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ORIGINAL PAPER

Production of alkyl esters from macaw palm oil by a sequential hydrolysis/esterification process using heterogeneous biocatalysts: optimization by response surface methodology

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Abstract The present study deals with the enzymatic synthesis of alkyl esters with emollient properties by a sequential hydrolysis/esterification process (hydroesterification) using unrefined macaw palm oil from pulp seeds (MPPO) as feedstock. Crude enzymatic extract from dormant castor bean seeds was used as biocatalyst in the production of free fatty acids (FFA) by hydrolysis of MPPO. Esterification of purified FFA with several alcohols in heptane medium was catalyzed by immobilized *Thermomyces lanuginosus* lipase (TLL) on poly-hydroxybutyrate (PHB) particles. Under optimal experimental conditions (mass ratio oil:buffer of 35 % m/m, reaction temperature of 35 °C, biocatalyst concentration of 6 % m/m, and stirring speed of 1,000 rpm), complete hydrolysis of MPPO was reached after 110 min of reaction. Maximum ester conversion percentage of 92.4 ± 0.4 % was reached using hexanol as acyl acceptor at 750 mM of each reactant after 15 min of reaction. The biocatalyst retained full activity after eight successive cycles of esterification reaction. These results show that the proposed process is a promising strategy for the synthesis of alkyl esters of industrial interest from macaw palm oil, an attractive option for the Brazilian oleochemical industry.

Keywords Alkyl esters · Hydroesterification · Macaw palm oil · Heterogeneous biocatalysts

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Introduction

Alkyl esters are valuable compounds which have many applications from the industrial point of view. In recent years, these compounds have gained international attention as a source of alternative fuels (biodiesel) and additives in food, detergent, cosmetic, and pharmaceutical industries due to their emollient properties [1–7]. Moreover, alkyl esters are also used as low temperature plasticizers for polyvinyl chloride, vinyl chloride, copolymers, polystyrene, ethyl cellulose, and synthetic rubber, and also in the manufacture of water-resistant lubricants or as solvents [7]. These compounds have been preferentially synthesized by transesterification reactions of triacylglycerols with several alcohols using homogeneous alkali catalysts such as hydroxides and alkoxides (potassium and sodium) [3, 6, 8]. Currently, free fatty acids (FFA) from unrefined feedstocks react with alkali catalysts to form soaps and water molecules that can accelerate the hydrolysis of triacylglycerols to mono- and diacylglycerols [4, 6]. Thus, feedstocks with low FFA content are required to minimize possible emulsification and separation problems. Acid catalysts are important alternative to the traditional alkali catalysts because they allow the application of unrefined feedstocks to produce alkyl esters. The reactions are normally carried out in a homogeneous phase in the presence of several acids such as sulfuric, sulfonic, chloride, and phosphoric. However, large amounts of alcohols and complete water molecules removal from the reaction medium are required [3, 6].

To overcome these problems, some strategies have been proposed for the synthesis of alkyl esters of industrial interest from triacylglycerols with high FFA content such as application of heterogeneous catalysts to mediate simultaneous esterification/transesterification reactions [6], and sequential hydrolysis/esterification process, so-called

hydroesterification [9–15]. The latter process involves two steps to produce alkyl esters—complete hydrolysis of triacylglycerols to FFA and glycerol, followed by purification of FFA from the hydrolysis step and esterification reaction with several alcohols. In this process, unrefined triacylglycerols from several sources can be used as feedstocks [13–15], and a short-time reaction can be obtained because esterification reaction proceeds faster than transesterification reaction, mainly for those reactions catalyzed by lipases [16–18]. Since the first step, the water content and the FFA content of the feedstock do not interfere in the production of FFA by hydrolysis reaction. Additionally, the aqueous glycerol produced presents higher purity than that obtained from transesterification reaction catalyzed by alkali catalysts [9–13]. Moreover, economic analysis performed in previous study reported in the literature shows that the hydroesterification can be as attractive as or more so than the transesterification process mediated by alkali homogeneous catalysts for the synthesis of alkyl esters (biodiesel) [11]. Different catalysts have been used to mediate alkyl esters synthesis by sequential hydrolysis/esterification process, including lipases from several sources. The production of FFA by hydrolysis step has been preferentially performed by homogeneous biocatalysts, followed by esterification catalyzed by several heterogeneous catalysts (chemical and biochemical catalysts) [11, 13, 15]. However, the production of alkyl esters using heterogeneous biocatalysts in both hydrolysis and esterification steps still is few reported by the literature [10].

The goal of this work was to produce alkyl esters with emollient properties by a sequential hydrolysis/esterification process using acid vegetable oil from macaw palm, so-called macauba (*Acrocomia aculeata*), a native oleaginous palm tree typically Brazilian. This palm has a potential to produce up to 30 ton of fruits/hectare year, presenting oil content between 23 and 34 % on dry mass basis [19]. Response surface methodology, a collection of mathematical and statistical techniques, was used to evaluate the relative significance of the affecting variables and to indicate the parameters for an optimized process of FFA production by hydrolysis of MPPO catalyzed by crude enzymatic extract from dormant castor bean seeds. After hydrolysis optimization step, esterification reaction with purified FFA and linear and branched-chain alcohols (isoamyl alcohol, hexanol, and 2-ethyl-hexanol) at different concentrations in heptane medium was performed. In this study, the experimental conditions to produce alkyl esters by esterification reaction were previously optimized in our lab using immobilized TLL by physical adsorption on PHB particles as biocatalyst [20]. Finally, reusability tests after eight successive cycles of esterification reaction in batch systems for the selected acyl acceptor were also performed. Recently, Aguieiras et al. [10] also reported the enzymatic synthesis of alkyl esters (ethyl esters–biodiesel) from acid MPPO by sequential hydrolysis/esterification process mediated by low-cost heterogeneous biocatalysts. In the present study, it was proposed a similar process for the synthesis of alkyl esters of industrial interest using MPPO as feedstock. However, we reported the synthesis of alkyl esters with emollient properties (hexyl esters), which has not been reported by the literature yet.

