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Article

Effect of the Temperature and Molar Ratio of Water-Oil on the Enzymatic Hydrolysis Kinetics of the Soybean Oil: Experimental and Mathematical Modeling

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The objective of this paper was to evaluate the kinetics of the hydrolysis of soybean oil by the action of the Lipozyme® TL IM enzyme varying the operational conditions of molar ratio water/oil (9:1-60:1) and temperature (40-64 °C). To describe the experimental data, a mathematical model based on the kinetic mechanism of Ping-Pong Bi Bi (PPBB) was proposed, in which the following steps were not considered formation of the complex enzyme/oil substrate, and formation of the acylated enzyme/oil substrate complex. The results of enzymatic hydrolysis of soybean oil indicated a yield in free fatty acids of 76% at the molar ratio of 46:1 and temperature of 52 °C. Furthermore, based on the values of the determination coefficient and root mean square error, the mathematical model based on the kinetic mechanism of PPBB showed good agreement with the experimental data in a relatively wide temperature range. Thus, it can be a useful tool for the optimization and assessment of the mechanisms of enzymatic hydrolysis of vegetable oil.

Keywords: enzymatic catalysis, soybean oil, Lipozyme® TL IM, Ping-Pong Bi Bi mechanism

Introduction

Several industry sectors such as chemical, pharmaceutical, food, and bioenergy, have been demonstrating interest in products based on oils, fats, and their derivatives, resulting in a large number of high-added value products, such as lubricating oils and biofuels.[1,2] The production of free fatty acids (FFA), monoacylglycerols (MAG), and diacylglycerols (DAG), by the hydrolysis of triacylglycerols, may thus stimulate the exploration of renewable raw materials such as vegetable oils, animal fat, and oils originated from industrial wastes. Besides, such materials present not only the advantage of being renewable but also technical and economic benefits.[1]

In general, vegetable oils are composed of acylglycerols, among which triacylglycerol (TAG) is the major compound. However, it is commonly necessary to modify the characteristics of the oils according to the required application. In this sense, the hydrolysis process stands out as a widely used method, consisting of the breakage of TAG ester bonds to generate FFA, DAG, MAG, and glycerol (GL). In the hydrolysis process, the reactions may be catalyzed by enzymes or by chemical agents (acids or bases).[3] The use of enzymatic catalysts has been gaining relevance due to its wide range of benefits, including the use of mild temperatures and atmospheric pressure during the process. Besides, enzymatic technology and biocatalysis are promising tools for the synthesis of high-added value compounds.[1]

The lipases, which are enzymes classified as hydrolases, act by breaking down ester bonds of several compounds, mainly acylglycerols, which are their best substrates.[1] The industrial use of lipases is still expensive though because the enzymes must be active and stable at specific conditions of pH and temperature, which can be optimized for each process. Possible options to reduce these costs are the employment of immobilized enzymes or even their reuse.[1] Lipozyme® TL IM (EC 3.1.1.3) is a lipase-specific enzyme for 1-3 TAG bond immobilized on silica gel, which has become relevant in the food industry, mainly due to several studies involving lipids. The enzymes originated from the

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fungus *Thermomyces lanuginosus*, including the Lipozyme® TL IM, take precedence for hydrolyzing medium-chain TAGs (C10-C12) and present high thermal stability.[4,5] There are several reports[3,4,6,7] on the application of this enzyme, involving mainly the processes of hydrolysis, esterification, transesterification, and glycerolysis.

The kinetic behavior of the acylglycerol enzymatic hydrolysis can be affected by some variables, such as temperature, pH, stirring speed, enzyme/oil mass ratio, and water/oil molar ratio (W/O). The initial substrate concentration affects positively the reaction velocity up to a determined value, above which the velocity remains nearly constant.[8,9] Besides that, the reaction may be influenced by the proportion of substrates (molar ratio). Regarding the enzymatic hydrolysis of vegetable oils, it can be said that the reaction kinetics is favored with a slight increase in the amount of water in the reaction medium. However, adding water beyond a critical value may decrease the initial reaction rate and thus the yield. In addition, reaction velocity also increases with temperature, since at elevated temperatures more molecules acquire enough energy to reach the transition state. Nevertheless, in reactions catalyzed by enzymes, this increase takes place up to a certain temperature, in which the enzyme provides the highest catalysis efficiency. The operation at elevated temperatures (above 70 °C) may cause the denaturation of the enzyme.[10]

Considering that, from economic and operational points of view, it is desirable to achieve favorable reaction kinetics, it is worth investigating the temperature along with the W/O molar ratio since both are relevant factors to the increase in the velocity of the reaction.[3,11,12] Given that context, it is important to investigate the effects of variations on the operational conditions of enzymatic hydrolysis. This can be supported by adequate mathematical models that allow the optimization of operational conditions since modeling is an important tool for parameters estimation, optimization, and simulation of catalytic processes.[13-15]

Among the mechanisms that can describe processes such as esterification, transesterification, and hydrolysis, Ping-Pong Bi Bi (PPBB) stands out. In the studies of Veny *et al.*,[16] and Raita *et al.*,[17] models based on the PPBB mechanism were applied to describe the experimental data of the jatropha oil enzymatic transesterification by the Lipozyme® RM IM and the esterification of palmitic acid catalyzed by glycine-based crosslinked protein-coated microcrystalline lipase, respectively. Additionally, Chesterfield *et al.*,[18] assessed the transesterification of waste cooking oil by using the lipase *Candida antarctica*, supported on the macroporous acrylic resin (Novozym 435), and used a kinetic model based on the complete PPBB mechanism. In these studies, the additional step of inhibition by methanol was considered, and the proposed models were capable to describe adequately the experimental data.

Zulkeflee *et al.*[19] described the esterification process catalyzed by the lipase of *Candida rugosa* in a batch reactor equipped with a temperature and water activity control system by using the PPBB mechanism. It was observed that the model could show a good fit for the experimental data. Besides, the PPBB mechanism was also used by Gómez *et al.*,[20] whose assessment evaluated the development of rate equations for two enzymatic Ping-Pong reactions in series with application in the biosynthesis of bis(2-ethylhexyl) azelate. A high determination coefficient was obtained, validating thus both the kinetic equations and the reactor design.

Furthermore, the PPBB mechanism is also widely used for modeling of hydrolysis of fats and oils. Al-Zuhair *et al.*[21] and Feng *et al.*[22] applied the referred model to describe experimental enzymatic hydrolysis data of palm oil by using lipase from *Candida rugosa* and tributyrin by the Amano lipase derived from *Pseudomonas fluorescens*, respectively. It is worth mentioning that in several works reported in the literature[16-18] the hypothesis of a pseudo-steady state was assumed. This assumption makes that several constants can be grouped into one only constant (e.g., K_m-Michaelis-Menten).[23] In all mentioned studies, the PPBB mechanism could adequately describe the experimental data. It must be highlighted that the complete PPBB mechanism embraces a series of consecutive steps that lead to a model composed of 24 kinetic constants. Nevertheless, estimating parameters for such a model is not a trivial task.[24,25]

The main objective of the present work was thus to increase the free fatty acids (FFA) yield by working with different operational conditions of temperature and molar ratio water/oil (W/O) in the enzymatic hydrolysis of soybean oil. Besides that, we aimed to develop a single mathematical model based on the PPBB mechanism that could be able to estimate the FFA yield as well as the yield of other fatty acid components (TAG, DAG, and MAG), within a temperature range.

