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Effects of the combined use of *Thermomyces lanuginosus* and *Rhizomucor miehei* lipases for the transesterification and hydrolysis of soybean oil

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ABSTRACT

The effects of the combination of immobilized lipases from *Thermomyces lanuginosus* (TLL) and *Rhizomucor miehei* (RML) on the transesterification (ethanolysis) and hydrolysis of soybean oil, a heterogeneous substrate composed of different fatty acids, were investigated. The influences on the yields of conversion of the substrate molar ratio, enzyme content, and the ratio of TLL and RML in the mixture of the biocatalyst were analyzed using the central composite design and the response surface methodology. The optimal conditions for transesterification obtained were: substrate molar ratio of 7.5:1 ethanol:soybean oil; enzyme content of 25% (weight of oil); and 80% of TLL in the mixture of biocatalysts. For hydrolysis, the optimal conditions were: substrate molar ratio of 3:1 water:soybean oil; enzyme content of 25% (weight of oil); and 65% of TLL in the mixture of biocatalysts. Under the optimal conditions, the yields of conversion were 90% for transesterification and 95% for hydrolysis. Time courses of the reactions showed that when using the optimal mixture of lipases, the yields were higher than those obtained using only one of the enzymes, approximately 15% higher than using only TLL and more than twice than using only RML. Enzyme activities remained unaltered for both transesterification and hydrolysis, even after ten reaction cycles in which the immobilized enzymes were washed with n-hexane at the end of each batch. The use of a mixture of immobilized lipases seems to be a promising technology in order to improve the enzymatic synthesis of biodiesel and hydrolysis of oils.

1. Introduction

Biodiesel consists of alkyl esters obtained from the transesterification of triglycerides with short chain alcohols such as methanol and ethanol. Biodiesel has become commercially attractive due to its environmental appeal and to the fact that it is produced from renewable natural resources, especially oils from soybean and other plants [1]. The conventional catalysis of this process requires a high input of energy and produces a mixture of the desired esters, mono- and diglycerides, glycerol, water and the alkaline catalyst (usually CH_3ONa, NaOH or KOH), among other by-products [2]. Sustainable alternatives for biodiesel production are being researched with the use of enzymes, which allow for mild reaction conditions and easier recovery of glycerol, preventing the drawbacks of the chemical synthesis [3]. However, these bioprocesses are costly and need to be optimized in order to economically replace the chemical synthesis [2].

Lipases (EC 3.1.1.3) have a wide range of applications, catalyzing reactions of hydrolysis, acidolysis, esterification, transesterification, and interesterification [4–6]. The main industrial application of lipases is in the hydrolysis of fats and oils [7], although their use in the transesterification of oils for the synthesis of biodiesel is increasing [8,9].

Some enzymatic systems catalyzed by lipases that have been tried in the biodiesel synthesis include: the reaction in the presence of organic solvents [10–12] or enzymes in solvent-free environments [13–15]. There are many researches focused on finding optimal lipases to catalyze this process. However, it must be remembered that the raw materials, natural oils, are not homogeneous substrates, containing triglycerides formed by very different fatty acids. Moreover, the reaction mixture will be formed by triglycerides, regio-isomers of diglycerides or monoglycerides, and even free fatty acids, meaning that the enzymes need to be active for a wide range of different substrates, making it difficult to find an optimal lipase for all likely substrates. Thus, the combined use of several lipases with different specificities could be a way to get an optimal biocatalyst. In the literature, there are reports on the use of a single lipase from several different microorganisms [16–18]; different biocatalysts of the same lipase (i.e., one single enzyme immobilized in different supports) [19]; and the use of a mixture of two or more lipases from different sources [20–22]. The use of immobilized lipases in different supports allows for systems with different stabilities and specificities depending on the support used, the intensity of the enzyme–support interaction, and the orienta-

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tion of the enzyme molecule on the support surface [23–25]. In this context, it is possible to propose that the simultaneous use of two different lipases, from different sources or immobilized on different supports, could improve the biocatalyst action over the different fatty acids present in natural oils.

Apart from the transesterification, the hydrolysis of vegetable oils is also industrially important. The complete hydrolysis of triglycerides will produce fatty acids and glycerol. These fatty acids find several applications such as in the manufacture of soaps, surfactants and detergents, and in the food industry. Since natural substrates are very heterogeneous, the high specificity and selectivity of the enzymes used in the hydrolysis reaction will lead to products of better quality [26–28].