Materials and methods

Materials

Castor bean seeds were purchased from BRSeeds Ltd. (Araçatuba, SP, Brazil). Enzymatic extract from *Thermomyces lanuginosus* lipase (TLL) was supplied by Sigma-Aldrich Co. (St. Louis, MO, USA) and used without further treatment (Lot# 051M1810 V). TLL is a liquid preparation with specific activity of 220 IU/mg of protein and 18.0 mg protein/mL of lipase solution [20]. Mesoporous PHB particles (average particle diameter of 75–90 μm, surface area of 17.1 m^2/g and porous diameter of 3.1 nm) were acquired from PHB Industrial (São Paulo, Brazil). Macaw palm oil from pulp seeds (MPPO), with acid value of 9.3 ± 1.9 mg KOH per gram of oil, was purchased from Paradigma Óleos Vegetais (Carmo do Parnaíba, MG, Brazil). The fatty acid composition (% m/m) was as follows: 17.6 % palmitic, 4.0 % palmitoleic, 2.0 % stearic, 58.6 % oleic, 16.0 % linoleic, and 1.0 % linolenic with 865.1 g/mol average molecular mass. 2-ethyl-hexanol (purity ≥ 99.6 %) and hexanol (purity ≥ 99.0 %) were acquired from Sigma-Aldrich Co. Isoamyl alcohol (purity ≥ 99.5 %) was purchased from Synth® (São Paulo, SP, Brazil). All other chemical reagents such as heptane (purity ≥ 99.0 %), acetone (purity ≥ 99.5 %), sodium hydroxide (purity ≥ 99.0 %), acetic acid (purity ≥ 99.0 %), and sodium acetate (purity ≥ 99.0 %) were supplied by Synth® and Vetec Química Ltda (São Paulo, SP, Brazil).

Preparation of crude enzymatic extract from castor bean seeds

Endosperm tissues from dormant castor bean seeds were carefully removed and the shells of the seedling discarded. The endosperms (20 g) were then triturated in a knife-mill during 10 min by adding chilled acetone (5 mL). After, the samples were mixed with chilled acetone (ratio 1:5 m/v) under stirring at 150 rpm and 4 °C, according methodologies previously described [21, 22]. Then, the suspension was filtered under vacuum via a Buchner funnel and washed with chilled acetone in excess. The delipidated

extract prepared was sieved to obtain average particles size of 75–90 μm. The product was defined as crude enzymatic extract and used to catalyze the hydrolysis of MPPO.

Immobilization of TLL by physical adsorption on PHB particles

The immobilization of TLL on wet PHB particles was performed in 5 mM buffer sodium phosphate pH 7.0 on a proportion 1:20 m/v (support:enzyme solution) by offering 40 mg protein/g of support [20]. The suspension was kept under continuous stirring in an orbital shaker (200 rpm) by 12 h at 25 °C, followed by filtration (Whatman filter paper 41) and washing with Milli-Q water. The biocatalyst prepared presented immobilized protein amount and hydrolytic activity of 24.7 ± 1.1 mg/g and 1,240 ± 30 IU/g of PHB particles, respectively [20].

Hydrolysis of MPPO in stirred-tank reactors

Hydrolysis reaction of MPPO was carried out in stirred-tank reactors (plastic flasks of 350 mL) containing 50 g of substrate at 100 mM buffer sodium acetate pH 4.5 under continuous stirring (1,000 rpm) [21, 22]. Samples (1 g) were periodically removed from the reaction medium via a syringe, weighed and transferred to 125 mL conical flasks. Ten milliliters of ethanol were then added to the sample to denature the enzyme, thus effectively freezing the reaction. Phenolphthalein indicator was added and the mixture titrated with NaOH solution (20 mM). The hydrolysis percentage was calculated using the Eq. 1 [23].

$$\text{Hydrolysis}\ (\%) = \left(\frac{V \times 10^{-3} \times M \times \text{MM}_{\text{FA}}}{\text{Wt} \times f}\right) \times 100 \quad (1)$$

where V is the volume of NaOH solution required during titration (mL), M is the NaOH solution concentration (20 mM), MM_{FA} is the average molecular mass of fatty acids from MPPO (285.1 g/mol), Wt is the mass of the sample taken (g) and f is the fraction of oil at start of reaction (% m/m).

Optimization of enzymatic hydrolysis of MPPO by response surface methodology

A 2^3 full factorial design with four assays at the center point was used to evaluate the reaction parameters on the enzymatic hydrolysis of MPPO in an emulsifier-free system catalyzed by crude enzymatic extract from dormant castor bean seeds. Star points were added to the experimental design to compose a second-order model. Eighteen experiments were performed in triplicate in a random order. The levels of each independent variable were chosen based on the importance of the experiments. The parameters were mass ratio oil:buffer (x_1), reaction temperature (x_2) and biocatalyst concentration (x_3). The coded and corresponding uncoded values are given in Table 1. The hydrolysis percentage was taken as response of the factorial design. The results were analyzed using software Statistica version 7.0 (StatSoft Inc., Tulsa, OK, USA) to fit the following second-order polynomial, as shown in Eq. 2.

$$Y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} x_i x_j + \varepsilon \quad (2)$$

where Y is the response variable; b_0, b_i, b_{ii}, and b_{ij} are the constant coefficients, x_i and x_j are the uncoded independent variables and ε is the random error.