Experimental

Reagents

Commercial soybean oil (Toledo, Brazil) was used as a substrate for the hydrolysis reactions, whereas commercial enzyme Lipozyme® TL IM (Novozymes, Frederiksberg, Denmark), was employed as a catalyst. Buffer solution (50 mM, pH 6.1) was prepared with distilled water,

monobasic (Vetec, Duque de Caxias, Brazil), and dibasic sodium phosphate (Synth, Diadema, Brazil). To determine the FFA content by titration, ethylic alcohol (Anidrol, Diadema, Brazil), ether (Sigma-Aldrich 99.0% P.A., Missouri, USA), and NaOH (Synth, Diadema, Brazil) were utilized.

In addition, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), heptane (Anidrol, Diadema, Brazil), and chromatographic standards of monoolein, diolein, and triolein (Sigma-Aldrich, Missouri, USA) were used to determine the MAG, DAG, and TAG contents.

Enzymatic hydrolysis reaction

To obtain kinetic experimental data for the soybean oil hydrolysis, the experimental conditions of W/O molar ratio (9:1, 20:1, 46:1, and 60:1) and temperature (40, 52, and 64 °C) were varied, within a time interval from 0 to 24 h. The reactions were conducted in a glass batch reactor of 250 mL, in which temperature and stirring speed (250 rpm) were controlled. The operating range of the soybean oil buffer solution mole ratio was defined from the Zenevicz *et al*.[3] study, while the operating range of temperature was limited by the thermal range of the Lipozyme® TL IM enzyme.

Firstly, the reagents (buffer solution and soybean oil with a predefined molar ratio) were placed into the reactor and the catalyst (1% m/m concerning the soybean oil) was added. After a predetermined reaction time, the mixture was centrifuged (3000 rpm, 5 min) and the FFA content was determined. The concentration of the intermediate compounds (TAG, DAG, and MAG) was determined for the kinetic curves that achieved the best FFA yields with the best molar ratio W/O for each temperature.

Quantification of free fatty acids

The standard method Ca5a-40, recommended by the American Oil Chemist's Society (AOCS),[26] was used to quantify FFA. Therefore, 1 g of the sample (oil phase) was diluted in 15 mL of ethylic alcohol/ether solution (1:1 v/v), which was titrated with a solution of NaOH (0.1 mol L^{-1}) by using phenolphthalein as an indicator.

Equations 1 and 2 were applied to determine acidity content (%) and FFA yield, respectively.

$$FFA_t = 100 \times \frac{V\, M_{NaOH}\, MM_{FFA}}{m} \quad (1)$$

$$FFA(\%) = 100 \times \frac{FFA_t}{TAG_0} \quad (2)$$

where FFA_t is the FFA content concerning the mixture of fatty acids in the soybean oil (%); V is the NaOH aqueous solution volume used for the titration (L); M_{NaOH} is the molar concentration of the NaOH aqueous solution (mol L^{-1}); MM_{FFA} is the weighted molar mass of fatty acids present in the soybean oil (g mol^{-1}), m is the mass of the oil sample (g) and TAG_0 corresponds to the triacylglyceride content present in the soybean oil. The FFA molar mass was calculated as a weighted average (based on the molar fraction) of the fatty acids molar mass present in the soybean oil (MM_{FFA} = 275.62 g mol^{-1}).[3,27]

Determination of TAG, DAG, MAG

The samples were analyzed in a gas chromatograph (Shimadzu, GC-2010 Plus, Kyoto, Japan) equipped with a flame ionization detector (FID). The MAG, DAG, and TAG contents were determined using a capillary column ZB-5HT inferno™ (Zebron, 10 m × 0.32 mm × 0.10 μm) and a column injector. The samples were previously derivatized with MSTFA (room temperature and 15 min) as described by Trentini *et al*.,[28] and injected (1 μL) using the following oven temperature gradient: initially, the column was held at 50 °C for 1 min, followed by heating to 180 °C at 15 °C min^{-1}, then to 230 °C at 7 °C min^{-1} and then to 380 °C at 10 °C min^{-1}, this temperature being held for 5 min. The detector temperature was 380 °C and the injector heating programming was: initial temperature of 60 °C held for 1 min followed by heating to 380 °C at 10 °C min^{-1}, this temperature being held for 10 min. Chromatographic standards of triolein, diolein, and monoolein were injected for the identification of the compounds and to construct the calibration curve used in the quantification of the compounds in the samples. To calculate the formation of MAG and DAG and the conversion of TAG, the molar mass of the compounds elected as representatives were used (monoolein, diolein, and triolein), with the values of 356.547, 621.000, 885.432 g mol^{-1}, respectively.

Mathematical modeling

The kinetics of the soybean oil enzymatic hydrolysis, catalyzed by the enzyme Lipozyme® TL IM in batch operation mode was modeled considering the following hypothesis for both tested models: (*i*) each step of the kinetic mechanism follows an elementary reaction law; (*ii*) the system is well stirred, allowing the limitations of mass transfer to be neglected; (*iii*) the water/oil substrate interface is saturated with enzymes, so the lipase adsorption step at the interface can be neglected (*iv*) isobaric and isothermal process (*v*). For the simplified

PPBB model, the kinetic mechanism shown in Table 1 was considered.

The complete PPBB mechanism (Table 1) is characterized by the interaction between the free enzyme and the first substrate (TAG) which results in the formation of an enzyme-substrate intermediate (ETAG). After that, this complex (ETAG) results in a new intermediate formed by the acylated enzyme and the oil substrate (EADAG). As the product (DAG) is formed, a nucleophilic attack from the second substrate water (W) on the acyl-enzyme (EA) occurs, forming the complex acyl enzyme-water (EAW). This, in turn, becomes the enzyme-free fatty acid intermediate (EFFA), resulting in the formation of the product (FFA) and free enzyme (E).[29,30] Sequentially, the same reaction steps describe the conversion of the compound DAG to MAG, and later MAG to GL (Table 1).

The simplified PPBB kinetic mechanism assumes that the formation stages of the acylated enzyme/oil substrate complexes and the formation stages of the enzyme/oil substrate complex occur quickly, and thus can be neglected in the mechanism. It should be noted that the formation of the acylated enzyme is considered, as well as its reaction with the water molecule. All those assumptions and simplifications result in a mathematical model with 8 kinetic constants.

The mathematical models used to describe the kinetics of soybean enzymatic hydrolysis were built by applying the mass conservation law (molar basis) for all compounds. For the simplified PPBB model, the molar balances for TAG, DAG, MAG, W, GLI, E and FFA are given by the equations 3-10:

$$\frac{dC_{TAG}}{dt} = -k_1 C_{TAG} C_E + k_2 C_{EA} C_{DAG} \quad (3)$$

$$\frac{dC_{DAG}}{dt} = k_1 C_{TAG}\ C_E - k_2 C_{EA}\ C_{DAG} - k_3 C_{DAG} C_E + k_4 C_{EA}\ C_{MAG} \quad (4)$$

$$\frac{dC_{MAG}}{dt} = k_3 C_{DAG} C_E - k_4 C_{EA}\ C_{MAG} - k_5 C_{MAG} C_E + k_6 C_{EA}\ C_{GL} \quad (5)$$

$$\frac{dC_W}{dt} = -k_7 C_{EA} C_W + k_8 C_{FFA} C_E \quad (6)$$

$$\frac{dC_{FFA}}{dt} = k_7 C_{EA} C_W - k_8 C_{FFA} C_E \quad (7)$$

$$\frac{dC_{GL}}{dt} = k_5 C_{MAG} C_E - k_6 C_{EA} C_{GL} \quad (8)$$

$$\frac{dC_{EA}}{dt} = k_1 C_{TAG}\ C_E - k_2 C_{EA}\ C_{DAG} + k_3 C_{DAG} C_E - k_4 C_{EA}\ C_{MAG} + k_5 C_{MAG} C_E - k_6 C_{EA}\ C_{GL} - k_7 C_{EA} C_W + k_8 C_{FFA}\ C_E \quad (9)$$

$$\frac{dC_E}{dt} = -k_1 C_{TAG}\ C_E + k_2 C_{EA}\ C_{DAG} - k_3 C_{DAG} C_E + k_4 C_{EA}\ C_{MAG} - k_5 C_{MAG} C_E + k_6 C_{EA}\ C_{GL} + k_7 C_{EA} C_W - k_8 C_{FFA}\ C_E \quad (10)$$

where k_i (i = 1 to 8) corresponds to specific reaction rate constants; C_a (a = TAG, DAG, MAG, GLI, FFA, W, E and

