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CHEMICAL SCIENCES

Hydrolysis of macauba kernel oil: ultrasound application in the substrates pre-emulsion step and effect of the process variables

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Abstract: The main goal of the present work was to evaluate the application of ultrasound as a previous step to promote the substrates pre-emulsion in the hydrolysis reaction of macauba kernel oil (MKO). The ultrasound effect on the substrates pre-emulsion was evaluated on the free fatty acid (FFA) content, as well as the process variables (reaction time, percentage of catalyst Lipozyme® RM IM, and buffer solution). Reactions carried out with the substrates pre-emulsion presented higher FFA production, up to a 40 wt% increase in 1 hour of reaction, yielding 80 wt% of FFAs in 8 hours. The use of catalyst in the reaction medium, from 5 to 15 wt%, favored the FFAs production in 2 hours of reaction. Addition of 25 to 100 wt% of buffer solution led to 86 wt% of FFAs in 4 hours of reaction. Enzyme recycling resulted in a slight decrease in the FFA content, although the catalyst had maintained 85% of its initial activity after 30 h of use. Therefore, the ultrasound pre-emulsion previous step allowed a more efficient hydrolysis reaction of MKO, leading to an increase of up to 40 wt% on the FFA content, when compared to the hydrolysis without such step.

Key words: enzymatic catalysis, free fatty acid, hydrolysis, ultrasound.

INTRODUCTION

Fatty acids are important raw materials for oleochemical industries due to their high demands for diverse applications. Vegetable oils conversion into high value-added products, such as free fatty acids (FFAs) and derivatives, has been of great commercial interest, as in the industries of soap, surfactant, lubricant, plastic, ink, coating, cosmetic, self-care, food, biodiesel, among others (Huang et al. 2010, Satyarthi et al. 2011, Pinyaphong et al. 2012, Avelar et al. 2013, Takisawa et al. 2013, Aguieiras et al. 2014, Wancura et al. 2019a, b), besides being used as substrates for other reactions of interest. The industrial production of monoacylglycerols (MAG) and diacylglycerols (DAG), for example, emulsifiers widely applied in the food, cosmetic and pharmaceutical industries, can occur through the partial esterification of glycerol and FFAs (Watanabe et al. 2005, Fregolente et al. 2009, Wang et al. 2011, Fiametti et al. 2011, 2012). Also, fatty acid esters can be produced from FFAs such as stearic, oleic and linoleic, and carbohydrates (glucose, fructose, sucrose and sorbitol), which have wide application as emulsifiers, in addition to having a preservative effect, and having been associated as anti-tumor agents and plant growth inhibitors (Castro et al. 2004). Fatty acids can also be esterified with methanol or ethanol, obtaining high-purity methyl or ethyl esters (biodiesel) (Aranda et al. 2008, M.M.R. Gomes, unpublished data, Feiten et al. 2014, Zaher & Soliman 2015, Wancura et al. 2018, Rosset et al. 2019).

Fatty acids production occurs mainly through hydrolysis of oils or fats (Babicz et al. 2010, Awadallak et al. 2013). Hydrolysis of triacylglycerols occurs at the oil and water interface, since these substrates are immiscible with each other. The contact and mixture of oil and water phases are commonly promoted by mechanical agitation, but recently there has been an attempt to reduce the mass transfer limitations through the application of ultrasound (Feiten et al. 2014, Michelin et al. 2015, Ramón et al. 2015, Remonatto et al. 2015, Awadallak et al. 2016, Zenevicz et al. 2016, 2018, Dal Prá et al. 2019). The cavitation (bubble formation, growth and implosive collapse in the reaction medium) generated in these reactions act through three main mechanisms, in combination or isolated. The first mechanism involved is the thermal effect, due to the high heat generation during cavitation which increases the temperature; the second is the free radicals formation by sonolysis of water; and the third is due to the mechanical forces (shear stress) generated due to the shock waves produced by cavitation (Sinisterra 1992, Mason et al. 2005, Mason 2007, Liu et al. 2008, Waghmare & Rathod 2016). Collapse of cavitation bubbles breaks the phase partition and promotes emulsification by ultrasonic jets. Thus, there is an increase in the reaction rates and, consequently, high yields are achieved applying only a small amount of catalyst, resulting in lower energy consumption when compared to the mechanical agitation process (Gasparotto et al. 2015, Luft et al. 2019).

