (0)	50 (0)	15 (1,68)	253.81
15	50 (0)	50 (0)	10 (0)	201.84
16	50 (0)	50 (0)	10 (0)	203.03

The linear, quadratic and interaction effects of the variables amplitude (X_1), duty cycle (X_2), and pulse time (X_3) on the transesterification reaction, as well as the regression coefficients, p-value and standard errors, are shown in Table 3. These variables will be statistically significant at 95 % confidence level if the effects have a p-value < 0.05. The linear effects are the most important because they represent the average change in the response, the positive effect representing an increase in the response and vice-versa (Poppe et al., 2015).

In Table 3 it is possible to verify that the amplitude and pulse time were statistically significant for the reactions. Amplitude was the most impacting variable, showing a negative effect, whereas pulse time had a positive effect. This means that higher reaction rates will be obtained with low amplitudes and high pulse time for any value of duty cycle in the range of 30 – 70 %. Duty cycle was not statistically significant. However, it is important to stress that values over 70 % were tested in a previous study and they proved to be inappropriate for this enzymatic reaction; thus, temperatures above 70 °C are not recommended in combination with the enzymes used in this study (Rodrigues et al., 2008). In enzymatic reactions, the pulse mode is an interesting alternative to continuous ultrasound irradiation because it can decrease possible enzyme deactivation and save energy consumption (Kumar et al., 2011; Adewale et al., 2015; Subhedar and Gogate, 2016). In comparison, the ultrasonic amplitude, ultrasonic cycle, and the interaction of these variables were significant in the transesterification reaction of waste tallow (Adewale et al., 2015).

Equation 2 describes the second-order polynomial model for the transesterification reaction, obtained by adjusting the experimental data, considering only the significant variables.

Table 3. Statistical analysis of CCD.

Variable	Effect	Regression coefficient	Standard error	p-value
Mean*	202.705	202.705	0.593	0.002
Linear				
X_1*	-26.899	-13.449	0.227	0.011
X_2	5.219	2.610	0.227	0.055
X_3*	7.996	3.998	0.227	0.036
Quadratic				
X_1X_1	0.936	0.4681	0.276	0.339
X_2X_2*	-28.230	-14.115	0.276	0.012
X_3X_3*	39.035	19.517	0.276	0.009
Interaction				
X_1X_2*	-13.902	-6.951	0.297	0.027
X_1X_3	6.772	3.386	0.297	0.058
X_2X_3*	-13.432	-6.716	0.297	0.028

*Significant effects obtained in this study.

$$Y = 202.70 - 26.89X_1 + 7.99X_3 - 28.23X_2^2 + + 39.03X_3^2 - 13.90X_1X_2 - 13.43X_2X_3 \quad (2)$$

where Y represents the initial reaction rate (mmol.L^{-1}. h^{-1}), X_1, X_2 and X_3 are the coded values of amplitude, duty cycle and pulse time, respectively.

The optimal working conditions for ultrasound lipase-catalyzed biodiesel syntheses were 30 % amplitude, 50 % duty cycle and 15 s pulse time, obtained from the response desirability profile, determined with the software Statistica 10.0. Under these conditions, the theoretical value for the highest initial reaction rate, predicted by the model, was 265.32 mmol.L^{-1}. h^{-1}. The experimental validation of the predicted model was performed using the optimized conditions, with two repetitions, and resulted in an initial reaction rate of 257.40 mmol.L^{-1}. h^{-1}. This value shows a satisfactory correlation between the experimental results and the predicted model.

As described above, the optimum amplitude for this reaction was 30 %, which corresponds to 40 W of power, as indicated by the equipment. In the production of biodiesel using canola oil and methanol catalyzed by CALB, the optimal conditions were defined as a power of 40 W, using an ultrasonic probe of 3.5 cm in diameter, methanol:oil molar ratio of 5:1, and enzyme concentration of 0.23 % (mass fraction) (Bhangu et al., 2017). The authors concluded that low intensity ultrasound was best for the enzymatic synthesis, since high intensities tended to inactivate the biocatalyst, reducing process efficiency. The authors also observed that low intensity ultrasonication reduced reaction time from 22 h to 1.5 h. On the other hand, different ultrasonic amplitudes varying from 30 to 50 % were studied for the transesterification of waste tallow catalyzed by immobilized CALB, with results showing increased yields proportional to the amplitudes. This behavior could be attributed to the high viscosity of waste tallow (Adewale et al., 2015). These results show that, under appropriate conditions, ultrasound is an interesting tool to be applied in enzymatic reactions. Some studies report that, under low intensity and suitable frequency, the micro-convection generated by ultrasound energy leads to enhancement of the enzymatic reaction due to conformational changes in its secondary structure (Borah et al., 2016; Huang et al., 2017). This physical effect causes unfolding of the enzyme proteins with exposure of inner hydrophobic groups, resulting in the enhancement of the reaction rate and enzyme-substrate affinity (Borah et al., 2016; Pellis et al., 2016).