To validate the optimization of FFA production in stirred-tank reactor, enzymatic hydrolysis of MPPO was performed in triplicate under optimal experimental conditions to confirm the results from the analysis of the response surfaces.

Hydrolysis reaction of MPPO under optimal conditions

Under optimal conditions defined in our study, it was verified the effect of stirring speed varying from 100 to 1,500 rpm and addition of calcium chloride at different concentrations (0–10 mM) on the hydrolysis percentage of MPPO by 30 min of reaction in stirred-tank reactors. Finally, hydrolysis reactions at different mass ratio oil:buffer varying from 35 to 46.8 % m/m was also carried out in triplicate by a maximum period of 120 min to verify the reaction time required to reach maximum hydrolysis percentage. Samples from the reaction medium were then removed at different times and determined the hydrolysis percentage, according to Eq. 1.

Separation of FFA from enzymatic hydrolysis of MPPO

An excess of hexane (volume ratio 1:2) was added to the reaction medium after complete hydrolysis of MPPO. The mixture was centrifuged at 5,000 rpm and 25 °C by 20 min. The superior phase, consisting of hexane and FFA, was separated from the aqueous phase (glycerol, biocatalyst, and buffer solution) at the bottom. The organic phase was then filtered under vacuum via a Buchner funnel and hexane was removed by a rotatory evaporator. Finally, anhydrous sodium sulfate was added to the purified FFA to remove any trace of water molecules.

Table 1 Matrix of factorial design used to investigate the influence of the independent variables on the hydrolysis percentage of MPPO in stirred-tank reactor catalyzed by crude enzymatic extract from dormant castor bean seeds

Assays	Coded (actual) variables			Hydrolysis (%)	
	Mass ratio oil:buffer (% m/m)	Reaction temperature (°C)	Biocatalyst concentration (% m/m)	Experimental	Predicted
1	−1 (20)	−1 (30)	−1 (3)	21.4 ± 1.3	21.2
2	+1 (40)	−1 (30)	−1 (3)	30.3 ± 2.1	26.9
3	−1 (20)	+1 (45)	−1 (3)	17.5 ± 0.6	15.0
4	+1 (40)	+1 (45)	−1 (3)	23.1 ± 1.5	20.7
5	−1 (20)	−1 (30)	+1 (7)	33.8 ± 3.5	30.3
6	+1 (40)	−1 (30)	+1 (7)	36.1 ± 0.5	36.1
7	−1 (20)	+1 (45)	+1 (7)	21.9 ± 1.3	24.1
8	+1 (40)	+1 (45)	+1 (7)	32.1 ± 2.6	29.9
9	−1.68 (13.2)	0 (37.5)	0 (5)	32.9 ± 1.5	32.3
10	+1.68 (46.8)	0 (37.5)	0 (5)	40.1 ± 1.9	42.0
11	0 (30)	−1.68 (24.9)	0 (5)	20.7 ± 1.1	23.5
12	0 (30)	+1.68 (50.1)	0 (5)	11.6 ± 0.8	13.1
13	0 (30)	0 (37.5)	−1.68 (1.64)	11.7 ± 1.1	15.4
14	0 (30)	0 (37.5)	+1.68 (8.36)	30.2 ± 0.9	30.8
15	0 (30)	0 (37.5)	0 (5)	37.2 ± 1.1	37.2
16	0 (30)	0 (37.5)	0 (5)	37.4 ± 0.7	37.2
17	0 (30)	0 (37.5)	0 (5)	36.5 ± 0.8	37.2
18	0 (30)	0 (37.5)	0 (5)	35.3 ± 2.6	37.2

The reactions were performed at 100 mM buffer sodium acetate pH 4.5 by 30 min of reaction under continuous stirring (1,000 rpm)

Alkyl esters synthesis by esterification reaction in heptane medium

Esterification of purified FFA with linear and branched-chain alcohols (isoamyl alcohol, hexanol, and 2-ethyl-hexanol) at equimolar ratio alcohol:FFA and different concentrations of each reactant in heptane medium was carried out according methodology previously described by Miranda et al. [20], with slight modifications. The reaction medium (10 mL) was incubated with 1.6 g of immobilized TLL (16 % m/v) at 32.5 °C by a maximum period of 30 min under continuous stirring in an orbital shaker at 200 rpm. The ester conversion was quantified by measurements of the concentration of residual fatty acids in the reaction medium [20, 24]. Samples were withdrawn (0.50 mL), diluted in 10 mL of an ethanol/acetone 1:1 (v/v) mixture and titrated with NaOH solution (20 mM) using phenolphthalein as indicator. All the experiments were carried out in triplicate. The control experiment (reactants incubated in the presence of PHB particles) was performed and no conversion was observed after 30 min of incubation.

Reusability tests in hexyl esters synthesis

Reusability tests of immobilized TLL in successive esterification reactions of purified FFA with hexanol at 750 mM of each reactant in heptane medium were performed. At the end of each cycle (eight batch reactions of 15 min each), the immobilized TLL was removed from the reaction medium and washed with ice heptane in excess to remove any reactant and product retained in the biocatalyst microenvironment. After this, the biocatalyst was introduced into a fresh medium. The reaction was periodically monitored by assessing the ester conversion percentage [20, 24].

Results and discussion

Optimization of the enzymatic hydrolysis of MPPO

The production of FFA from macaw palm oil using crude enzymatic extract from dormant castor bean seeds as biocatalyst was performed. The choice of the biocatalyst was based on previous studies carried in our lab aiming the production of FFA from several vegetable oils [21, 22]. The reactions were conducted in emulsifier-free systems, thus a vigorous mechanical stirring (1,000 rpm) was required to allow a good homogeneity in the reaction medium and accessibility of reactant molecules to the biocatalyst microenvironment. The reactions were performed in 100 mM buffer sodium acetate pH 4.5. Under these conditions, the selected biocatalyst exhibits maximum hydrolytic activity [22].