Lipases from *Thermomyces lanuginosus* (TLL) and *Rhizomucor miehei* (RML) have been widely researched in biocatalysis and they are currently commercialized in their soluble and immobilized forms. Recently, the use of these two lipases has been reviewed for their main characteristics and applications, and while recognized as 1,3-specific lipases, their fatty acid specificities are not coincident [29–31]. These enzymes have been selected as model enzymes to show that the mixture of two lipases, although apparently similar, may become a powerful tool to improve the biodiesel synthesis or oil hydrolysis due to the heterogeneity of the substrate. As a model substrate, we have selected one of the most used oils, soybean oil. As any natural oil, it is a heterogeneous compound, and is composed of palmitic acid (11.9%), palmitoleic acid (0.3%), stearic acid (4.1%), oleic acid (23.2%), linoleic acid (54.2%) and linolenic acid (6.3%) [32].

In a previous work of our group, TLL has been modified by chemical amination and it was immobilized by multipoint covalent attachment on glyoxyl-agarose [33], producing a biocatalyst that was more stable than the soluble enzyme, with a high recovery of activity (70%). The same protocol was used to immobilize aminated TLL on Lewatit-aldehyde and this preparation has been used in the biodiesel synthesis showing a good activity in a two-step process [19]. Lipase of *R. miehei* (RML) was also tested for the enzymatic biodiesel synthesis with interesting results, but producing lower yields than those obtained with TLL [18].

Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes. The main advantages of RSM are the reduced number of experiments needed to provide sufficient information for statistically acceptable results, and to be a faster and cheaper method for gathering research data than the classical method of one-variable-at-a-time [34]. The use of RSM has been reported for hydrolysis and biodiesel enzymatic synthesis [15,35–38].

The objectives of the present study were to assess and optimize the lipase-catalyzed transesterification of soybean oil and ethanol using a mixture of immobilized TLL and RML in a solvent-free system, using central composite design (CCD) and response surface methodology. The tested reaction parameters were: the substrate molar ratio; enzyme concentration; TLL/RML ratio (defined as the percentage of TLL in the enzyme mixture). Other parameters such as reaction temperature and water content were not included in this study, since they have been optimized in previous works [15,18]. The dependent variable being measured was the yield of conversion. Additionally, we also tested the hydrolysis catalyzed by the mixture of lipases in order to determine the effects of this strategy in this important reaction.

2. Materials and methods

2.1. Materials

Lipases from *T. lanuginosus* (TLL, Lipolase 100L, soluble form) and *R. miehei* (RML, Lipozyme RM-IM, immobilized form) were purchased from Novozymes (Brazil). TLL was multipoint-covalently immobilized in Lewatit VP OC 1600 (Bayer, Germany) modified to obtain aldehyde groups as described elsewhere [19,33], and the prepared biocatalyst was named Lew-TLL. Activity of the immobilized TLL was adjusted to coincide with the lipase activity of the commercial preparation of RML in the reaction of hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB), as described elsewhere [19]. Therefore, the amounts of enzymes used in the experiments were based on grams of biocatalyst with the same activities over *p*-NPB. Refined soybean oil was purchased at a local market. Ethanol and other chemicals were of analytical or HPLC grade.

Table 1
Process variables and their levels used in the CCD.

Variables	Name	Coded Levels				
		−1.68	−1	0	1	1.68
X_1	Substrate molar ratio (ethanol:soybean oil)	3	4.8	7.5	10.2	12
X_2	Enzyme content (% as oil wt.)	5	9	15	21	25
X_3	TLL/RML ratio (% of TLL in the mix)	0	20	50	80	100

2.2. Methods

Except for the experimental design, all experiments in this research were carried out as triplicates and the calculated standard error was always lower than 5%. In order to avoid any problems related to volume modification during sampling, each experimental point represents one reaction flask that was used as "sample", collected at the desired times.

2.2.1. Transesterification reaction

Varying amounts of ethanol were added to 2.75 mmol of soybean oil, and 4% of water (wt. by oil mass) into 50 mL Erlenmeyer flasks to get different molar ratios, followed by the addition of varying amounts of biocatalysts (Lew-TLL and Lipozyme RM-IM), according to the experimental design. The mixtures of soybean oil, ethanol, water, and lipase were stirred in an orbital shaker (200 rpm) for 10 h at 30 °C.