After obtaining the optimized ultrasound conditions, the next stage of this work was evaluating the influence of the following reaction parameters: oil:alcohol molar ratio, biocatalyst concentration and the addition of solvent and a mechanical stirring combined with ultrasound system, as these variables directly influence the reaction of transesterification.

Effect of Enzyme Concentration

The concentration of biocatalyst required for the reaction is an important factor for the successful industrial application, taking into account the high enzyme cost (Subhedar et al., 2015). Moreover, this variable influences directly the reaction rate. Thus, in order to obtain the optimum amount of catalyst able to promote higher ethyl esters yields, enzyme concentrations were varied from 5, 15 to 25 % (oil mass), and the results are presented in Figure 1.

When enzyme concentration increased from 5 to 15 %, the ethyl ester yields increased from 9.17 to 33.16 %. Further increments, however, led to small gains in yields (10 %), which does not justify an increase in production costs, related to enzyme amount. The same behavior was observed by Michelin et al. (2015) and Batistella et al. (2012) who obtained higher biodiesel yields at higher enzyme concentration, using ultrasonic technology for the transesterification reaction of macauba and soybean oil, catalyzed by Novozym 435. These authors obtained the best conditions of enzyme concentration as 20 % and 27.1 %, respectively (Batistella et al., 2012; Michelin et al., 2015). Adewale et al. (2015) also observed an increase in biodiesel yield with increasing enzyme catalyst concentration. Under ideal conditions, there is an increase in enzyme

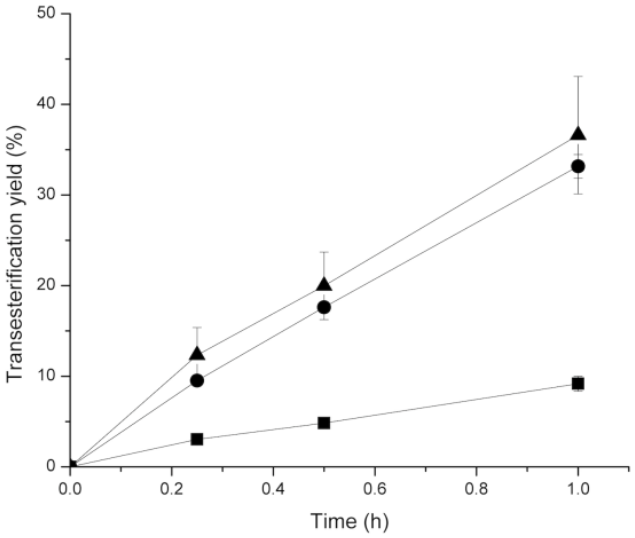

Figure 1. Effect of different enzyme concentrations in the ultrasound-assisted transesterification of soybean oil using *combi-lipase* biocatalysts. (–■–) 5 %, (–●–) 15 %, (–▲–) 25 % in relation to the oil mass. Reaction conditions: ethanol:soybean oil molar ratio of 6:1; temperature of 40 °C; *combi-lipase* composition of 75 % Novozym 435 + 10 % Lipozyme TL-IM and 15 % Lipozyme RM-IM. Results are the mean of triplicates.

concentration promoting higher reaction rates up to a point where the reaction interface between biocatalyst and substrate becomes saturated, causing the reaction rate to become constant (Liu et al., 2008).

Effect of substrates molar ratio

Substrate molar ratio is another important variable affecting the biodiesel production (Adewale et al., 2015). The stoichiometry of this reaction requires 3 mol of alcohol to 1 mol of triglyceride, but because this is a reversible reaction, an excess of alcohol is usually used in order to favor the reaction towards the products (Subhedar et al., 2015). However, under the effect of ultrasound, it is possible to reduce the excess alcohol required (Kumar et al., 2011). Thus, experiments were performed using an ethanol:oil molar ratio of 3:1, 6:1, and 9:1 to investigate the effect of molar ratio in an ultrasound-assisted transesterification reaction.