In the present study, it was proposed a full factorial design to find the variables of the process that have a

Table 2 Estimated coefficients, standard errors, and p values for the enzymatic hydrolysis of MPPO obtained by factorial design

Variable	Coefficients	Standard error	p
Mean	37.16	±0.38	0.00002
x_1	2.86	±0.26	0.00154
x_2	−3.10	±0.26	0.00122
x_2^2	−6.67	±0.26	0.00013
x_3	4.59	±0.26	0.00038
x_3^2	−4.97	±0.26	0.00032

Table 3 Analysis of variance (ANOVA) for the model that represents the enzymatic hydrolysis of MPPO

Source	Sum of squares	Degree of freedom	Mean square	F-value	p (prob $<F$)
Regression	1318.64	5	263.73	36.59	7.35×10^{-7}
Residues	77.61	12	6.47		
Lack of fit	74.91				
Pure error	2.70				
Corr. total	1,396.25	17			

$R^2 = 94.44$ %, $F_{5,12,0.05} = 3.11$

significant influence on the enzymatic hydrolysis of MPPO, and to find the setting values of the principal parameters for setting the results of the process on the desired values. Table 1 shows the independent variables, levels, and factorial design in terms of coded and actual, together with the experimental and predicted results. The hydrolysis percentage varied from 11.5 % (assays 12 and 13) to 40 % (assay 10) after 30 min of reaction. The results from Table 1 revealed also good correspondence between predicted and experimental values, implying that the empirical model derived from the factorial design can be used to adequately describe the relationship between the factors and response (hydrolysis percentage). These results were used to estimate the main variable effects and their interactions (Table 2). The statistical analysis showed significant linear effects for mass ratio oil:buffer (x_1), reaction temperature (x_2), and biocatalyst concentration (x_3) and quadratic effects for reaction temperature (x_2^2) and biocatalyst concentration (x_3^2) at 95 % of confidence level. From these results, statistical model could be composed with the coefficients correspondent to the significant effects. The coefficients related to non-significant effects were excluded from the model. The best fitting response function can be described by the Eq. 3.

$$\text{Hydrolysis}\ (\%) = 37.16 + 2.86x_1 - 3.10x_2 - 6.67x_2^2 + 4.59x_3 - 4.97x_3^2 \quad (3)$$

where x_1, x_2, and x_3 represent the coded values for mass ratio oil:buffer, reaction temperature, and biocatalyst concentration, respectively.

The analysis of variance (ANOVA), presented in Table 3, indicated that the second-order polynomial model was an adequate representation of the actual relationship between the response and the significant variables, with very small p value (<0.05) and satisfactory coefficient of determination ($R^2 = 94.44$ %). Thus, the quadratic polynomial model given by the Eq. 3 can be used to predict the hydrolysis reaction, create and explore the response surfaces and find the optimum experimental conditions of hydrolysis reaction.

To study the effects of the three variables as well as their interactions on the enzymatic hydrolysis of MPPO, response surfaces and contour plots were generated using software Statistica version 7.0, as shown in Fig. 1a–c. Figure 1a shows the interaction between mass ratio oil:-buffer and reaction temperature and their effects on the hydrolysis percentage. The enzymatic hydrolysis of MPPO was improved with the increase of the temperature up to 39 °C and drastic reduction was observed after 45 °C due to inactivation of enzyme molecules. Optimum reaction temperature of hydrolysis reaction was around 33–39 °C. The increase of mass ratio oil:buffer increased also the hydrolysis percentage and at highest mass ratio oil:buffer (46.8 % m/m) maximum hydrolysis percentage of MPPO was observed. The interaction between the variables was not significant at 95 % of confidence level.

The effect of biocatalyst concentration and mass ratio oil:buffer and their interaction on the hydrolysis of MPPO is shown in Fig. 1b. In accordance to Fig. 1a, maximum hydrolysis percentage was reached at highest mass ratio oil:buffer (46.8 % m/m). The increase on the hydrolysis percentage of MPPO by increasing the biocatalyst concentration from 3 to 6 % m/m was observed, as expected. However, biocatalyst concentration above 6 % m/m exhibited slight reduction on the hydrolysis percentage due to possible bad dispersion of the biocatalyst in the reaction medium [22]. It was possible to observe that maximum hydrolysis percentage may be reached at 6 % m/m of biocatalyst concentration. Similarly to Fig. 1a, the interaction between the variables was not also significant at 95 % of confidence level.

In Fig. 1c is showed the effect of the biocatalyst concentration and reaction temperature on the hydrolysis reaction. The response surface plot also shows that maximum hydrolysis percentage was reached between 33–39 °C and biocatalyst concentration at 6 % m/m, as previously shown in Fig. 1a, b. Similarly to Fig. 1a, b the interaction between the variables was not significant at 95 % of confidence level.