Table 1. Complete and simplified Ping-Pong Bi Bi mechanisms

Complete PPBB mechanism	Simplified PPBB mechanism
$E + TAG \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ETAG$	
$ETAG \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EADAG$	
$EADAG \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EA + DAG$	$TAG + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA + DAG$
$EA + W \underset{k_{20}}{\overset{k_{19}}{\rightleftharpoons}} EAW$	$EA + W \underset{k_8}{\overset{k_7}{\rightleftharpoons}} FFA + E$
$EAW \underset{k_{22}}{\overset{k_{21}}{\rightleftharpoons}} EFFA$	
$EFFA \underset{k_{24}}{\overset{k_{23}}{\rightleftharpoons}} E + FFA$	
$E + DAG \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EDAG$	
$EDAG \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EAMAG$	
$EAMAG \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} EA + MAG$	$DAG + E \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA + MAG$
$EA + W \underset{k_{20}}{\overset{k_{19}}{\rightleftharpoons}} EAW$	$EA + W \underset{k_8}{\overset{k_7}{\rightleftharpoons}} FFA + E$
$EAW \underset{k_{22}}{\overset{k_{21}}{\rightleftharpoons}} EFFA$	
$EFFA \underset{k_{24}}{\overset{k_{23}}{\rightleftharpoons}} E + FFA$	
$E + MAG \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} EMAG$	
$EMAG \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} EAGL$	
$EAGL \underset{k_{18}}{\overset{k_{17}}{\rightleftharpoons}} EA + GL$	$MAG + E \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EA + GL$
$EA + W \underset{k_{20}}{\overset{k_{19}}{\rightleftharpoons}} EAW$	$EA + W \underset{k_8}{\overset{k_7}{\rightleftharpoons}} FFA + E$
$EAW \underset{k_{22}}{\overset{k_{21}}{\rightleftharpoons}} EFFA$	
$EFFA \underset{k_{24}}{\overset{k_{23}}{\rightleftharpoons}} E + FFA$	

E: enzyme; EA: enzyme acylated; TAG: triacylglycerol; ETAG: enzyme-triacylglycerol complex; DAG: diacylglycerol; EDAG: enzyme-diacylglycerol complex; EADAG: enzyme acylated- diacylglycerol complex; MAG: monoacylglycerol; EMAG: enzyme-monoacylglycerol complex; EAMAG: enzyme acylated-monoacylglycerol complex; GL: glycerol; EAGL: enzyme acylated-glycerol complex; W: water; EAW: water-enzyme acylated complex; FFA: free fatty acid; EFFA: enzyme-free fatty acid; k_i (i = 1 … 24): reaction specific rate constants.

EA) corresponds to the concentrations of the compounds, namely triacylglycerol, diacylglycerol, monoacylglycerol, glycerol, free fatty acid, water, enzyme and acylated enzyme, respectively.

The initial conditions used in the solution procedure of the differential algebraic equations system were as follows: $C_{TAG}(0)$ = 1.11588, $C_{DAG}(0)$ = 0.01548, $C_{MAG}(0) = 1.0000 \times 10^{-6}$, $C_{FFA}(0) = 0.01971$, $C_W(0) = 51.3305$, $C_E(0) = 3.125 \times 10^{-4}$, $C_{EA}(0) = 0.0$, $C_{GLI}(0) = 0.0$, in which concentrations value are expressed in mol kg^{-1}. The values were calculated based on total oil and water masses used in the hydrolysis reactions for the best molar ratio condition (water/oil) at each temperature assessed. The models were solved by the numeric Rosenbrock method,[31] code in the software Maple®.[32]

Parameters estimation procedure

The experimental data sets used to estimate the model parameters were obtained as described using hydrolysis enzymatic reaction of the soybean, for the compounds of FFA, TAG, DAG, and MAG in the W/O 46:1 molar ratio condition and at temperatures of 40, 52, and 64 °C. Thus, to determine a single set of parameters estimated by the model (pre-exponential factor and activation energy), the specific reaction rate constants (k), which vary with temperature, were replaced by Arrhenius' Law. The values of pre-exponential factor (A) and activation energy (E_a) were estimated for the experimental data of the enzymatic hydrolysis kinetics of soybean oil. The minimization of the objective function (OF) is given by equation 11. In the search for the minimum OF, the Powel method (available in the DirectSearch from Maple®[32]) was applied to a set of arbitrary values counted as an initial estimate to the Simplex Downhill method, developed by Nelder and Mead.[33]

$$OF = \sum_{k=1}^{nt}\sum_{j=1}^{nc}\sum_{i=1}^{nd}\left(\frac{c_{k,j,i}^{exp} - c_{k,j,i}^{mod}}{c_{k,j,i}^{exp}}\right)^2 \quad (11)$$

where nc is the number of components of the reaction; nd the number of experimental data; nt the number of assessed temperatures; $c_{k,j,i}^{exp}$ the experimental concentration i of the component j at the temperature k; and $c_{k,j,i}^{mod}$ is the concentration i of the component j at the temperature k calculated by the model. The quality of the models' fits was evaluated by the determination coefficient (R^2) and by the root mean square error (RMSE) which considers the number of different parameters of each model.[34]

Results and Discussion

Kinetics of the soybean oil enzymatic hydrolysis

The kinetic behavior of the soybean oil enzymatic hydrolysis for the molar ratios (W/O) of 9:1, 20:1, 46:1, and 60:1 at the temperatures of 40, 52, and 64 °C, respectively, can be observed in Figures 1a-1c. It can be noticed that there is a similarity in the FFA yield profile for the kinetic curves in the studied temperatures, in which the presence of three regions can be highlighted. In the first region (between 0 and 1 h), there is a high increase in the FFA yield. While in the second region (from 1 to 21 h) a decrease in the slope of the line that represents the FFA yield was observed. Finally, the third region (from 21 to 24 h) shows a tendency towards equilibrium in most of the tests carried out. Furthermore, the tendency towards equilibrium is better observed in tests