2.2.2. Hydrolysis reaction

Hydrolysis reaction followed similar protocol and experimental design as for transesterification. Different molar ratios of water were added to 2.75 mmol of soybean oil into 50 mL Erlenmeyer flasks, followed by varying concentrations of biocatalysts (Lew-TLL and Lipozyme RM-IM), according to the experimental design. The mixtures of soybean oil, water and lipase were stirred in an orbital shaker (200 rpm) for 10 h at 30 °C.

2.2.3. HPLC analysis

After the completion of reactions, 5 mL of distilled water was added, followed by centrifugation at 2500 × *g*, 15 min, 4 °C, and the lower phase, containing glycerol, was analyzed by HPLC with its concentration determined using a refractive index (RI) detector (Perkin Elmer Series 200, USA) and a Phenomenex RHM monosaccharide column (300 mm × 7.8 mm) at 80 °C, using ultrapure water as the eluent, flow of 0.6 mL min^{-1}, and sample volume of 20 μL. The yield of conversion was calculated as follows:

$$\text{Conversion yield}\,(\%) = \left[\frac{\text{mmol glycerol}}{\text{mmol initial soybean oil}}\right] \times 100 \quad (1)$$

As glycerol is just formed when the three positions of the triglyceride are attacked, we checked the yield conversions by measuring the ethanol consumption.

2.2.4. Experimental design

A central composite design with three variables was carried out in order to obtain the optimal conditions for transesterification and hydrolysis reactions. The variables and their coded and uncoded values are presented in Table 1. Table 2 shows 18 treatments of the three variables, each at five levels. The design was constructed of eight factorial points, six axial points (two axial points on the axis of design variable), and four replications at the central point. In each case, the percentage of yield of conversion for transesterification and for hydrolysis was determined. The second-order polynomial equation for the variables was as follows:

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

where Y is the response variable, β_0 is the constant, β_i, β_{ii}, β_{ij} are the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j are the coded level of variables x_i and x_j. The above quadratic equation was used to plot surfaces for all variables.

2.2.5. Statistical analysis

The experimental design and analysis of results were carried out using Statistica 7.0 (Statsoft, USA). The statistical analysis of the model was performed as analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities, $p(t)$, was determined by Student's t-test; the second order model

Table 2
Experimental design and results of the CCD.

Treatment	X_1	X_2	X_3	Transesterification conversion (%)	Hydrolysis conversion (%)
1	−1	−1	−1	22.91	33.25
2	−1	−1	1	45.68	41.84
3	−1	1	−1	52.68	59.96
4	−1	1	1	70.66	86.13
5	1	−1	−1	36.19	17.12
6	1	−1	1	49.11	38.67
7	1	1	−1	60.78	34.89
8	1	1	1	78.30	65.12
9	−1.68	0	0	35.16	42.47
10	1.68	0	0	48.37	35.44
11	0	−1.68	0	24.30	13.01
12	0	1.68	0	76.53	73.16
13	0	0	−1.68	36.78	44.62
14	0	0	1.68	63.27	48.04
15 (C)	0	0	0	43.97	38.54
16 (C)	0	0	0	46.48	42.39
17 (C)	0	0	0	45.84	43.30
18 (C)	0	0	0	47.60	41.99

equation significance was determined by Fisher's *F*-test. The variance explained by model is given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots (2D).

2.2.6. Enzyme reuse

After the transesterification or the hydrolysis reaction, the immobilized enzymes were separated from the reaction medium by a simple filtration. The biocatalyst was washed with 20 mL of n-hexane and then dried for 24 h at 40 °C. A parallel experiment was carried out without solvent washing as a control [18].