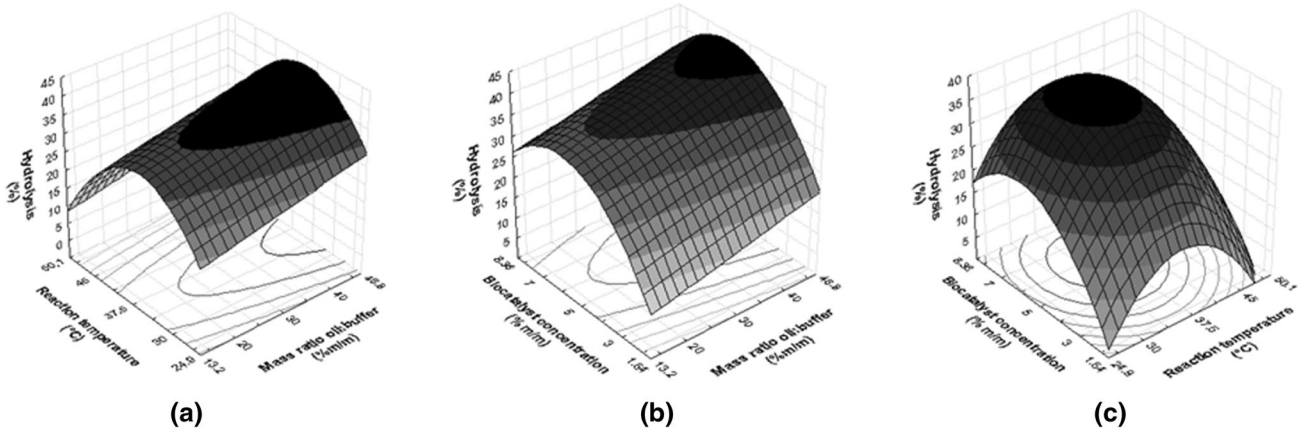

Fig. 1 Response surface plots of hydrolysis percentage of MPPO predicted from the quadratic model. Effect of the reaction temperature and mass ratio oil:buffer (**a**), mass ratio oil:buffer and biocatalyst concentration (**b**), and reaction temperature and biocatalyst concentration (**c**)

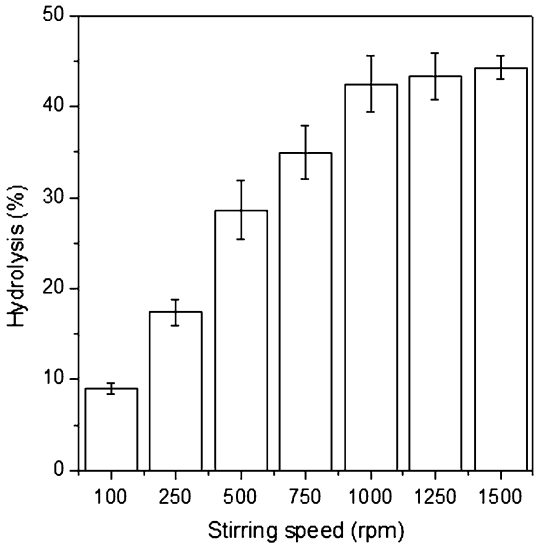

Fig. 2 Effect of stirring speed on the enzymatic hydrolysis of MPPO. The reactions were performed for 30 min of reaction at 46.8 % mass ratio oil:buffer, biocatalyst concentration of 6 % m/m, and 35 °C in stirred-tank reactors

The optimal conditions for the enzymatic hydrolysis of MPPO were as follow: mass ratio oil:buffer of 46.8 % m/m, reaction temperature of 35 °C, and biocatalyst concentration of 6 % m/m. Then, hydrolysis of MPPO in triplicate was performed under these conditions to verify the predicted value, determined by applying regression analysis (Eq. 3). The predicted value was 42.1 % and the experimental was 42.5 ± 3.1 % at 30 min of reaction. The results indicate that the generated model gave an adequate prediction of FFA production by enzymatic hydrolysis of MPPO.

The application of crude enzymatic extract from dormant castor bean seeds as biocatalyst is a promising strategy to produce FFA by hydrolysis of several triacylglycerols, including MPPO, because lipase molecules are "naturally" immobilized on solid materials from the seeds [22]. The reaction system studied is composed by a liquid phase, containing oil and buffer, and solid phase, containing crude enzymatic extract from dormant castor bean seeds. Thus, the kinetic reaction could be affected by diffusion limitations of reactant molecules from the reaction medium to the biocatalyst microenvironment. Thus, the effect of stirring speed on the hydrolysis reaction was also performed varying from 100 to 1,500 rpm, as shown in Fig. 2. The results show an increase on the hydrolysis percentage as the stirring speed is increased up to 1,000 rpm (hydrolysis percentage = 42.5 ± 3.1 % after 30 min of reaction). However, stirring speed above 1,000 rpm was not observed significant increase on the hydrolysis percentage. Based on these results, stirring speed of 1,000 rpm was enough to allow good diffusion of oil droplets from the liquid phase to the microenvironment of immobilized lipase molecules. This indicated that there was no mass transfer limitation above 1,000 rpm.

To verify the effects of external mass transfer and intraparticle diffusion resistances, theoretical calculations were performed according methodology previously described [16, 25, 26]. Initially, the diffusion coefficient (D_{AB}) was calculated by the Wilke–Chang correlation, as shown in Eq. 4 [20, 27]. In this study, it was assumed that the mass transfer process is controlled by the diffusion of MPPO molecules in the reaction medium.

$$D_{AB} = \frac{7.4 \times 10^{-8}(\phi M_B)^{0.5} T}{\eta_B V_A^{0.6}} \quad (4)$$

where D_{AB} is the diffusion coefficient of MPPO molecules (cm^2/s), ϕ is the association factor of the water ($\phi = 2.6$,

dimensionless), M_B is the molecular mass of the water (18 g/mol), T is the absolute reaction temperature (308 K), η_B is the dynamic viscosity of buffer solution (0.78 mPa s) and V_A is the molecular volume of MPPO at boiling point (1,160.2 cm^3/mol). The latter parameter was determined by the relation between molecular mass of MPPO (893.3 g/mol) and its specific mass at boiling temperature (0.77 g/cm^3 at 235 °C).