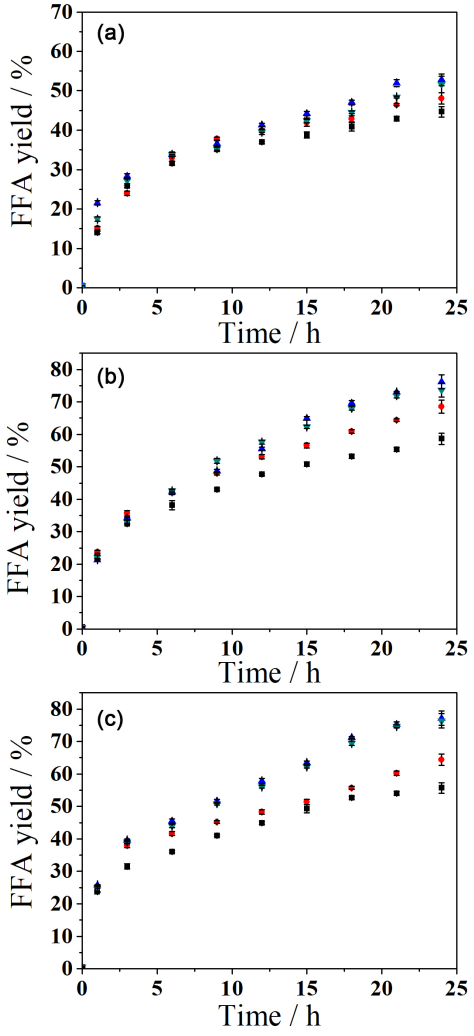

Figure 1. Kinetics of the soybean oil enzymatic hydrolysis for the molar ratios W/O of: 9:1 (■), 20:1 (●), 46:1 (▲), and 60:1 (▼). Experimental conditions: 250 rpm, molar ratio enzyme/oil 1% m/m, 40 °C (a), 52 °C (b) and 64 °C (c).

with a molar ratio O/W 9:1, when compared with tests with a molar ratio O/W 64:1.

Table 2 presents the FFA yields along with the initial reaction rates for all the conditions tested. Analysis of variance (ANOVA) was performed for all results (FFA yields and initial reaction rates), in which a significant difference could be observed for both the temperature variation and the variation of the W/O molar ratio, with a 95% confidence interval.

From Tukey's test (Table 2), it was possible to observe that the highest initial rate is 1.67×10^{-5} mol mL^{-1} h^{-1} obtained in the molar ratio W/O 46:1 and temperature 64 °C. As for the FFA yields, no significant difference was observed neither for the temperature increase from 52 to 64 °C nor for the molar ratio increase from 46:1 to 60:1. Therefore, it can be said that the better operational condition achieved was the temperature of 52 °C and the W/O molar ratio of 46:1, considering that the same FFA yield (77%) was achieved by lower consumption of water and energy to heat the reactional mixture, compared with the highest conditions of temperature and molar ratio tested (64 °C and 60:1, respectively).

Moreover, the results obtained in the present study showed the same yield (FFA yield higher than 30% after 3 h) in a shorter reaction time when compared to the study of Freitas *et al.*,[27] (30% after 4 h) (Table 3), in which the enzymatic hydrolysis of soybean oil using lipase from different sources was evaluated. On the other hand, one may notice by observing Table 3 that the FFA yield values were lower than those reported by Cavalcanti-Oliveira *et al*.,[35] (89% after 48 h). However, in the latter case, a liquid lipase was used, which may have favored the reaction due to the absence of enzymatic support (silica). In addition, despite the highest yield achieved by Cavalcanti-Oliveira *et al*.,[35] it should be mentioned that the reaction time was expressively longer compared to the one used in the present study.

One factor that restricts the use of enzymes for the hydrolysis of vegetable oils is that low reaction rates are commonly observed.[1,37] In the present study, the higher initial rates $\left(\left. \frac{dC_{FFA}}{dt} \right|_{t=0} \right)$ were observed, in descending order, for the conditions of W/O molar ratio of 46:1, 60:1, and 20:1, at the temperature of 64 °C. By comparing these initial rates with the ones reported in previous studies, one may notice that Tavares *et al*.,[11] achieved a higher value (3.5×10^{-4} mol mL^{-1} h^{-1}). Nevertheless, in that study, the high stirring speed used (790 rpm) may

Table 2. Initial rates and free fatty acids yield (after 24 h) of the soybean oil enzymatic hydrolysis

Molar ratio W/O	Initial rates ± dp / ($\times 10^{-5}$ mol mL^{-1} h^{-1})			Free fatty acids yield ± dp / %		
	Temperature / °C					
	40	52	64	40	52	64
9:1	1.02 ± 0.003^{h}	1.37 ± 0.004^{e}	1.4 ± 0.004^{d}	45 ± 1.3^{E}	59 ± 1.7^{CD}	56 ± 1.6^{CD}
20:1	1.00 ± 0.00^{i}	1.4 ± 0.004^{d}	1.61 ± 0.004^{c}	48 ± 1.4^{DE}	69 ± 2.0^{AB}	65 ± 1.8^{B}
46:1	1.2 ± 0.003^{f}	1.41 ± 0.004^{d}	1.67 ± 0.005^{a}	53 ± 1.5^{CD}	76 ± 2.1^{A}	77 ± 2.2^{A}
60:1	1.14 ± 0.003^{g}	1.4 ± 0.004^{d}	1.64 ± 0.005^{b}	52 ± 1.5^{CD}	74 ± 2.1^{AB}	77 ± 2.2^{A}

The indexes (a-i) correspond to Tukey's test; dp: standard deviation. Mean and standard deviation results were provided by experimental duplicate. Initial rates and standard deviation are presented in the same order of magnitude.

Table 3. Free fatty acid yields were achieved for several vegetable oils and enzymes

Oil substrate	Catalyst	Operational conditions	Yield / %	Reference
Soybean oil	*Thermomyces lanuginose*	T = 40 °C, M_w/Mo = 4:1, me/mo = 1% (m/v), rpm = 200	30 (after 4 h)	27
Palm oil	Lipozyme® TL IM	T = 55 °C, M_w/M_o = 4:1, me/mo = 3.5% (m/m), rpm = 200	28 (after 6 h)	36
Soybean oil	*Thermomyces lanuginose*	T = 60 °C, M_w/M_o = 26:1, me/mo = 2.3% (m/v)	89 (after 48 h)	35
Soybean oil	Lipozyme® TL IM	T = 40 °C, M_w/M_o = 20:1, me/mo = 10% (m/m), rpm = 300, ultrassonic power = 132 W	60 (after 2 h)	3
Crambe oil	Lipozyme® RM IM	T = 50 °C, M_w/M_o = 10:1, me/mo = 2.7% (m/m), rpm = 790	74 (after 40 h)	11
Soybean oil	Lipozyme® TL IM	T = 52 °C, M_w/M_o = 46:1, me/mo = 1% (m/m), rpm = 250	77 (after 24 h)	present work

T: temperature; M_w/M_o: molar ratio of water/oil; me/mo: mass ratio enzyme/oil; rpm: rotation of minutes.

have favored the reaction, thus increasing the initial rates. On the other hand, Huang *et al.*[38] obtained initial rates of 5.3×10^{-6} mol mL^{-1} h^{-1}, which are lower than the ones observed in the present work. This can be explained by the fact that relatively low conditions of stirring speed and temperature were used (200 rpm and 45 °C, respectively). Although high initial rates have been observed, it is suggested that by increasing the stirring speed of the reaction medium, as well as the temperature, the hydrolysis reaction might be favored, resulting in higher FFA yields.

In a general way, molar ratio and temperature affected both the reaction initial rate (see Figure 2a) and FFA yield (see Figure 2b) of the soybean enzymatic hydrolysis. It can also be said that the temperature shows a more pronounced effect.