The ultrasound system has been considered an eco-friendly green technology due to its high efficiency, reduction or absence of organic solvents use in several reactions, and possibility of reducing the amount of reagents added. Besides the significant decrease in reaction or processing times compared to other conventional techniques and its low instrumental requirements, the ultrasound system presents an economically viable performance, with increased yield and selectivity, favoring reactions that normally do not occur under normal conditions (Martines et al. 2000, Li et al. 2005, Mason 2007, Silva et al. 2013, 2014, Feiten et al. 2014, Remonatto et al. 2015, Dal Prá et al. 2019).

Ultrasound systems have been satisfactorily applied to various biochemical and enzymatic reactions (Batistella et al. 2011, Silva et al. 2013, 2014, 2015, Balen et al. 2015, Trentin et al. 2014, 2015, Awadallak et al. 2016, Santin et al. 2017, Zenevicz et al. 2016, 2018, Coppini et al. 2019). Lipase-catalyzed hydrolysis of triacylglycerols may be performed under mild conditions (Lerin et al. 2011, Feiten et al. 2014, Kabbashi et al. 2015, Gasparotto et al. 2015, Luft et al. 2019), allowing the development of a more efficient, economical and ecologically correct process for hydrolysis of vegetable oils or fats, which can be conducted at milder temperatures, and reach higher hydrolysis yields (Awadallak et al. 2013, 2016, Coppini et al. 2019).

The main features of immobilized enzymes are the ease in separating the catalyst from the reaction medium, non-generation of effluents, purity of the glycerol generated as a by-product, in addition to the possibility of reuse (Ranganathan et al. 2008, Jerbaek et al. 2009, Santin et al. 2014, Fernandez-Lafuente 2014, Facin et al. 2019). They perform satisfactorily at mild temperatures (Feiten et al. 2014, Kabbashi et al. 2015, Santin et al. 2017), therefore preventing product degradation and reducing energy costs (Antczak et al. 2009, Wancura et al. 2020). However, utilization of these immobilized catalysts presents the mass transfer limitation as inconvenient, since the supports can hinder the substrates access to its catalytic site (Jerbaek et al. 2009, Santos et al. 2015, Facin et al. 2019), which may be solved by ultrasound application (Leaes et al. 2013, Lerin et al. 2014, Trentin et al. 2015).

The ultrasound utilization in enzymatic reactions may be a simple and important tool to control the aggregation and/or dispersion of particles in heterogeneous systems and to increase the solubility of homogeneous mixtures without the need to add organic solvents or surfactants, hence reducing the substrate-enzyme mass transfer limitations without waste generation. Furthermore, the ultrasound system is an interesting tool to be applied in order to disturb weak bonds and induce favorable conformational changes in the structure of proteins, increasing their catalytic activity (Babicz et al. 2010, Fiametti et al. 2012, Leaes et al. 2013, Silva et al. 2013, Michelin et al. 2015, Ramón et al. 2015, Feiten et al. 2016, Santin et al. 2017). Nevertheless, the application of ultrasound during the reaction can weaken the enzyme’s conformation (Bezbradica et al. 2006, Feiten et al. 2016), reducing its reuse. Therefore, ultrasound may be applied as a step prior to the reaction.