3. Results and discussion

3.1. Transesterification reaction

3.1.1. Model fitting and ANOVA

As stated in Section 1, in order to be applied in large-scale production, the enzymatic biodiesel synthesis needs to be improved to be competitive with conventional chemical routes. The experiments using the mixture of immobilized TLL and RML were designed to meet these challenges. The results of the central composite design used for optimization of the reaction parameters are presented in Table 2. Among the treatments, the highest conversion (78.3%) was obtained for treatment 8 (10.2 ethanol:soybean oil molar ratio; 21% by oil wt. of enzyme; 80% of TLL in the mixture TLL/RML). The experimental data have been adjusted to the proposed model by the second-order polynomial Eq. (2), and the adequacy of the model was performed by analysis of variance and the parameters R and R^2. The second-order polynomial model to transesterification reaction is presented in Eq. (3).

$$T = 45.75 + 4.00X_1 - 0.50{X_1}^2 + 14.37X_2 + 2.35{X_2}^2 + 8.47X_3 + 2.41{X_3}^2 - 0.12X_1X_2 - 1.28X_1X_3 - 0.02X_2X_3 \quad (3)$$

where T is the percentage of yield of conversion for transesterification reaction, and X_1, X_2, and X_3, are the coded values of substrate molar ratio, enzyme content, and TLL/RML ratio, respectively. Statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA). The computed *F*-value (37.09) was highly significant ($p < 0.0001$). The goodness of a model can be checked by the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient ($R^2 = 0.97$) implies that the sample variation of 97% for biodiesel production is attributed to the independent variables, and can be explained by the model. The closer the value of R (correlation coefficient) is to 1, the better the correlation between the experimental and predicted values. Here, the value of R (0.99) suggests a high representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation.

3.1.2. Effect of parameters on transesterification rates

The linear, quadratic, and the interaction effects of the variables substrate molar ratio, enzyme content, and TLL/RML ratio are presented in Table 3. All linear effects were statistically significant and positive, which indicates that an increase in these variables should positively affect the yields of conversion, with the content of enzyme showing the highest effect. The positive effect of the mixture of TLL/RML suggests that higher amounts of TLL in the mixture will work to enhance the reaction. Although TLL is considered to be more efficient for transesterification, the results showed that a small addition of RML improves the yields of this conversion. This may be explained by the different specificity of each lipase for the diverse fatty acids presented in the soybean oil. Apparently, TLL is more active than RML over most of the tri-, di- and mono-glycerides present in the reaction with soybean oil, but RML could exhibit higher activities on some of them, producing a mixture of lipases much more efficient than the individual ones. For example, Lipozyme RM-IM displays high specificity towards linolenic acid [31], present in soybean oil [32]. The substrate molar ratio was the variable with the lower effect, but it is still important and a high substrate molar ratio (over 7.5:1 alcohol:soybean oil) improves the reaction yields because it ensures high reaction rates and minimizes diffusion limitations [15,18]. The relationship between reaction variables and response can be better understood by examining the series of contour plots depicted in Fig. 1a–c, which were generated from the predicted model. Fig. 1a shows that increasing enzyme content and substrate molar ratio will have a positive effect in the yield of reaction. However, at high substrate molar ratio (over 7.5:1 alcohol:soybean oil) and enzyme content over 15%, an increase in the substrate molar ratio does not enhance the reaction yield. It can be observed in Fig. 1b that the combined increase of enzyme content and TLL/RML ratio caused a correspondent increase in the yields of conversion. Finally, the reaction yields can also be improved with the combination of high substrate molar ratios and increased TLL/RML ratio, as can be seen in Fig. 1c.

3.1.3. Optimal conditions for transesterification and the model validation

The optimal conditions for the transesterification reaction catalyzed by the mixture of TLL and RML were found to be as: 7.5:1 ethanol:soybean oil; 25% as oil wt. of enzyme content; and 80% TLL/RML ratio. Under these conditions, the theoretical value for the yield of reaction predicted by the model is 88.1%, which is higher than the obtained using only Lipozyme TL-IM (immobilized TLL from Novozymes) [39]. Validation of the proposed model was conducted under the optimized conditions. The test was carried out

Table 3
Statistical analysis of the CCD.