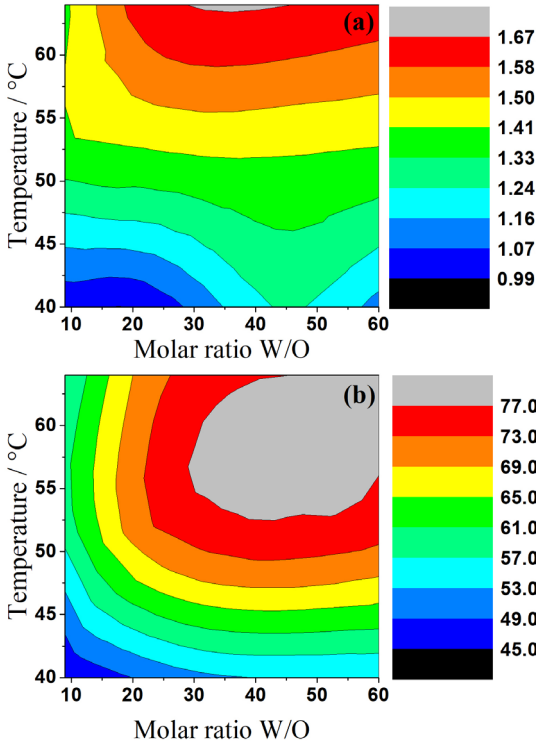

Figure 2. The initial reaction rate (a) and FFA yield (b). Experimental conditions: temperatures of 40, 52 and 64 °C, and molar ratios W/O of 9:1, 20:1, 46:1 and 60:1.

According to literature reports.[1,2] the reaction of soybean enzymatic hydrolysis starts with the adsorption of the lipase at the water/oil interface. The enzyme is then bound to the substrate molecule (TAG) forming an enzyme/oil substrate complex. Thereafter, a series of reactions occur and the soluble products (FFA and GL) may diffuse in the water layer. The excess of water in the system may promote a shifting in the reaction in the direction of the formation of products, improving thus the hydrolysis yield (since it is well known that an excess of water beyond the stoichiometric value (1:3) may displace the reaction equilibrium to product formation).[3]

Literature reports[3] indicate that a slight increase in the water amount in the reaction media leads to an enzymatic activity increase due to the greater interface area and the better arrangement. However, above a maximum amount (critical point), inhibition of the lipase's catalytic activity may occur, caused by the high-water content.[37,39] This effect can be observed by analyzing the FFA yields for all assessed temperatures. At each temperature, FFA yields increased as the W/O molar ratio was increased (from 9:1 to 46:1). Nevertheless, the addition of water up to an amount beyond the critical point may increase the thickness of the water layer around the enzyme.[40] This may impair the diffusion of low solubility reagents and products, like oils, through the water layer to the enzymes' active sites.[41,42] Thus, it was observed that the increase of the water amount in the reaction media to molar ratio W/O values above 46:1 impaired the contact between oil substrate and enzyme, which explains the fact that FFA yields for the molar ratios W/O of 46:1 and 60:1, which were the highest ones tested, were statistically equal at a 95% confidence interval (see Table 2).

Furthermore, the manipulation of the operational condition molar ratio W/O influences the initial reaction rate. As previously mentioned, the initial reaction rates of the vegetable oils enzymatic hydrolysis are commonly low, which could be even a limiting factor for the process feasibility.[1] According to Voll *et al.*,[43] low initial reaction rates are also related to the restricted miscibility from water in oil. However, as the reaction occurs, intermediate compounds are formed (DAG and MAG), which present surfactant properties that reduce the interfacial tension and the energy at the surface between the phases, promoting more contact.[44] This phenomenon may enhance the reaction rate after a certain period. Phuah *et al.*[45] reported that a low initial reaction rate could be noted also when a low water content was used, indicating that the number of water molecules was insufficient to make the reaction kinetics favorable. Zaks and Klibanov[46] have additionally suggested that the water molecules are responsible for the activation of the biocatalysts, which affect the specific arrangement for the enzymatic reactions.[18] The addition of water is thus essential to make the reaction kinetics more favorable.

By definition, an emulsion is a heterogeneous system, which consists of at least one immiscible liquid (internal discontinuous phase) disperse into another (external continuous phase) in form of small drops with a final diameter usually higher than 0.1 μm. Such systems show low stability, which can be enhanced by surfactant additives. These additives reduce the interfacial tension and

the energy at the surface between the phases, avoiding the coalescence of the particles by the formation of barriers.[47] The compounds DAG and MAG (intermediate products of the hydrolysis), present emulsifying properties, which after a relatively long time may help to stabilize the reaction medium.[43]

The influence of temperature on the chemical reactions was also evidenced by the kinetic studies, in which by increasing the temperature the reaction initial rate increased (see Figure 2). This could be explained by the fact that when the temperature increases, more kinetic energy is provided to the substrate molecules (water and oil). Hence, if the process is endothermic, the number of collisions becomes higher as well as the energy involved in each collision, increasing the number of molecules that react (reaction velocity). Besides that, high temperatures cause a reduction of the viscosity of both oil and water, reducing the mass transfer resistance thus improving the interaction between enzyme and oil substrate.[45] According to Brock *et al*.,[48] each temperature increase of 10 °C leads to a reduction of the oil viscosity at the ratio of 1.36. On the other hand, at higher temperatures (above 70 °C), denaturation of enzymes may occur.[10]

By analyzing the tested temperatures separately, it can be noticed that at 40 °C, the initial reaction rates were, in general, lower than the rates observed at the higher temperatures (52 and 64 °C), due to the higher viscosity of the mixture (water/oil) at the lower temperature. Studies by Coelho *et al*.[49] show that tests conducted at temperatures equal to and/or below 40 °C reduce the hydrolysis rate of vegetable oils due to the increased viscosity of the emulsion. According to Chen and Tao,[50] usually, an abnormal increase in temperature is avoided because it tends to coagulate the particles, thereby causing a deterioration of the emulsions. However, the present work brings one of the kinetics of the enzymatic hydrolysis of soybean oil, different from that proposed by Chen and Tao.[50] Thus, parameters such as the number of intermediate compounds and agitation cannot be correlated. In addition to the fact, the reaction yield was improved with the temperature factor.

In summary, the results of the soybean oil enzymatic hydrolysis by the Lipozyme® TL IM showed an FFA yield of nearly 77%, with an initial rate of 1.67×10^{-5} mol mL^{-1} h^{-1}. Overall, it could be noticed that the W/O molar ratio and mainly temperature have influence over the initial reaction rate as well as over the FFA yield, and the best conditions achieved were a W/O molar ratio of 46:1 and the temperatures of 52 and 64 °C. Hence, the optimization of these operational variables is extremely necessary to enhance the hydrolysis yield, which could be performed by mathematical modeling of the process in further studies.

Mathematical modeling

The PPBB mechanism is reported in several studies[16,17,19,51] whose reaction steps are observed as described in Table 1 (complete PPBB mechanism). Mechanisms based on enzymatic reactions with various substrates, such as the PPBB mechanism, can be complex and result in equations with dozens of parameters. Estimating those parameters is not a trivial task, since the procedure may be susceptible to different errors due to the strong correlation between the estimated parameters.[12,24,25] Thus, to reduce the number of reaction steps (consequently the number of estimated parameters) present in the complete PPBB mechanism a simplification was proposed. The simplified PPBB is an alternative model to the complete PPBB since the simplified PPBB model has a smaller number of parameters estimated by the model (8 kinetic constants), and the computational effort required is lower compared to the complete PPBB (24 kinetic constants). Furthermore, the simplifications proposed for the simplified PPBB allow all compounds (TAG, DAG, MAG, GL, W, and FFA) to be measured experimentally except for the EA complex. It should be noted that compound EA should not be simplified since it is this compound that characterizes the model as sequential (see Table 1).