Results in Figure 2 show the optimum molar ratio of 3:1, indicating that ultrasound irradiation favored the reaction, requiring only the exact stoichiometric amount of oil and ethanol. This behavior can be attributed to the enhancement of mass transport favored by ultrasound, which is crucial for this process, since the enzyme is immobilized and the reactants constitute immiscible phases. As explained before, cavitation possibly increased emulsion formation and better mixing throughout the reaction (Sivakumar et al., 2014; Subhedar et al., 2015).

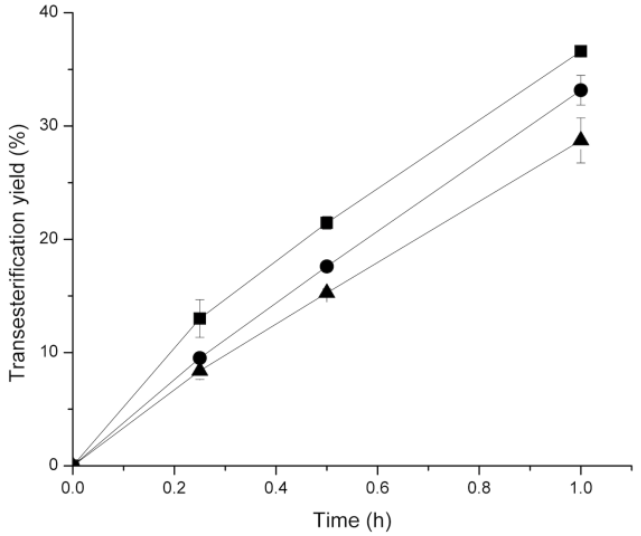

Figure 2. Effect of different substrate molar ratio on the ultrasound-assisted transesterification of soybean oil using *combi-lipase* biocatalysts. (–■–) 3:1, (–●–) 6:1, (–▲–) 9:1 soybean oil:ethanol. Reaction conditions: temperature of 40 °C; *combi-lipase* composition of 75 % Novozym 435 + 10 % Lipozyme TL-IM and 15 % Lipozyme RM-IM; biocatalysts concentration of 15 % in relation to the oil mass. Results are the mean of triplicates.

Batistella et al. (2012) reported the optimum molar ratio of ethanol to soybean oil as 3:1 for the transesterification reaction catalyzed by CALB and RML lipases in an ultrasonic bath. Similar results were found by Adewale et al. (2015) and Kumar et al. (2011), who obtained the highest yields of biodiesel at a molar ratio of 4:1 for methanol:waste tallow and methanol:Jatropha oil, respectively. Both reactions were conducted using an ultrasound probe. However, Yu et al. (2010) reported optimal conditions of a molar ratio of 6:1 methanol:soybean oil for the transesterification reaction using an ultrasonic bath combined with a vibration system. The best molar ratio for a transesterification reaction depends on several factors, including frequency and intensity of the ultrasound system, the type of sonicator (bath or probe), the chemical composition of triglycerides and alcohols in the reaction, as well as reactor size and operational characteristics (Kalva et al., 2009; Adewale et al., 2015), explaining the large variations reported in the literature.

Effect of Solvent Tert-Butanol

The use of solvents in enzymatic transesterification reactions promotes multiple effects on reactants and products, such as increased reactant solubility, protecting the lipase from denaturation, increased homogeneity, thus easing mass transfer and reaction rates. It also provides a reduction in viscosity and stabilization of immobilized lipases (Dossat et al., 2002; Yu et al., 2010). However, the use of this reagent makes the process more expensive, toxic, and flammable, requiring higher investment costs in order to reach safety measures of an industrial plant (Dossat et al., 2002; Liu et al., 2008).

In this sense, the ultrasound system is an alternative method that could be used to reduce the limitations of mass transfer in a solvent-free system. Therefore, the influence of the *tert*-butanol solvent on the enzymatic transesterification reaction of soybean oil was evaluated in the presence of ultrasound irradiation. The results of these experiments are presented in Figure 3.