Variable	Transesterification			Hydrolysis		
	Effect	Standard error	*p*-Value	Effect	Standard error	*p*-Value
Mean	45.753*	0.758	<0.0001	41.347*	1.040	<0.0001
Linear						
X_1	8.006*	0.822	0.0023	−11.306*	1.128	0.0021
X_2	28.756*	0.822	<0.0001	31.686*	1.128	<0.0001
X_3	16.951*	0.822	0.0002	13.519*	1.128	0.0012
Quadratic						
X_1X_1	−1.004	0.854	0.3247	0.005	1.172	0.9963
X_2X_2	5.110*	0.854	0.0093	2.927	1.172	0.0879
X_3X_3	4.834*	0.854	0.0109	5.220*	1.172	0.0210
Interactions						
X_1X_2	−0.240	1.074	0.8373	−6.694*	1.473	0.0199
X_1X_3	−2.575	1.074	0.0961	4.253	1.473	0.0632
X_2X_3	−0.046	1.074	0.9680	6.561*	1.473	0.0210

* Statistically significant at 95% of confidence level.

with four repetitions and the average yield with the standard deviation obtained was 90.0 ± 2.4%, showing a very good correlation between the experimental results and the statistical predicted by the model.

3.2. Hydrolysis reaction

3.2.1. Model fitting and ANOVA

Based on previous studies [30,31] where RML showed better hydrolytic activity than transesterification, we tested the mixture of RML and TLL in the hydrolysis of soybean oil to assess whether a higher percentage of RML in the mix would improve yields. A similar CCD was carried out, with the same variables (substrate molar ratio – water:soybean oil; enzyme content; and TLL/RML ratio), and the results are presented in Table 2. The highest hydrolysis of 86.13% was obtained for treatment 4 (4.8 water:soybean oil molar ratio; 21% as oil wt. of enzyme; 80% of TLL in the mixture TLL/RML). As for the transesterification, the experimental data have been adjusted to the proposed model by the second-order polynomial Eq. (2) and the second-order polynomial model to hydrolysis reaction is presented in Eq. (4).

$$H = 41.34 - 5.65X_1 - 0.002{X_1}^2 + 15.84X_2 + 1.46{X_2}^2 + 6.75X_3 + 2.61{X_3}^2 - 3.34X_1X_2 + 2.12X_1X_3 + 3.28X_2X_3 \quad (4)$$

where H is the percentage of yield conversion for hydrolysis reaction, and X_1, X_2, and X_3 are the coded values of substrate molar ratio, enzyme content and TLL/RML ratio, respectively.

The computed F-value (7.60) was highly significant ($p = 0.0045$). The goodness of the model was checked by the determination coefficient ($R^2 = 0.89$) and correlation coefficient ($R = 0.94$) showing a satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation.

3.2.2. Effect of parameters on the hydrolysis rates

The effects of the variables on the hydrolysis are presented in Table 3. The relationship between reaction variables and response can be better understood in the series of contour plots depicted in Fig. 2, which was generated from the predicted model. As observed for transesterification, enzyme content was the variable that presented the highest effect on hydrolysis of soybean oil. TLL/RML ratio presented a positive effect, slightly lower than for transesterification, but still showing that a higher amount of TLL in the mixture enhances the reaction yield conversion. Multipointly immobilized TLL on Lewatit is highly active and stable [19], and is one of the best lipases for hydrolysis and transesterification [29]. However, the addition of amounts of RML in the reaction medium produced higher yields of conversion. As for the transesterification, the reasons for this positive effect may be due to the different specificities of these two lipases over tri-, di-, and mono-glycerides present in the soybean oil. Another possibility is the fact that RML preferentially hydrolyzes the ester bond at position $sn-1$ instead of position $sn-3$ [31], synergistically acting with TLL and increasing the reaction rate, while in transesterification, RML preferentially hydrolyzes the ester bond at position $sn-3$, thus competing for the substrate with TLL.

Another interesting result was the effect of substrate molar ratio. This variable presented a negative effect, meaning that an increase