Macauba (*Acrocomia aculeata*) is a palm species native to subtropical regions, distributed among Latin American countries and abundantly found in the Brazilian Cerrado (Berton et al. 2013, Lopes et al. 2013, Evaristo et al. 2016). Macauba fruits have great potential for obtaining oil (Silva & Andrade 2013, Michelin et al. 2015, César et al. 2015, Colonelli et al. 2017). Such species has oleaginous fruits in huge bunches that can weigh more than 90 kg under natural conditions (Pires et al. 2013, M.A. Lopes & S.P. Favaro, unpublished data). Currently, there is a growing interest in macauba oil production, because it is able to produce 10 times more oil per hectare when compared to soybeans (Roscoe et al. 2007). It is estimated that, under proper agronomic care, a commercial plantation can yield 16000-25000 kg ha^{-1} of fruit, and production of up to 6200 kg ha^{-1} of oil (Pires et al. 2013, Colombo et al. 2018). Researches indicate that macauba can annually produce around 4.5 tons of pulp oil and more than 600 kg of almond oil, surpassing the average of 3.5 tons of palm oil yield (M.A. Lopes & S.P. Favaro, unpublished data). Macauba kernel offers oil in excellent nutritional quality, referenced as a source of fatty acids, such as oleic and lauric (César et al. 2015, Colombo et al. 2018).

With that in mind, the present work aimed to perform the hydrolysis of macauba kernel oil, in order to evaluate the effect of the substrates pre-emulsion step, the effect of process variables (percentage of catalyst and buffer solution), the kinetics of free fatty acids production (FFAs) and the catalyst reuse.

MATERIALS AND METHODS

Materials

Macauba kernel oil was purchased from Cocal Brasil, which chemical composition was reported by Raspe et al. (2013). Immobilized enzyme Lipozyme® RM IM was employed as catalyst, provided by LNF Latin American. Sodium phosphate buffer solution pH 8.2 (Neon) was prepared according to Gomori (1955) and added to the reactions; *n*-hexane (Anidrol) and isopropyl alcohol (Anidrol) were applied to the catalyst washing step. For sample titration, ethyl ether (Anidrol), ethyl alcohol (Anidrol, 95%), sodium hydroxide (Anidrol, 97%) and phenolphthalein (Nuclear) were used.

Enzymatic hydrolysis of macauba oil after ultrasound pre-emulsification

Pre-emulsion was conducted in an experimental apparatus consisting of an ultrasound bath of operating frequency of 37 kHz and maximum rated electrical power output of 132 W (Ultronique, Q5.9/40A), a mechanical agitator (IKA®, RW20) and a three-neck flat-bottom flask. The reaction mixture pre-emulsion was carried out at a temperature of 55 °C, applying the ultrasound maximum electrical power and a mechanical agitation rate of 700 rpm for 1 hour.

Experiments without the pre-emulsion step were carried out in order to investigate its effect on the FFAs production. Prior to the reaction, the enzyme was kept at 40 °C for 1 h for activation and, after the pre-emulsion step, was added to the reaction mixture. The reaction was then carried out employing a hot plate with magnetic agitator (IKA®, RCT) at a constant agitation rate of 400 rpm. Buffer solution pH 8.2 and temperature of 55 °C were determined in a previous work (Raspe et al. 2013).

Reaction time started with the catalyst addition to the reaction medium. At the end of the reaction time, the enzyme was removed by filtration, with successive washings using *n*-hexane. The filtrate was centrifuged (Quimis, Q222TM1) at 3500 rpm for 10 minutes and the solvent present in the supernatant was eliminated in an oven with air circulation (Marconi, MA035) at 80 °C.

The free fatty acids (FFAs) content was determined following the Ca 5a-40 method (AOCS 1990), which is based on acid-base titration using an ethanol solution, and potassium hydroxide (KOH) previously standardized as the titrant. Each sample was titrated in duplicate, and the FFA content was calculated from the Equation 1:

$$FFA\,(wt\,\%) = \frac{C \times MM \times v}{(10 \times m)} \qquad (1)$$

where C is the concentration of sodium hydroxide (mol L^{-1}) used as titrant, MM corresponds to the average molar mass of FFA of macauba kernel oil (Raspe et al. 2013), v is the volume required for the titration (ml) and m is the mass of sample (g).