In the simplified PPBB mechanism, it was considered that the formation stages of the acylated enzyme/oil substrate complex and the formation stage of the enzyme/oil substrate complex occur quickly, being neglected in the simplified PPBB mechanism. Both simplifications result in a model composed of 8 kinetic parameters. The mathematical modeling (simulated curves from the estimated parameters) for the kinetics of enzymatic hydrolysis of soybean oil for a simplified PPBB model is shown in Figures 3-5. Both simulation and experimental data presented a point of maximum for the intermediate compounds, which is expected for a process in a closed-batch system described by a mechanism consisting of a series of consecutive reactions. Besides, it can be noted a reduction of TAG concentration (Figure 3d) whereas the FFA concentration increased (Figure 3a).

Furthermore, other points of maximum can be observed in the kinetic curves of DAG (Figure 3c) and MAG (Figure 3b), which are typical points for reaction intermediates. Similar profiles were also achieved for the temperatures of 52 °C (Figure 4) and 64 °C (Figure 5). In addition, it should be mentioned that the concentration of acylglycerols, water, and GL presented the expected profiles, considering that the results obtained satisfy the mass conservation law ($\sum_{i=1}^{6} m_i$ = constant, TAG, DAG, MAG, GL, W and FFA).

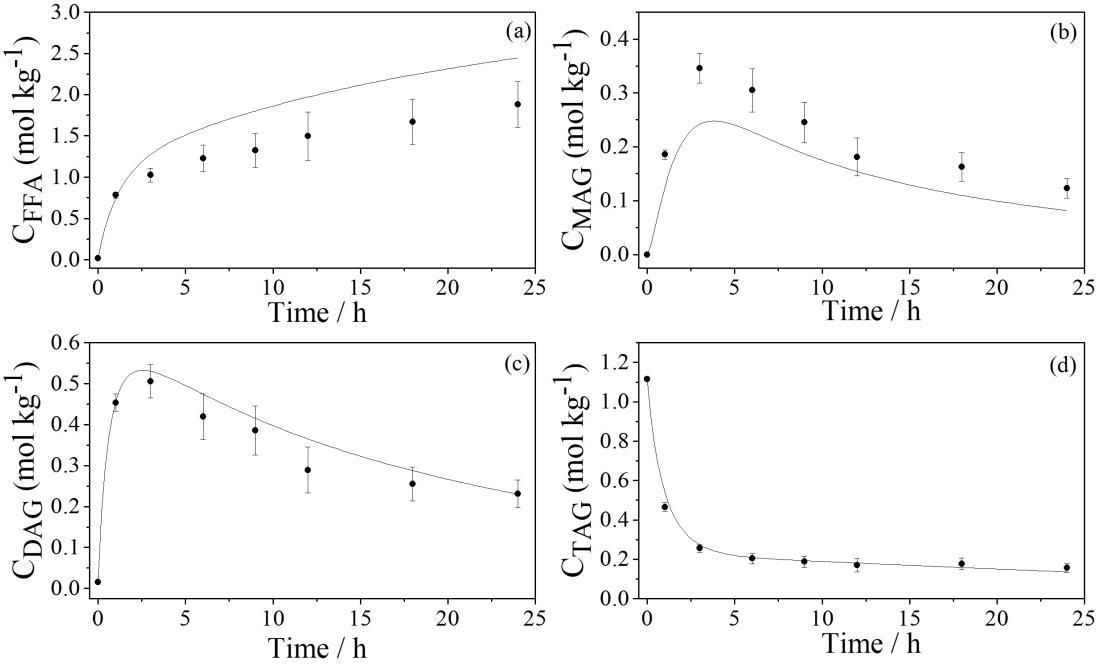

Figure 3. Simulation of the soybean oil enzymatic hydrolysis: (a) C_{FFA} (mol kg^{-1}); (b) C_{MAG} (mol kg^{-1}); (c) C_{DAG} (mol kg^{-1}); (d) C_{TAG} (mol kg^{-1}). Conditions: temperature = 40 °C; molar ratio W/O 46:1, enzyme/oil substrate ratio = 1% (m/m), stirring speed = 250 rpm; pH = 6.1. (●) Experimental data; (—) model.

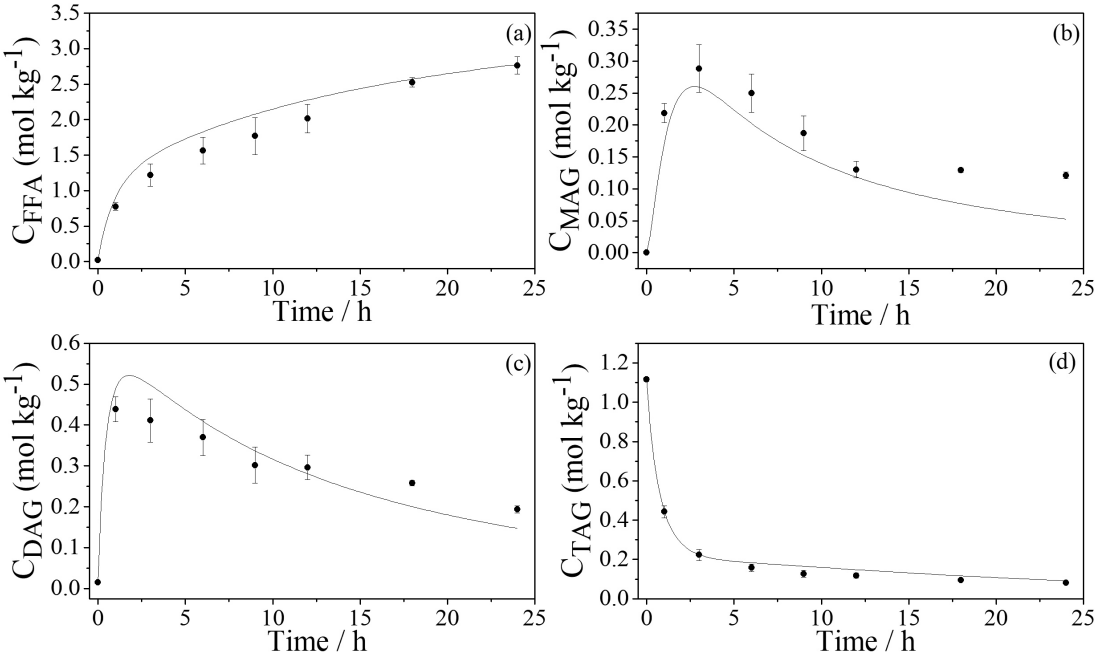

Figure 4. Simulation of the soybean oil enzymatic hydrolysis: (a) C_{FFA} (mol kg^{-1}); (b) C_{MAG} (mol kg^{-1}); (c) C_{DAG} (mol kg^{-1}); (d) C_{TAG} (mol kg^{-1}). Conditions: temperature = 52 °C; molar ratio W/O 46:1, enzyme/oil substrate ratio = 1% (m/m), stirring speed = 250 rpm; pH = 6.1. (●) Experimental data; (—) model.