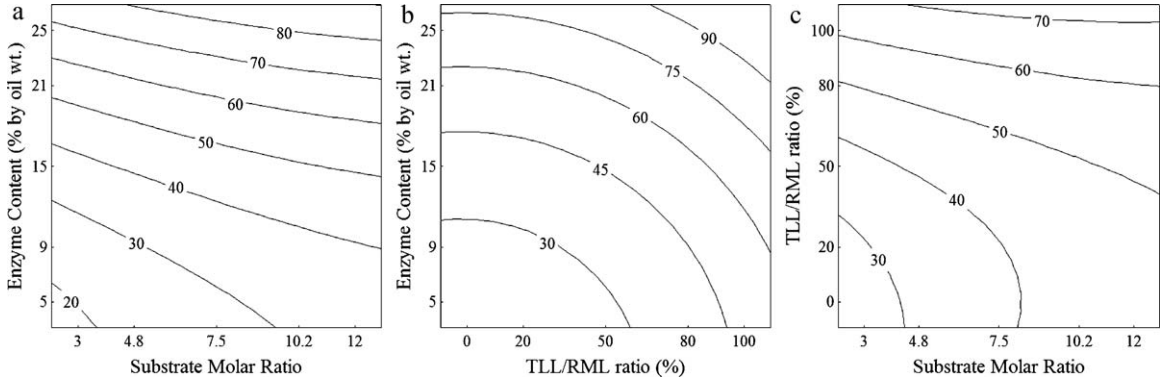

Fig. 1. Contour plots of yields of conversion of transesterification of soybean oil. (a) Substrate molar ratio versus enzyme content. (b) TLL/RML ratio versus enzyme content. (c) Substrate molar ratio versus TLL/RML ratio. The numbers inside the contour plots indicate yields of conversion (%) at given reaction conditions. In each figure, the missing variable was fixed at the central point.

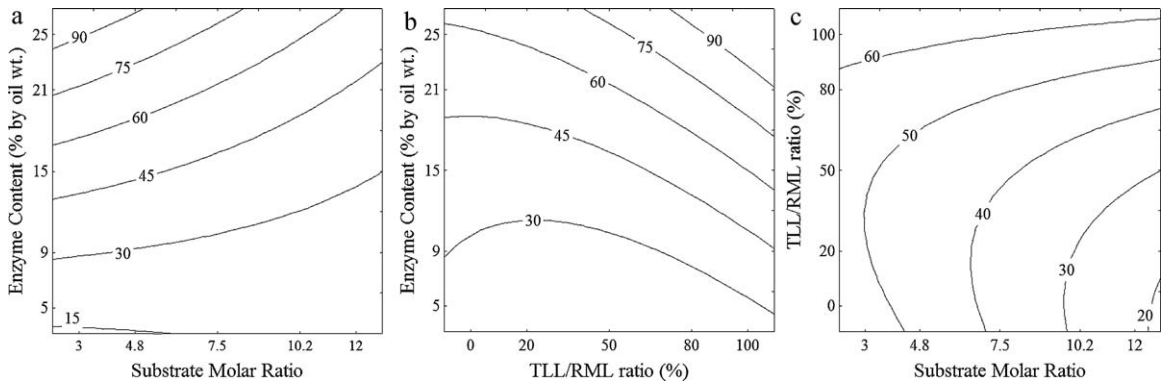

Fig. 2. Contour plots of yields of conversion of hydrolysis of soybean oil. (a) Substrate molar ratio versus enzyme content. (b) TLL/RML ratio versus enzyme content. (c) Substrate molar ratio versus TLL/RML ratio. The numbers inside the contour plots indicate yields of conversion (%) at given reaction conditions. In each figure, the missing variable was fixed at the central point.

in the concentration of water for hydrolysis decreases the reaction yield conversion, which can be seen in Fig. 2a and c. Increasing enzyme content or the TLL/RML ratio, will lead to increased hydrolysis yields. However, this effect is more accentuated for the enzyme content, since it occurs for any substrate molar ratio, while for TLL/RML ratio, the effect is mainly observed at low substrate molar ratio (near the stoichiometric ratio).

3.2.3. Optimal conditions for hydrolysis and model validation

The optimal conditions for hydrolysis reaction catalyzed with the mixture of TLL and RML were found to be as 3:1 water:soybean oil; 25% as oil wt. of enzyme content; and 65% TLL/RML ratio. Under these conditions, the theoretical value for the yield of reaction predicted by the model is 96.4%. The optimal hydrolysis yield was higher than that of transesterification, requiring different substrate molar ratios and TLL/RML ratios, while keeping the same enzyme content for both reactions. Experimental validation of the proposed model was conducted under optimized conditions with four repetitions and the average yield with the standard deviation obtained was $95.7 \pm 3.2\%$, showing excellent correlation between experimental results and the statistical predicted by the model.