The diffusion coefficient of MPPO molecules was 2.90×10^{-6} cm^2/s. This parameter was used to determine the external mass transfer coefficient (k_{SL}) (Eq. 5) [16, 25–27].

$$t_d = \frac{D_{AB}}{(k_{SL})^2} \therefore k_{SL} = \frac{2D_{AB}}{d_p} \quad (5)$$

$$t_r = \frac{C_0}{r_0} \quad (6)$$

where t_d is the diffusion time (s), t_r is the reaction time (s), k_{SL} is the solid–liquid mass transfer coefficient (cm/s), d_p is the average diameter of particle of the biocatalyst (82.5×10^{-4} cm), C_0 is the initial reactant concentration in the reaction medium (mM) and r_0 the initial rate of reaction (mM/s).

The value of solid–liquid mass transfer coefficient (k_{SL}) was calculated as 7.03×10^{-4} cm/s. To determine the reaction time (t_r) using the Eq. 6, both C_0 and r_0 were experimentally determined and their values were 524 mM and 0.108 mM/s, respectively. Based on these results, the diffusion time ($t_d = 5.87$ s) value was strongly lower than reaction time ($t_r = 4.86 \times 10^3$ s), showing that the reaction was not controlled by external mass transfer effect.

Subsequently, a comparison between the rate of substrate diffusion per unit interfacial area ($k_{SL}C_0$) and the reaction rate per unit area ($\varphi r_0/a$) was also performed [16, 25–27]. φ is defined as the phase volume ratio and a is the interfacial area per volume of reaction medium. Assuming that crude enzymatic extract from dormant castor bean seeds has spherical shape, then $\varphi/a = R_p/3$, where R_p is the average radius of the particle of biocatalyst (41.25×10^{-4} cm). The rate of substrate diffusion per unit interfacial area ($k_{SL}C_0$) was calculated as 3.68×10^{-7} mol/cm^2.s and the reaction rate per unit area ($\varphi r_0/a$) was 4.45×10^{-10} mol/cm^2 s. It is clear that the rate of substrate diffusion per unit area was strongly higher than the reaction rate per unit area. These results show that the enzymatic hydrolysis of MPPO performed under vigorous stirring (1,000 rpm) was not controlled by diffusion effects, as above described (Fig. 2).

In another study, it was verified the influence of calcium ions (calcium chloride) varying from 0 to 10 mM on the hydrolysis percentage of MPPO (Fig. 3). The addition of calcium ions in enzymatic hydrolysis reactions of triacylglycerols from several sources has been widely reported due to its stimulatory effect on the catalytic activity of lipases [28, 29], and the formation of insoluble Ca-salts of the FFA released during hydrolysis reaction, which avoids possible product inhibition [28]. According to Fig. 3, no influence of calcium ions on the hydrolysis percentage of MPPO was observed. These results are in accordance with previous study reported by Su et al. [30] that also verified no influence of calcium ions on the hydrolytic activity of purified lipase from several Chinese castor bean varieties.

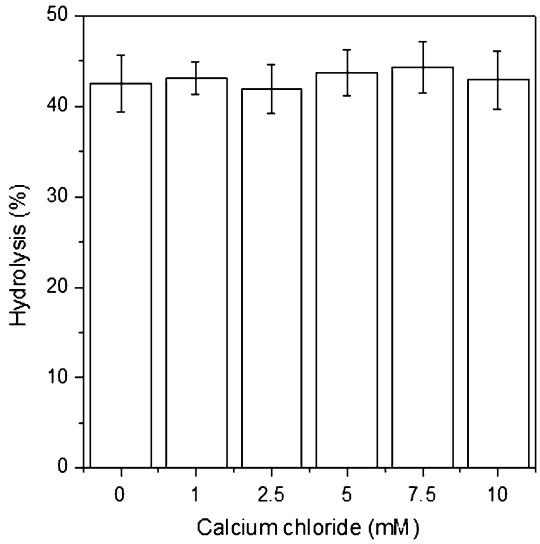

Fig. 3 Effect of calcium chloride concentration on the enzymatic hydrolysis of MPPO. The reactions were performed for 30 min of reaction at 46.8 % mass ratio oil:buffer, biocatalyst concentration of 6 % m/m, and 35 °C in stirred-tank reactors under continuous stirring

Under optimal conditions (reaction temperature of 35 °C, biocatalyst concentration of 6 % m/m and stirring speed of 1,000 rpm), enzymatic hydrolysis of MPPO at different mass ratio oil:buffer varying from 35 to 46.8 % m/m was performed by a maximum period of 120 min, as shown in Fig. 4. At highest mass ratio oil:buffer (46.8 % m/m), previously selected by response surface methodology (see Fig. 1a–c), maximum hydrolysis percentage was 74.1 ± 1.5 % after 100 min of reaction. Thus, hydrolysis reactions at lowest mass ratio oil:buffer were then performed aiming complete hydrolysis of MPPO. From results shown in Fig. 4, it was possible verify that at 40 % m/m a maximum hydrolysis percentage of 87.0 ± 1.8 % after 120 min of reaction was observed. However, complete hydrolysis of MPPO was reached after 110 min of reaction performed at 35 % m/m oil:buffer. This decrease of hydrolysis percentage by increasing MPPO concentration is due to the increase of FFA concentration in the interface oil/water that leads to changes of the ionization state of the enzyme, thus affecting its catalytic activity [21]. Based on these results, enzymatic