Our results show a similar behavior for the reactions with and without solvent under ultrasound application, suggesting that ultrasound itself was sufficient to improve the miscibility of the reaction mixture, favoring mass transfer between the substrate and the active site of the enzyme, turning irrelevant the use of the organic solvent (Trentin et al., 2015), and allowing the recovery of products without further complex purification processes (Dossat et al., 2002; Liu et al., 2008). The majority of reports in the literature in which the ultrasonic technology was employed in the enzymatic synthesis of biodiesel did not use solvents in the reactions, usually reporting excellent yields of conversion (Liu et al., 2008; Subhedar et al., 2015; Bhangu et al., 2017; Santin et al., 2017).

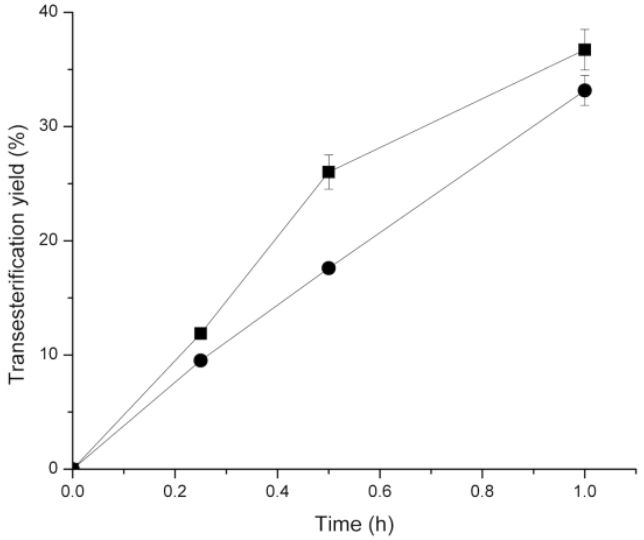

Figure 3. Evaluation of the use of the solvent *tert*-butanol in the ultrasound-assisted transesterification of soybean oil using *combi-lipase* biocatalysts. (–■–)20 % (in relation to the oil mass) of *tert*-butanol, and (–●–) without solvent. Reaction conditions: ethanol:soybean oil molar ratio of 6:1; temperature of 40 °C; *combi-lipase* composition of 75 % Novozym 435 + 10 % Lipozyme TL-IM and 15 % Lipozyme RM-IM; biocatalyst concentration of 15 % in relation to the oil mass. Results are the mean of triplicates.

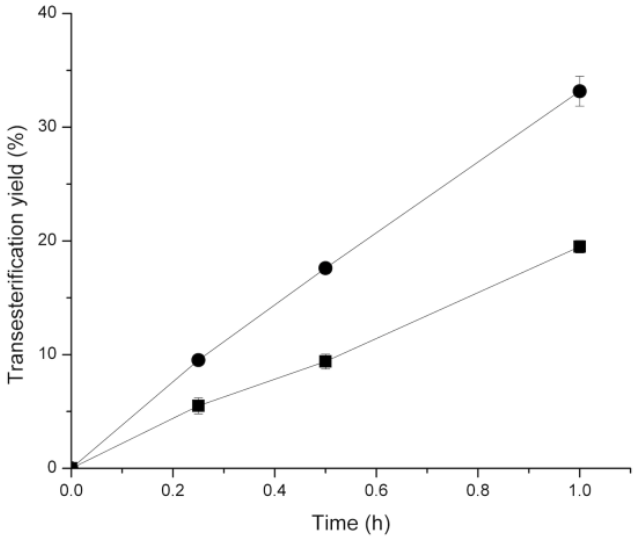

Figure 4. Comparison of the ultrasound-assisted transesterification of soybean oil using *combi-lipase* biocatalysts combined with a mechanical stirring system (–■–), and without mechanical stirring (–●–). Reaction conditions: ethanol:soybean oil molar ratio of 6:1; temperature of 40 °C; *combi-lipase* composition of 75 % Novozym 435 + 10 % Lipozyme TL-IM and 15 % Lipozyme RM-IM; biocatalysts concentration of 15 % in relation to the oil mass. Results are the mean of triplicates.

Effect of mechanical stirring combined with ultrasound system

The ultrasound-assisted enzymatic-transesterification reactions were carried out using the ultrasonic pulse mode, which means that the biocatalyst particles remained agitated only when the ultrasound was switched on. Therefore, it was decided to evaluate the influence of a mechanical stirring, working continuously, combined with the ultrasound system, in order to keep the lipases mobile throughout the reaction of transesterification. The result of this study is presented in Figure 4. In the literature, it is also possible to find studies stating that the use of ultrasound energy for biodiesel production can eliminate the need of an external agitation (Tan et al., 2019).