3.3. Time course of enzymatic reactions

In order to compare the use of combined or pure lipases, an experiment using the optimal conditions previously defined for transesterification and hydrolysis was carried out with the mixture of TLL/RML and using only either TLL or RML. The time course for transesterification and hydrolysis of soybean oil catalyzed by TLL, RML and the mixture of them is presented in Figs. 3 and 4, respectively. Clearly, the mix of lipases gave the best results for both reactions, around 10% higher than that of TLL and 50% than that of RML for transesterification, and 20% higher than that of TLL and 60% than that of RML for hydrolysis. This result is in agreement with that obtained for the CCD. Comparing the initial reaction rates, it seems again that TLL and RML attack different fatty acids in the oil, because for both transesterification and hydrolysis, yields of con-

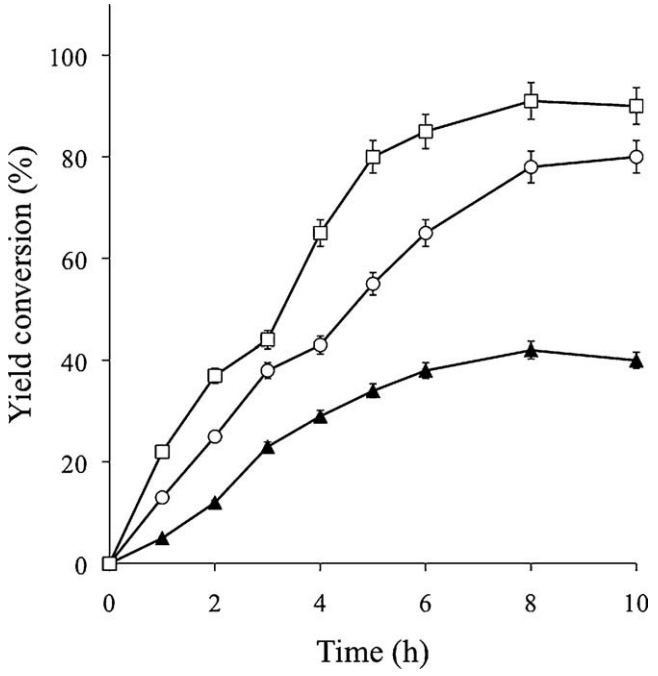

Fig. 3. Time course of transesterification of soybean oil catalyzed by (○) TLL, (▲) RML, (□) and the mixture of TLL/RML. Reaction conditions: substrate molar ratio, 7.5:1 ethanol:soybean oil; enzyme content, 25% as oil wt.; TLL/RML ratio, 80%; and water content, 4% as oil wt.

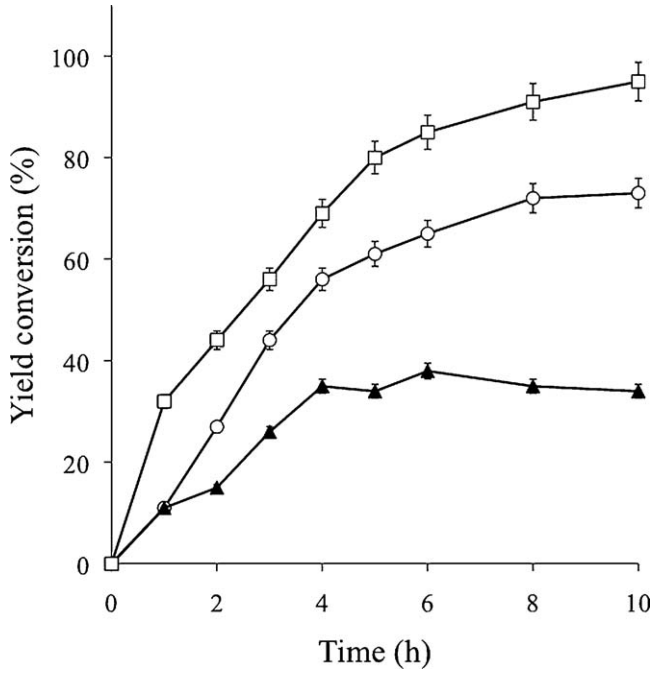

Fig. 4. Time course of hydrolysis of soybean oil catalyzed by (○) TLL, (▲) RML, (□) and mix of TLL/RML. Reaction conditions: substrate molar ratio, 3:1 water:soybean oil; enzyme content, 25% as oil wt.; TLL/RML ratio, 65%.