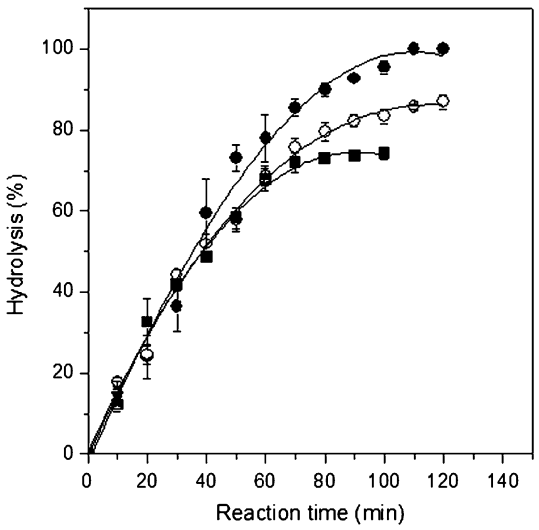

Fig. 4 Kinetic profiles of hydrolysis of MPPO catalyzed by enzymatic extract from dormant castor bean seeds at 35 (*filled circle*), 40 (*unfilled circle*), and 46.8 % (*filled square*) mass ratio oil:buffer. The reactions were performed at 6 % m/m of biocatalyst and 35 °C in stirred-tank reactors under continuous stirring (1,000 rpm)

hydrolysis of MPPO may be successfully performed at 35 % m/m under experimental conditions previously optimized by response surface methodology.

Esterification reaction of FFA by immobilized TLL on PHB particles

Although crude enzymatic extract from dormant castor bean seeds has presented high catalytic activity in aqueous medium (hydrolysis reaction), preliminary studies carried out in our lab demonstrated its low activity to catalyze esterification and transesterification reactions in organic medium (data not shown). Thus, an alternative biocatalyst prepared by immobilizing TLL by physical adsorption on PHB particles was tested in alkyl esters synthesis by direct esterification in heptane medium. This biocatalyst exhibits high catalytic activity in esterification and transesterification reactions [20, 31]. In this study, alkyl esters synthesis by esterification reactions of purified FFA from the enzymatic hydrolysis of MPPO and several alcohols was performed at equimolar ratio alcohol:FFA, reaction temperature of 32.5 °C, biocatalyst concentration of 16 % m/v in an orbital shaker at 200 rpm by a maximum period of 30 min, as previously described by Miranda et al. [20]. As can be seen in Fig. 5, immobilized TLL exhibited a high specificity toward linear-alkyl alcohol (hexanol). Ester conversion percentage of FFA with isoamyl alcohol at different concentrations around 84 % was reached after 15 min of reaction, as shown in Fig. 5a. The reactions performed using 2-ethyl-hexanol as acyl acceptor at different concentrations exhibited maximum ester conversion percentage around 70 % after 30 min of reaction (Fig. 5b). However, esterification percentage above 90 % was reached after 15 min of reaction using hexanol, a linear-chain alcohol, as acyl acceptor at different reactants concentration, as shown in Fig. 5c. Maximum ester conversion of 92.4 ± 0.4 % was reached for the reaction performed at 750 mM of each reactant. At 1,000 mM, a decrease on the ester conversion percentage was observed due to possible substrate inhibition or acidification of the reaction medium [20, 24]. These results show that the biocatalyst prepared by physical adsorption of TLL on PHB particles, a highly hydrophobic, eco-friendly and low-cost support, is highly promising to catalyze esterification reactions at high reactant concentrations.

In this study, mass transfer effects were also determined for the esterification reaction of purified FFA and hexanol in heptane medium ($\phi = 1$) catalyzed by immobilized TLL on PHB particles. The diffusion coefficient of FFA molecules in heptane (D_{AB}) at 32.5 °C was calculated as 1.42×10^{-5} cm^2/s using the Eq. 4. The value of solid–liquid mass transfer coefficient (k_{SL}) was 3.44×10^{-3} cm/s (Eq. 5). From these values, time constants for reaction (t_r) and diffusion (t_d) were, respectively, 192.3 and 1.2 s, indicating that no external diffusion effect was also observed for esterification reaction. The rate of FFA molecules diffusion per unit interfacial area ($k_{SL}C_0$) and the reaction rate per unit area ($\varphi r_0/a$) were calculated as 2.58×10^{-6} mol/cm^2 s and 1.61×10^{-8} mol/cm^2 s, respectively. These results indicate that the rate of reactant diffusion per unit area was around 160-fold higher than the reaction rate per unit area, which clearly show that the intraparticle diffusion of reactant molecules did not influence the esterification reaction rate. Similarly to hydrolysis reaction performed by crude enzymatic extract from dormant castor bean seeds, the biocatalyst prepared by immobilizing TLL on PHB particles allowed also good accessibility of FFA molecules from the reaction medium to the biocatalyst microenvironment under continuous stirring at 200 rpm. These results are in accordance with previous study performed in our lab for the alkyl oleate synthesis catalyzed by the same biocatalyst tested in the present study [20].