Catalyst reuse

For enzyme (Lipozyme® RM IM) recycling studies, fixed conditions were established for the hydrolysis reaction: time of 6 hours, temperature of 55 °C, stirring rate of 400 rpm, 75% buffer solution (based on the oil mass), and enzyme concentration of 10% (based on the substrates mass). Enzyme was then recovered by washing it with isopropyl alcohol (approximately 110 ml) and separated using filter paper. After filtering, the enzyme was taken to an oven at 40 °C for 1 hour. Then, it was left in a desiccator for 24 hours. After that, the enzyme was reused.

Data analysis

To verify the influence of the parameters evaluated in each step on the results obtained, analysis of variance (ANOVA; Excel® 2010 software) and the Tukey test were carried out with a 95% confidence interval. The evaluation was performed from data obtained in triplicates, and the results were presented by the mean ± standard deviation.

RESULTS AND DISCUSSION

Ultrasound pre-emulsion effect

The effect of the reaction mixture pre-emulsion on the production of FFAs is shown in Figure 1, in which it is possible to observe that in only 1 hour of reaction a 40% increase in the FFA content was obtained in the hydrolysate, and 25% in 8 hours, when ultrasound was applied for the reaction mixture pre-emulsification. Ultrasound application promotes greater dispersion of oil in water, leading to a larger interfacial area and smaller droplet size, thus favoring the hydrolysis initial rate (Huang et al. 2010). Ultrasound treatment promotes an increase in the microscopic droplet formation speed, an increase in the contact surface and in the cohesive forces, resulting in the formation

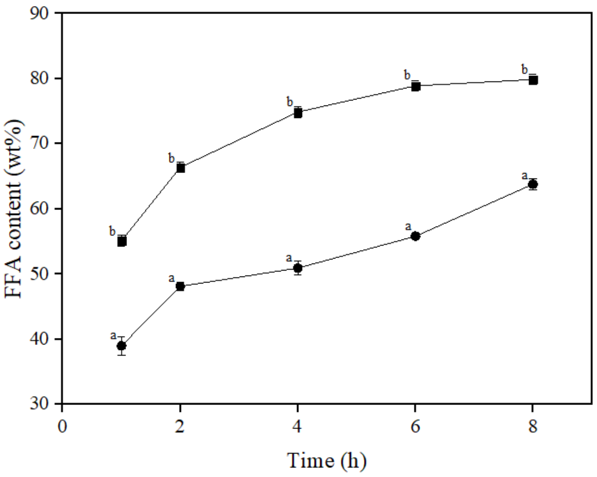

Figure 1. FFA content obtained in the reactions carried out at 55 °C, 5 wt% Lipozyme® RM IM, 50 wt% buffer solution, and stirring rate of 400 rpm —■— with substrates pre-emulsion and —●— without substrates pre-emulsion. Means followed by the same letter, for the same reaction time, do not statistically differ ($p<0.05$).

of microemulsions (Martines et al. 2000, Dal Prá et al. 2019).

Several authors argue that the use of ultrasound technology in chemical and biochemical reactions outlines advantages over conventional methods, including the reduction in reaction time, reduction in the amount of reagents added, and increased yields (Liu et al. 2008, Yu et al. 2010, Lerin et al. 2011, Feiten et al. 2014, Santin et al. 2014, 2017, Zenevicz et al. 2016, 2018, Coppini et al. 2019). It is well known in the literature that the enzymatic hydrolysis reaction takes place at the interface between oil and water (or buffer solution) (Pourzolfaghar et al. 2016). Therefore, the application of ultrasound promotes greater contact between the substrates molecules through their dissolution and homogeneity, allowing an increase in the interfacial area for the enzyme action (Bansode & Rathod 2017), enabling an increase in the hydrolysis rate. Higher yields, promoted by ultrasonic technology, have been attributed to its emulsification capacity, provided by the cavitations generated in the system (Waghmare & Rathod 2016). These findings were evidenced by Feiten et al. (2014) when investigating the soybean oil hydrolysis catalyzed by Lipozyme® TL IM in the presence and absence of ultrasound, showing a 50% reduction in the process time by means of ultrasonic irradiation, compared to simple mechanical agitation.