The values of the parameters estimated by the models (A and Ea) are presented in Table 4. From the parameters estimated by the model (A, Ea), it was possible to calculate the reaction specific rate coefficients (k) for the three temperatures studied (Table S1, Supplementary Information (SI) section). It should be highlighted that for the estimation of these parameters, three sets of experimental data with the same molar ratio W/O (46:1) were used at different temperatures (40, 52, and 64 °C). The estimated parameter uncertainties (Table 4) were calculated using a 1% variation in each parameter. Based on this, it was observed that the uncertainties

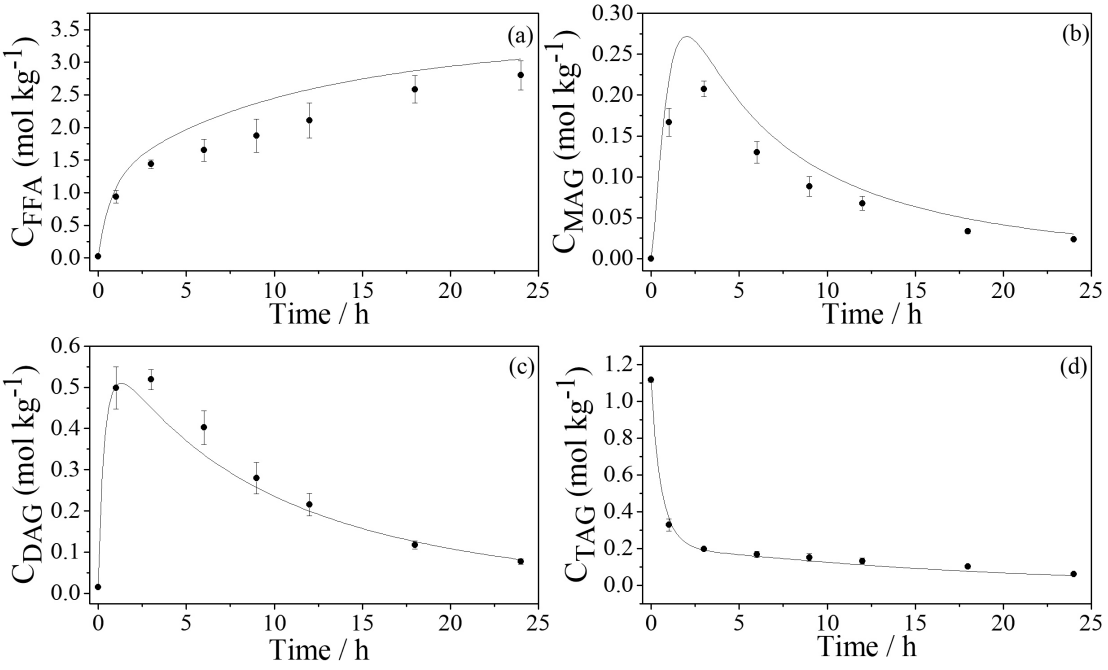

Figure 5. Simulation of the soybean oil enzymatic hydrolysis: (a) C_{FFA} (mol kg^{-1}); (b) C_{MAG} (mol kg^{-1}); (c) C_{DAG} (mol kg^{-1}); (d) C_{TAG} (mol kg^{-1}). Conditions: temperature = 64 °C; molar ratio W/O 46:1, enzyme/oil substrate ratio = 1% (m/m), stirring speed = 250 rpm; pH = 6.1. (●) Experimental data; (—) model.

were smaller when compared to the estimated parameter values, indicating that these are sensitive and no step can be discarded. The determination coefficients for the FFA, MAG, DAG, and TAG data, as well as the RMSE, are shown in Table 4. These data, along with the graphical interpretation (Figures 3-5), indicate the model adequately described the experimental data set. By analyzing Figures 3-5, one may also notice the influence of the temperature on the enzymatic hydrolysis of soybean oil, since at higher temperatures (52 and 64 °C) the simulated concentration profiles of the compounds (FFA, TAG, DAG, and MAG) are closer to the experimental data.

In general, it can be said that the PPBB mechanism has been used in several studies,[16-19] in which the models showed good agreement with the experimental data. It is worth mentioning that, in the present work, the determination of the parameters (A, Ea) allowed the simulation of the concentration profiles in the evaluated temperature range (40-64 °C) for the 46:1 molar ratio.

Furthermore, through the set of parameters estimated by the model, it was possible to predict FFA concentration profiles for the 9:1, 20:1, and 60:1 W/O molar ratios, shown in Figures 6-8. The simplified mathematical model studied in the present work allowed us to predict, with good correlation, the profiles of FFA concentration for the molar ratio W/O 20:1 (for all temperatures studied) and in the molar ratio W/O 60:1 for the temperatures of 52 and 64 °C. In the molar ratio W/O 9:1, it was observed that the values predicted by the model were distant from the experimental points, indicating that for this molar ratio it would be necessary to carry out an independent study and determine a new set of parameters (specific reaction rate constants).

Table 4. Quality and parameters estimated by the model

Indices (i)	Parameters estimated by the model	
	$A_i \pm dp$ / ($\times 10^7$ kg mol^{-1} h^{-1})	$Ea_i \pm dp$ / (J mol^{-1})
1	0.7601 ± 0.1634	$1.9807 \times 10^4 \pm 4258.5$
2	1.1890 ± 0.7313	$1.9059 \times 10^3 \pm 1172.1$
3	0.8206 ± 0.5744	$1.1261 \times 10^4 \pm 784.42$
4	1.2317 ± 0.1330	$2.5150 \times 10^4 \pm 2716.16$
5	1.9960 ± 0.1337	$2.4285 \times 10^4 \pm 1627.1$
6	1.8741 ± 0.3317	$1.0618 \times 10^3 \pm 187.94$
7	2.6466 ± 0.4235	$2.7929 \times 10^4 \pm 4468.6$
8	1.3007 ± 0.000	$9.2422 \times 10^7 \pm 0.000$

Quality parameter	Temperature / °C		
	40	52	64
R^2 FFA	0.993	0.991	0.991
R^2 MAG	0.975	0.965	0.998
R^2 DAG	0.992	0.970	0.982
R^2 TAG	0.998	0.998	0.998
OF		2.466	
RMSE		0.16	

A_i (i = 1 … 8): pre-exponential factor; Ea_i (i = 1… 8): activation energy; FFA: free fatty acid; MAG: monoacylglycerols; DAG: diacylglycerols; TAG: triacylglycerols; OF: objective function; RMSE: root mean square error; dp: estimated parameter uncertainty; R^2: correlation coefficient.

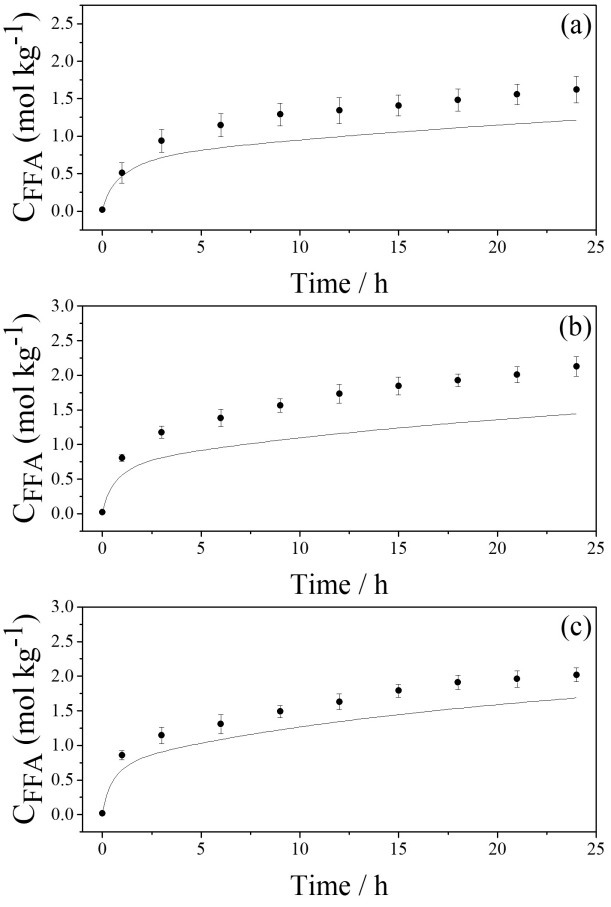

Figure 6. Prediction of the soybean oil enzymatic hydrolysis: (a) temperature 40 °C; (b) temperature 52 °C; (c) temperature 64 °C. Conditions: molar ratio W/O 9:1, enzyme/oil substrate ratio = 1% (m/m), stirring speed = 250 rpm; pH = 6.1. (●) Experimental data; (—) model.