It was expected that, keeping biocatalysts particles in movement during all the process, they would be better dispersed in the reaction medium, optimizing the contact between the substrate and the biocatalyst, resulting in higher biodiesel yields. Surprisingly, the use of the mechanical stirring in combination with ultrasound energy reduced yields (Figure 4).

Yu et al. (2010) evaluated the enzymatic transesterification reaction of soybean oil, using CALB as biocatalyst, and the influence of three reaction systems: mechanical stirring of 50 rpm, ultrasonic bath, and the two systems in synergy, with the highest yields (92 %) obtained in 4 h with the combination of systems. The authors suggested that this configuration produces rapid emulsification caused by the ultrasound system and the optimized contact between the enzyme and the substrate provided by the vibrational system, consequently increasing the reaction rate. Gharat and Rathod (2013) reported similar results, using mechanical stirring combined with an ultrasound bath for the enzymatic transesterification of waste cooking oil and dimethyl carbonate, achieving yields of 86.61 % in 4 h.

These results contrast with those found in the present work, possibly associated with the way the ultrasonic was irradiated through the system. Both cited authors used ultrasonic baths, which provide an indirect irradiation, much less efficient than the probe system used by us, requiring an auxiliary stirring system to promote better reaction conditions.

Comparisons of combi-lipase and individually lipases

In order to compare the transesterification reactions catalyzed by the *combi-lipase* biocatalysts and the lipases individually, reactions were carried out using the ultrasound under optimized conditions, changing only the catalyst in each reaction. The results of these experiments are presented in Figure 5, where it is possible to verify that, in the first hour of reaction, the enzyme TLL showed faster formation of ethyl esters, followed by the *combi-lipase* biocatalyst.

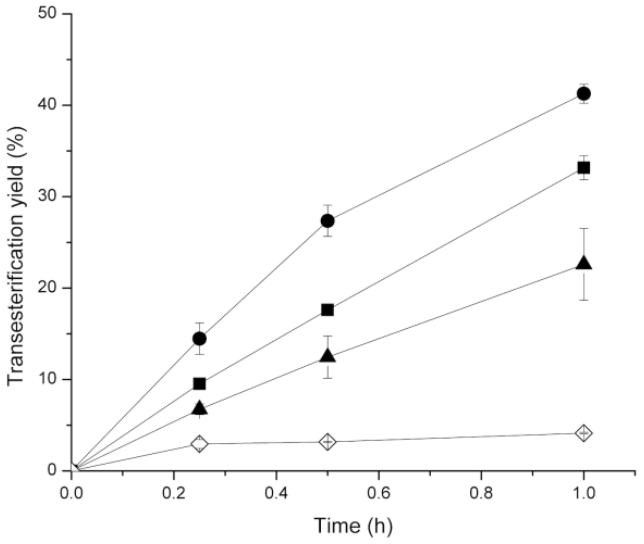

Figure 5. Comparison of the efficiency of the *combi-lipase* (–■–) compared to stand-alone enzymes (–●–) TLL, (–▲–) CALB, or (–◊–) RML, in the ultrasound-assisted transesterification of soybean oil. Reaction conditions: ethanol:soybean oil molar ratio of 6:1; temperature of 40 °C; biocatalyst concentration of 15 % in relation to the oil mass. Results are the mean of triplicates.

Based on these results, it was decided to evaluate the reaction kinetics using the *combi-lipase* and the lipase TLL individually, for 6 h of reaction, in order to evaluate the maximum conversion of ethyl esters, as well as the stability of these biocatalysts in a longer process, assisted by ultrasound irradiation (Figure 6).