Effect of enzyme loading

Figure 2 presents the effect of the catalyst percentage evaluated using 5, 10 and 15 wt% of enzyme (based on the substrates mass) in the pre-emulsified reaction mixture. It can be seen in Figure 2 that the increase in the catalyst percentage from 5 to 10 wt% led to an increase in the FFA production during the evaluated reaction time (10 h). However, when the percentage of catalyst increased from 10 to 15 wt%, there was no increase in the process yield at most of the time, assuming that the use of excessive enzyme concentration in the reaction medium promoted saturation at the substrate interface, directly affecting the reaction rate. Rusli et al.

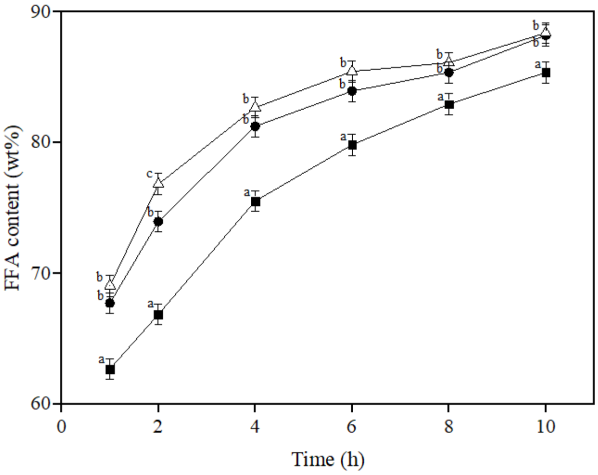

Figure 2. Effect of catalyst percentage (—■— 5 wt%, —●— 10 wt% and —△— 15 wt% based on the substrates mass) on the FFA content (reactions performed at 55 °C, 50 wt% buffer solution, and stirring rate of 400 rpm). Means followed by the same letter, for the same reaction time, do not statistically differ ($p<0.05$).

(2020) explain that once saturation has been reached and the interface is completely covered by enzyme molecules, additional enzymes do not have access to the substrates and, therefore, there is no increase in the reaction rate. In addition, the feasibility of the process in high amounts of catalyst becomes questionable (Waghmare & Rathod 2016).

Similar to this work, Chen et al. (2014) found that the increase in the concentration of *Candida rugosa* catalyst in the hydrolysis of linseed oil system did not influence the production of FFAs, reporting hydrolysis ratio of linseed oil in the order of ~95% with sufficient 1.0 wt% of catalyst. Using the Lipozyme® TL, Mello et al. (2015) reported that an increase in the catalyst percentage from 7.5 to 15 wt% did not increase the hydrolysis yield of crambe oil during 4 hours of reaction. Likewise, addition from 1.25 to 1.50 wt% in the concentration of Novozyme® 435 catalyst in the hydrolysis of residual frying oil, investigated by Waghmare & Rathod (2016), had no influence on the yield of ~75% of FFAs, after 180 min of reaction. When investigating the hydrolysis of virgin coconut oil, Nguyen et al. (2017) found that increasing the concentration of *Candida rugosa* catalyst from 1.5 to 2.5 wt% did not increase the ~60% of FFAs produced in the reaction.

It is noteworthy to point out that the use of ultrasonic irradiation influences the reactions catalyzed by enzymes, due to the intensification promoted in the substrates contact, because of the cavitation process (Bansode & Rathod 2017). This allows the enzymatic load to be used in the reaction not to be excessive, due to the fact that the mechanical shock waves and liquid jets of the process renew the catalyst contact surface, increasing the substrates diffusion to the enzyme active sites (Waghmare & Rathod 2016). As a result, high reaction yields at low catalyst concentration and without loss to the protein structure are verified (Zenevicz et al. 2016, Feiten et al. 2016), in addition to the possibility of its reuse (Facin et al. 2019).