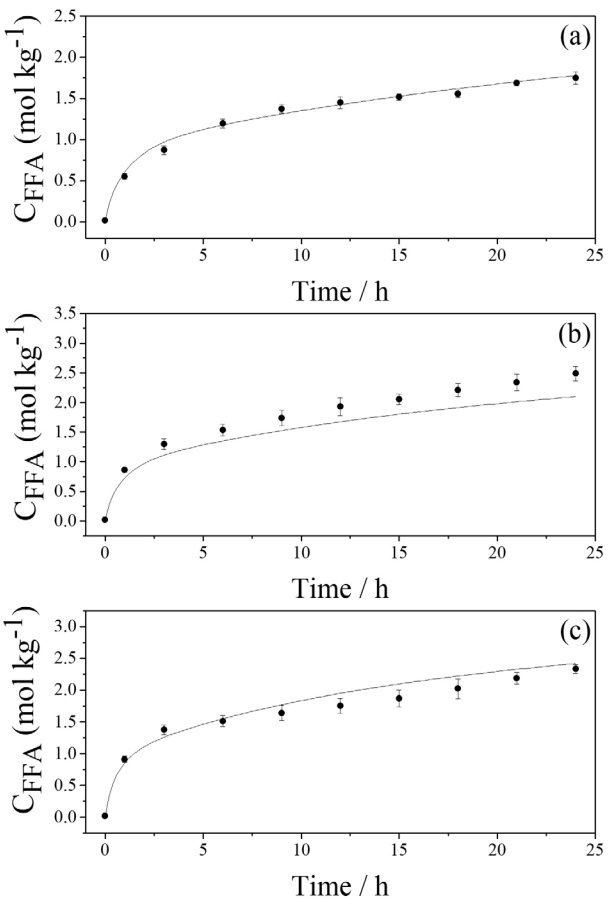

Figure 7. Prediction of the soybean oil enzymatic hydrolysis: (a) temperature 40 °C; (b) temperature 52 °C; (c) temperature 64 °C. Conditions: molar ratio W/O 20:1, enzyme/oil substrate ratio = 1% (m/m), stirring speed = 250 rpm; pH = 6.1. (●) Experimental data; (—) model.

To simulate data in another W/O molar ratio condition it would be necessary to use a new set of experimental data, as the kinetic constants carry an experimental error that may depend on the reaction system used.

Overall, the simplified PPBB model showed good agreement with the experimental data for all assessed temperatures. Besides that, it was capable of well describing the concentration profiles for all compounds (TAG, DAG, MAG, and FFA). Thus, the simplified PPBB model can be used to spare extensive experimentation, as well as the material, energy, and time required to study the process of soybean oil enzymatic hydrolysis. Therefore, the model could support the minimization of the costs related to the number of enzymes to be added. Besides, the employment of such a model leads to well-defined velocity laws, which is an extremely important factor to the applicability in continuous flow processes like a fixed bed reactor, as long as the hydrodynamic conditions in which the velocity laws were determined to remain unchanged.

In short, the application of mathematical modeling to describe the soybean oil enzymatic hydrolysis process by the enzyme TL IM can be considered a useful tool to understand the reaction mechanisms as well as support the phenomenological studies and the process optimization

Conclusions

The present work evaluated the effect of the operational conditions of water/oil molar ratio and temperature on the soybean oil hydrolysis by the action of the Lipozyme® TL IM enzyme's action. It was concluded that using constant stirring speed and enzyme dosage (250 rpm and 1% m/m, respectively), the molar ratios of 46:1 and 60:1 presented the best FFA yields, at temperatures of 52 and 64 °C, which was 77% after 24 h of reaction. Regarding the initial rates, it was observed that the highest temperature within the range tested (64 °C) provided the highest initial rate, for both the best molar ratio conditions achieved (46:1 and 60:1).

Furthermore, a mathematical model based on the simplified Ping-Pong Bi Bi mechanism was tested to describe the experimental kinetic data. By analyzing the

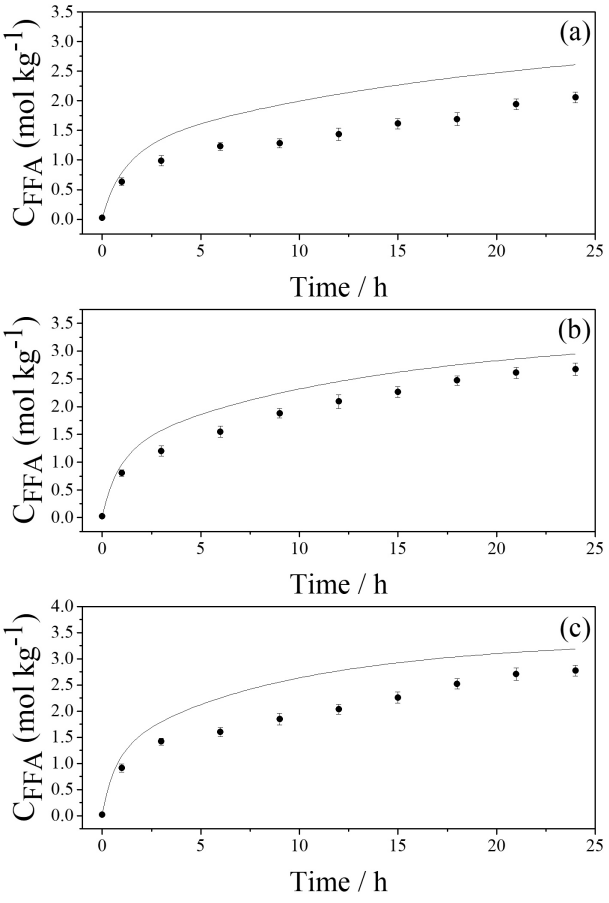

Figure 8. Prediction of the soybean oil enzymatic hydrolysis: (a) temperature 40 °C; (b) temperature 52 °C; (c) temperature 64 °C. Conditions: molar ratio W/O 60:1, enzyme/oil substrate ratio = 1% (m/m), stirring speed = 250 rpm; pH = 6.1. (●) Experimental data; (—) model.

quality parameters, it was noticed that the simplified PPBB model presented a good agreement with the experimental data. Overall, it was observed in the present study that the soybean oil enzymatic hydrolysis is favored by the temperature and by molar ratios with a greater water content within the tested ranges, providing a positive effect on both reaction yield and initial rate. In addition, the proposed models showed a good prediction capacity and thus may be considered useful tools for the optimization of vegetable oil's enzymatic hydrolysis as well as for the phenomenological comprehension of the process mechanisms.

Supplementary Information

Supplementary information (Table S1 with values of the reaction speed coefficients for the temperatures used in the soybean oil hydrolysis process) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Meline G. Porcel was responsible for conceptualization, data curation, investigation, project administration, resources, software, validation, visualization, writing original draft, review and editing; Júnior Staudt for conceptualization, investigation, project administration, software, validation, visualization, writing original draft, writing-review and edition; Gustavo H. Spohr for conceptualization, investigation, project administration, software, validation, visualization, writing original draft, writing-review and editing; Camila da Silva for conceptualization, data curation, formal analysis funding acquisition, investigation, project administration, resources, software, validation, visualization, writing original draft, writing-review and editing; Carlos E. Borba for conceptualization, data curation, formal analysis funding acquisition, investigation, project administration, resources, software, validation, visualization, writing original draft, writing-review and editing.

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