The reusability of immobilized enzymes is very important for their application, especially in industrial scale. The reusability of the biocatalyst prepared (immobilized TLL by physical adsorption on PHB particles) was carried out on the alkyl esters synthesis by esterification reaction of purified FFA with hexanol at equimolar ratio (750 mM of each reactant) in batch system. Figure 6 shows that the biocatalyst prepared did not exhibit loss of its catalytic activity after eight successive cycles of esterification reaction. These results show that the biocatalyst

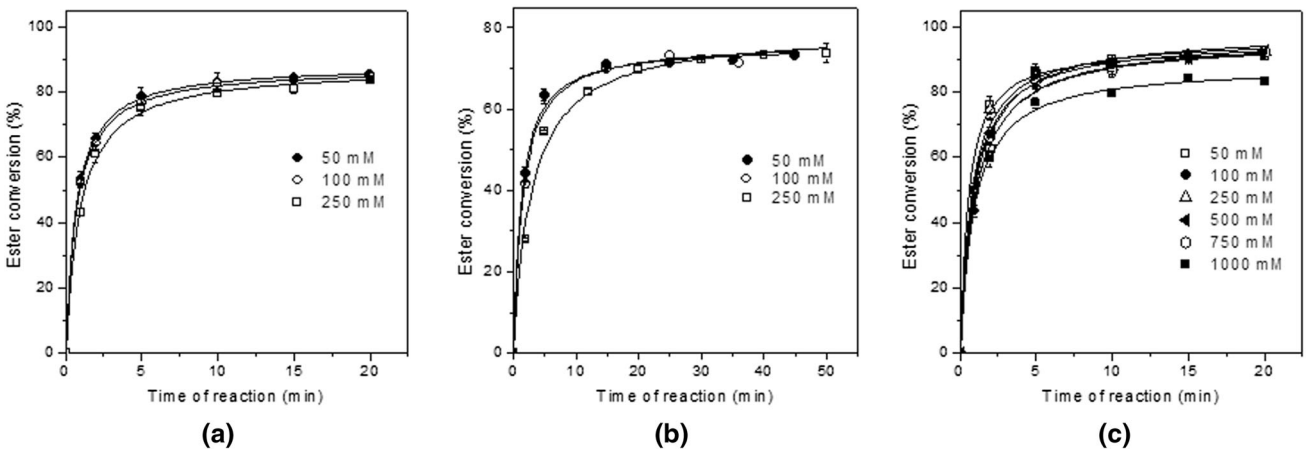

Fig. 5 Kinetic profile of esterification of purified FFA with isoamyl alcohol (**a**), 2-ethyl-hexanol (**b**), and hexanol (**c**) at different concentrations in heptane medium catalyzed by immobilized TLL on PHB particles. The reactions were performed at equimolar ratio alcohol:FFA, 16 % m/v of immobilized TLL at 32.5 °C under continuous stirring in an orbital shaker at 200 rpm in heptane medium

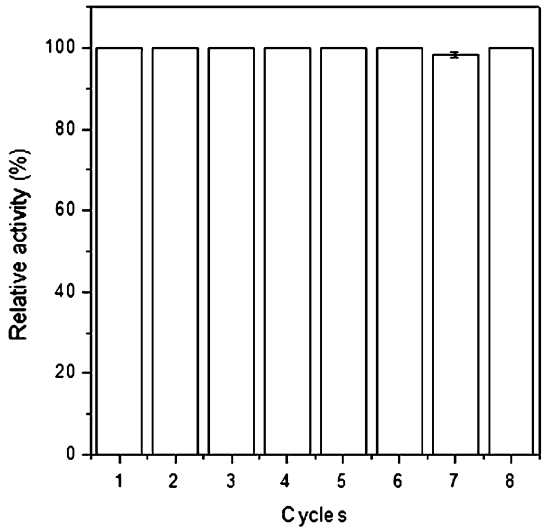

Fig. 6 Reusability tests of immobilized TLL on PHB particles in successive cycles of hexyl esters synthesis by esterification reaction in heptane medium. The reactions were performed at equimolar ratio alcohol:FFA (concentration of 750 mM of each reactant), 16 % m/v of biocatalyst at 32.5 °C under continuous stirring in an orbital shaker at 200 rpm

tested in esterification reaction presented better stability than previous studies reported by the literature concerning the enzymatic synthesis of alkyl esters by direct esterification [32–36].

The application of purified FFA from MPPO, a low-cost Brazilian vegetable oil, may be successfully used as feedstock to produce an important class of oleochemical compounds of industrial interest such as hexyl esters. Moreover, the biocatalysts used in each process step (hydrolysis and esterification reactions) exhibited high catalytic activity. According to our results, the synthesis of alkyl esters (hexyl esters) by sequential hydrolysis/esterification process seems to be attractive from the industrial point of view because required short-time reaction to reach high ester conversion of 92.4 ± 0.4 % after 125 min of reaction [hydrolysis reaction − 110 min (Fig. 4) + esterification reaction − 15 min (Fig. 5)]. These results are in accordance with previous studies reported in the literature concerning the enzymatic synthesis of alkyl esters by esterification and hydroesterification reactions [5, 9, 13, 37].

Moreover, the purified FFA generated in the hydrolysis reaction catalyzed by crude enzymatic extract from dormant castor bean seeds can also be successfully used as substrate or intermediate compounds to produce other compounds of industrial interest such as biolubricant agents [5, 13, 15], biofuels (biodiesel) [9, 10, 14, 37], additives as cold flow improvers in diesel fuels (alkyl methacrylates and polystyrene-*co*-alkyl methacrylates) [38] and fatty acid esters of sterols, a class of precursors of a wide variety of metabolites (prostaglandins, leukotrienes, and hydroxyl fatty acids) with critical biological functions [39].

Conclusions

In the present work, we proposed a promising process to produce alkyl esters of industrial interest from macaw palm oil. The process consists of sequential hydrolysis of this vegetable oil using a low-cost and highly active biocatalyst from dormant castor seeds to produce a mixture of FFA, followed by enzymatic esterification reaction with hexanol in heptane medium. The sequential process reached ester conversion of 92.4 ± 0.4 % around 2 h of reaction (hydrolysis + esterification reactions). According to the

results, the application of the biocatalyst from dormant castor bean seeds in hydrolysis step and immobilized TLL on PHB particles, a low-cost and eco-friendly support, in esterification step may be economically attractive for the production of compounds in industrial scale from vegetable oils broadly abundant in our country.

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