Effect of buffer percentage

Figure 3 shows the FFA content as a function of the buffer solution percentage of 25, 50, 75, and 100 wt% (based on the substrates mass) investigated in the hydrolysis reaction. The buffer solution addition in the reaction mixture, 25 wt% to 75 wt%, favored an increase in the FFAs formation of ~17% in 2 and 6 h of reaction. However, the increase in the buffer solution concentration from 75 to 100 wt% does not seem to have influenced the process. In such reaction system, water (or buffer solution) acts helping to increase the enzyme's catalytic activity and contributes to the hydrolysis process as a reagent (Waghmare & Rathod 2016). On one hand, when an excess of buffer solution is added to the system, the thickness of the substrates layer formed around the enzyme can increase, causing reagents and products with low solubility in aqueous media to diffuse with

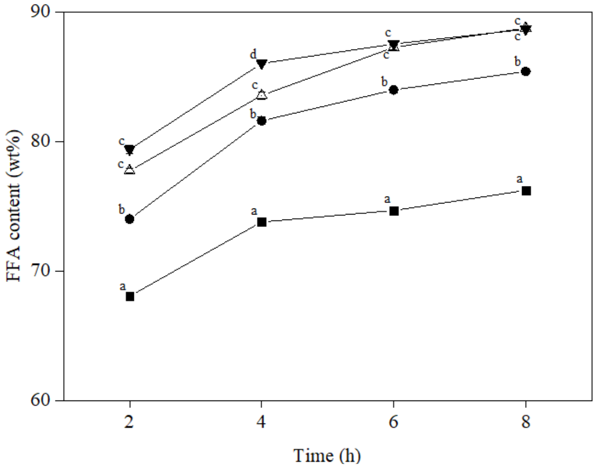

Figure 3. Effect of the buffer solution percentage (─■─ 25 wt%, ─●─ 50 wt%, ─△─ 75 wt% and ─▼─ 100 wt% based on the oil mass) on the FFA content (hydrolysis carried out at 55 °C, stirring rate of 400 rpm, and 10 wt% Lipozyme® RM IM). Means followed by the same letter, for the same reaction time, do not statistically differ (p<0.05).

difficulty to the active site of the enzyme (Yadav & Devi 2004). On the other hand, at low buffer concentrations, excess of oil in the system increases the viscosity of the reaction medium, leading to mass transfer limitations (Awadallak et al. 2013), also causing the catalyst surface coating, making it unavailable to react with the substrates (Waghmare & Rathod 2016).

Within the proposed process (in the range of variables evaluated), 75 wt% of buffer solution was the most suitable in the reaction medium. For Matuoog et al. (2017), the excess of water (70 wt%) in the fish oil hydrolysis reaction medium, catalyzed by lipase from *Thermomyces lanuginosus*, promoted lower yields than the percentage of 50% (based on the mass of oil), which resulted in a maximum of ~40% FFAs. Pongket et al. (2015) highlight that the relationship between oil and buffer is one of the parameters that most affect the hydrolysis degree, as it promotes limitations to the substrates interfacial area and ionization of the protein structure, reflecting on the enzyme catalytic capacity. In the hydrolysis of mustard oil catalyzed by porcine pancreas lipase, Goswami et al. (2012) found that when an excess of water is added to the process, a drastic reduction (over 100%) in yields is verified.

It is also noteworthy to point out that environmental conditions, such as pH and ionic strength (salt ions), affect the enzyme activity by their interaction with the enzyme residues or the support of immobilized enzymes (Lima et al. 2001, Feiten et al. 2016). Low salt concentrations may promote ions binding to some residues, improving the enzyme catalytic activity and stability in aqueous solutions by stabilizing its structure, effect so-called salting-in. Nevertheless, during the salting-out phenomenon, high salt concentrations may negatively affect the enzyme's activity, blocking the enzyme active site to access the substrates, or destabilizing the charged groups on its structure to the point that enzymes solubility substantially decreases (Dixon & Webb 1979, W.F. Azevedo Junior, unpublished data). Hence, addition of 25 wt% to 75 wt% of sodium phosphate buffer may have stabilized the catalyst in solution, favoring an improvement on its catalytic activity, whereas the increase from 75 to 100 wt% does not seem to have affected its behavior.