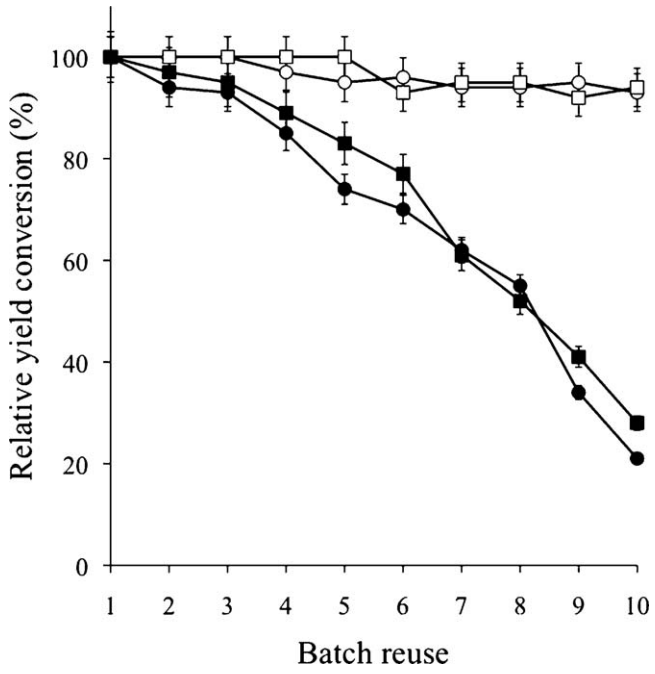

Fig. 5. Enzyme stability over repeated batches of (■, □) transesterification and (●, ○) hydrolysis of soybean oil catalyzed by the mix of TLL/RML. Open symbols are enzymes that were submitted to n-hexane wash. Closed symbols are enzymes without treatment.

versions were higher using the mix TLL/RML than the individual enzymes, which confirm our hypothesis of different specificities of these lipases. Final yield conversions considering the consumption of ethanol were 95% for the optimal mixture, and 83% and 62%, for TLL and RML, respectively, when these enzymes were used alone.

3.4. Enzyme reutilization

For the large scale use of a biocatalyst its stability in the medium is important, which could allow for several batches system reactions. In order to check the viability of this process, the mix of enzymes was submitted to several batches for transesterification and hydrolysis under the optimal conditions defined before. The results of 10 repeated batches are presented in Fig. 5, with their relative activities considering the first batch as 100%. It can be observed that, for both transesterification and hydrolysis, the reaction could be repeated for at least 10 runs without significant losses of activity when the immobilized lipases are washed with n-hexane. As reported before [18], this solvent helps to remove the layer of oil/biodiesel formed around the enzyme that causes loss of activity by limiting substrate and product diffusions [40]. When no washing was applied, the mix of enzyme could be reused 3 times with conversions of more than 90%, suggesting that the washing treatment could be performed after every three batches.

4. Conclusions

We compared the transesterification and hydrolysis of soybean oil catalyzed by a mixture of two lipases, from *T. lanuginosus* and from *R. miehei*. These lipases present good activities when used separately for these reactions [29–31]. The results of this paper show that immobilized TLL is much better than immobilized RML for both reactions. However, the central composite design has shown that the use of a mixture of biocatalysts, with a larger amount of TLL, results in higher yields of conversion than for the use of each enzyme separately. The mechanism action of this process is not clear, but a possibility is the different specificities of these two lipases towards different fatty acids of soybean oil. The combined use of a very active lipase (Lew-TLL) with another enzyme (Lipozyme RM-IM) significantly improved the results. The initial hypothesis regarding the complexity of finding an optimal biocatalyst for a complex raw material formed by different substrates seems to be confirmed. It can be expected that for heterogeneous substrates such as vegetable oils, a mixture of several lipases, two, three, or even more, showing different specificities, will be required for effective transesterification and hydrolysis. Further studies adding new lipases to the mixture of biocatalysts and other substrates (e.g., other vegetable oils, and even artificial homotriglycerides such as triolein and tripalmitin) will be performed to improve the understanding of the effect of this strategy. Our results suggest that the approach of mixing different lipases might be technologically feasible and the enhanced yields will have a positive reduction for the costs of the enzymatic synthesis of biodiesel and the hydrolysis of oils.

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