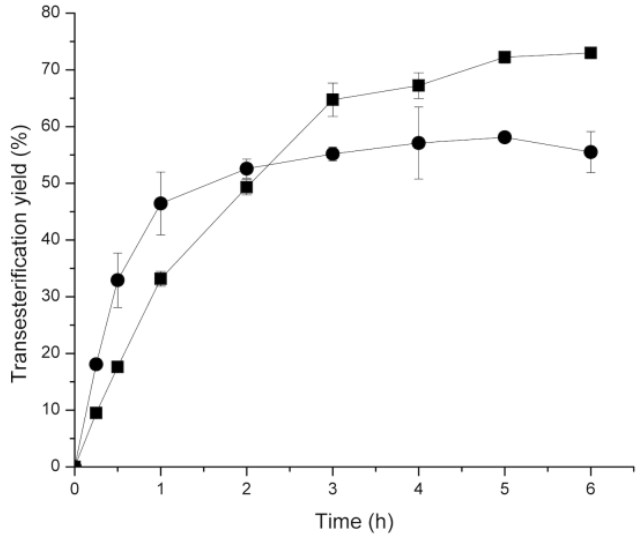

Figure 6. Kinetics of ultrasound transesterification reaction of soybean oil catalyzed by *combi-lipase* (–■–), and by TLL (–●–). Reaction conditions: ethanol:soybean oil molar ratio of 6:1; temperature of 40 °C; biocatalyst concentration of 15 % in relation to the oil mass. Results are the mean of triplicates.

The enzyme TLL has an initial reaction rate of 263.68 mmol $L^{-1}h^{-1}$, higher than that for the *combi-lipase* (239.76 mmol $L^{-1}h^{-1}$) in 1 h of reaction. However, a stabilization in the rate of ethyl ester formation was observed for TLL, around 55 % of conversion after 2 h of reaction, remaining constant to the end of the process. On the other hand, the combi-lipase showed a lower initial reaction rate, but throughout the process it achieved approximately 75 % of conversion. The *combi-lipase* was able to promote better yields in longer reaction times, as can be observed in this study, being a more attractive alternative than the use of the enzymes individually, mainly in reactions with heterogeneous substrates such as oils and fats. The enzyme TLL is sn-1,3-specific, hydrolyzing ester bonds at sn-1 and sn-3 positions of the triglycerides. The mixture of lipases, in this work, was composed of two specific lipases, TLL (Fernandez-Lafuente, 2010) and RML (Rodrigues and Fernandez-Lafuente, 2010) and a non-specific lipase (CALB) which acts in all positions of the substrate molecule (Anderson et al., 1998).

The results obtained in this work compared well with those reported by Poppe et al. (2015), who used a *combi-lipase* (CALB, TLL, and RML) for the transesterification reaction of olive and palm oil, and observed that the initial reaction rate for the individual lipases was similar to their combination, suggesting that from 4 h to the end of the reaction the mixture of lipase promoted higher yields than the individual enzymes (Poppe et al., 2015).

Evaluating the kinetics of the ultrasound assisted transesterification reaction catalyzed by *combi-lipase*, yields of conversion of ethyl esters of about 75 % in 5 h reaction were observed in the present work. Santin et al. (2017) and Gharat and Rathod (2013) analyzed the ultrasound transesterification reaction catalyzed by the enzyme CALB, obtaining conversions of about 88 % for soybean oil and 87 % for waste cooking oil in 4 h of reaction, respectively (Gharat and Rathod, 2013; Santin et al., 2017). However, one of the advantages of using the *combi-lipase* compared with CALB used individually is the reduction of the process cost, since this enzyme has a very high commercial price when compared to the others, costing 16 times more than TLL, as an example (Li et al., 2010).

CONCLUSIONS

The present work reported the transesterification of soybean oil using ethanol under the influence of ultrasound irradiation directly into the reaction and catalyzed by a mixture of enzymes (*combi-lipase*). Through the optimization of ultrasound parameters, it was possible to determine that the amplitude was the parameter that most affected the transesterification

reaction and mild ultrasound energies are the most indicated for reaction catalyzed by lipases. Furthermore, the use of a duty cycle proved to be more efficient than continuous energy application, as it can keep the temperature of the reaction mixture at low levels, protecting the enzyme from denaturation and reducing energy consumption. Results also showed that the use of an ultrasound system is a promising alternative to reduce the amounts of alcohol and biocatalyst required for the reaction when compared to the conventional process. It can also eliminate the need of solvents in this reaction. This study suggests that a mixture of lipases, in combination with the use of ultrasound, is an alternative to reduce the enzymatic biodiesel production costs. Further studies are required to improve the yields of conversion and to test the concept of ann ultrasound-assisted transesterification reaction, catalyzed by a mixture of lipases in a continuous reactor.

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