Enzyme recycle effect

The reuse of immobilized enzymatic catalyst is one of the factors that most attracts its applicability, being determined by its stability and maintenance of catalytic activity (Souza et al. 2020). Therefore, in this study, enzyme recycling was investigated under fixed reaction conditions: 75 wt% buffer solution (based on the oil mass), 10 wt% Lipozyme® RM IM catalyst (based on the substrates mass), for 6 hours at 55°C and mechanical agitation rate of 400 rpm which was evaluated for 5 cycles (Figure 4). It is possible to verify in Figure 4 that the catalyst maintained 85% of its initial activity after 30 h of use.

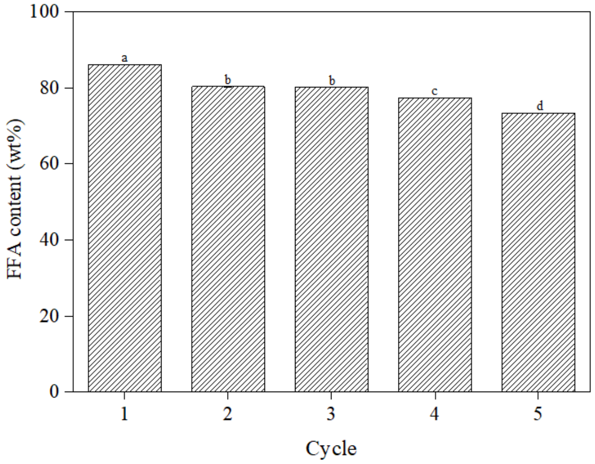

Figure 4. Effect of Lipozyme® RM IM recycling, in 6 hours of reaction, temperature of 55 °C, stirring rate of 400 rpm, 75% buffer solution (based on the oil mass), and an enzyme concentration of 10% (based on the substrates mass).

The catalytic activity maintenance after successive reuses suggests that the process follows the principles of green chemistry and sustainable development, enabling the catalyst reuse and providing better process performance and commercial viability (Sheldon & Woodley 2018), highlighting the relevance of its investigation. Hence, Phuah et al. (2016), when investigating the operational stability of the Lipozyme® RM IM catalyst in the partial hydrolysis of palm oil, verified the total maintenance of the catalytic activity for 10 consecutive cycles of 1 hour each. When evaluating the reuse of Lipozyme® RM IM catalyst combined with Novozym® 435 (in proportion of 80 and 20%, respectively), Alves et al. (2014) verified maintenance of about ~90% of the catalysts initial activity after 15 reuse steps of 4 hours each.

It is noteworthy that the catalytic activity loss of ~15% of Lipozyme® RM IM in this work may be attributed to the desorption of the enzyme from the support, linked to inactivation through its reuse (Kabbashi et al. 2015). Some reports highlight that Lipozyme® RM IM has low operational stability due to fractures in the acrylic resin in which it is immobilized, which may expose the enzyme structure to denaturation and conformational changes (Souza et al. 2020). Such evidence was verified in Souza et al. (2020) work, in which a ~50% reduction on the catalytic activity of a lipases cocktail (75% Lipozyme® RM IM and 25% Novozym® 435) was reported after 4 consecutive reactions of 3 hours each in the hydrolysis of coconut oil applying an ultrasound bath at low and high power (37 kHz and 300 W, respectively).

CONCLUSIONS

Hydrolysis of vegetable oils is the main reaction to produce FFAs. In this study, application of ultrasonic irradiation as a previous step for the substrates emulsification favored the FFAs production, pointing the promising use of an eco-friendly, green technique to overcome mass transfer limitations in water–oil mixtures. After substrates emulsification under ultrasound, great conversions where found in MKO hydrolysis, up to ~89 wt% of FFAs, when utilizing 10 wt% Lipozyme® RM IM, in 10 hours of reaction. The catalyst maintained 85% of its initial activity after 30 h of use. Therefore, ultrasonic irradiation is an interesting strategy to enhance the substrates miscibility prior to the reaction itself and, consequently, increase hydrolysis yields of vegetable oils compared to simple mechanical agitation.

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