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**_Combi-lipase_ for heterogeneous substrates: a new approach for hydrolysis of soybean oil using mixtures of biocatalysts**

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**Abstract**

It has been proposed the concept of *combi-lipase* biocatalyst. It is based on the combination of different lipases as biocatalysts in reactions using heterogeneous substrates. The hydrolysis of soybean oil was evaluated as a model substrate, and Novozym 435 (CALB), Lipozyme TL-IM (TLL), and Lipozyme RM-IM (RML) were used as biocatalysts. Results showed that, although individually TLL was the most active enzyme, whereas CALB was the less active one, the combination of 80 % of RML and 20 % of CALB was the best biocatalyst. Reaction parameters were optimized, allowing to obtain more than 80 % of hydrolysis in 24 h using the *combi-lipase*, up from less than 50 % when any individual lipase. Reusability of the *combi-lipase* showed that it could be used for at least 15 cycles without any significant decrease. The concept of *combi*-biocatalyst might be a useful technology for reactions including full modification of heterogeneous substrates.

Keywords: Oil hydrolysis; Novozym 435; Lipozyme TL-IM; Lipozyme TL-IM; soybean oil; combi-lipase biocatalyst.

**1. Introduction**

Fatty acids are important ingredients in the manufacture of coatings, adhesives, and surfactants, which are used in the production of soaps, industrial surfactants, and detergents, as well as in the food industry.[1] Therefore, the hydrolysis of oils and fats for the production of free fatty acids is an industrially relevant process. Traditionally, oil hydrolysis is carried out using chemical catalysts at high temperature and pressure (250 °C and 70 bar), which may produce undesirable reactions, such as oxidation, dehydration of the free fatty acids, or the interesterification of the triglycerides[2]

In this context, there is a great interest to explore the possibilities of lipases as biocatalysts for the production of free fatty acids. Oil hydrolysis catalyzed by lipases can be performed at low temperatures, saving energy, and exhibiting high selectivity, which leads to products with high purity and generating less by-products. There is a great body of research towards finding optimal lipases for the hydrolyses of different oils.[3-11] It has been proposed, for example, to combine 1,3-specific with non-specific lipases to increase the reaction rate by attacking the different positions of triglycerides in the oil composition.[12-14] One important hindrance for the application of this approach is the fact that the fatty acid composition of oils is diverse and usually the main fatty acid accounts for no more than 70 or 80 % of the oil nature, usually much less, meaning that there is an heterogeneous mixture of triglycerides. Other problems that will slow down hydrolyses are the production of diglycerides that may be not easily recognized by the used lipase and fatty acids inhibition. Finally, during oil hydrolysis the reaction pH is generally kept uncontrolled to prevent saponification and to avoid problems during purifications steps, thus, in conclusion, reaction conditions will be heterogeneous and will be changing along the reaction course. Therefore, it could be hypothesized that the full hydrolysis of complex substrates such as vegetable oils, could be better performed using a mixture of biocatalysts made up of different enzymes, with different specificities and activities. It was shown, for instance, that the combined use of 2 different 1,3-specific lipases from *Rhizomucor miehei* (RML) and *Thermomyces laguginosus* (TLL), improved the reaction rate and the yield of the synthesis of biodiesel using soybean oil as substrate.[15]

Some interesting lipases are commercially available. The probably most used biocatalyst by industry is Novozym 435, an immobilized preparation of the lipase B from *Candida antarctica* (CALB) on the hydrophobic resin Lewatit VP OC 1600.[16] Lipozyme TL-IM is another widely used lipase, originally produced by *Thermomyces lanuginosus* (TLL), but industrially obtained from a genetically modified strain of *Aspergillus oryzae.*[17, 18] TLL was immobilized on a cationic silicate via anion exchange[19, 20] and it has been used in multiple reactions.[21] Finally, Lipozyme RM-IM is prepared by the immobilization of the lipase from *Rhizomucor miehei* (RML) on Duolite ES 562, which is a weak anion-exchange resin based on phenol–formaldehyde copolymers.[22-24] RML has been reviewed for its uses, from chemical processes[25] to oils modification.[26]

In this context, the aim of this research was to test the enzymatic hydrolysis of oils based on the design of a "*combi-lipase* biocatalyst" formed by the mixture of the three most commonly used immobilized lipases Novozym 435, Lipozyme RM-IM, and Lipozyme TL-IM. As model substrate, it was chosen soybean oil, the most abundant and one of the cheapest vegetable oils, which has a heterogeneous composition of fatty acids. Central composite design and response surface methodology[27] were used in order to optimize reaction parameters, whereas reusability of the biocatalyst was tested in several batch reactions.

## 2. Material and methods

2.1. Enzymes and other materials

Lipases from *T. lanuginosus* (TLL, Lipozyme TL-IM), *R. miehei* (RML, Lipozyme RM-IM) and *C. antarctica* (CALB, Novozym 435) were kindly donated by Novozymes (Novozymes, Spain). The enzymes were in their immobilized form; TLL was immobilized on a silicate support, RML on an anion-exchange resin, and CALB on a macroporous resin. Refined soybean oil was purchased at a local market, with a reported composition of (as mass fraction): 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 %). All other chemicals were of analytical or HPLC grade.

## 2.2. Methods

Except for the experimental design, all the experiments in this research were carried out as triplicates and the calculated standard error was always under 5 %.

### 2.2.1. Hydrolysis of oil

Different molar ratios of water were added to 5 mmol of soybean oil into 50 mL Erlenmeyer flasks, added of varying concentrations of biocatalysts (TLL, RML, and CALB), according to the experimental design. The mixtures of soybean oil, water, and lipases were stirred in an orbital shaker (200 rpm) for the specific time and temperature. For each point of the experimental design or time course reactions, samples were collected at the desired times to measure the hydrolysis degree. The progress of hydrolysis was monitored by determination of the free fatty acid released by titration of 0.3 g samples using 0.01 M NaOH using phenolphthalein as pH indicator and 5 mL of ethanol as quenching agent.

### 2.2.2. Reactions using the combination of different lipases

In order to determine the optimal combination of lipases for the hydrolysis reaction, a 3-factor mixture design and triangular surface analysis was performed. The simplex-centroid design with interior points composed of 10 experiments is shown in Table 1. The reaction conditions were: substrate molar ratio, 3:1 (water: oil); temperature, 40 ºC; biocatalyst content 10 % (as the oil mass); and the reaction time was of 4 h. The biocatalyst content corresponds to individual or mixtures of lipases according to Table 1.

2.2.3. Central composite design

After selecting the best lipase mixture, a central composite design of 3 variables was carried out in order to obtain the optimal conditions for the hydrolysis reaction. The variables and their coded and uncoded values are presented in Table 2. Table 3 shows 18 treatments of the 3 variables, each at 5 levels. The design was constructed of 8 factorial points, 6 axial points (2 axial points on the axis of design variable), and 4 replications at the central point. In each case, the percentage of conversion for hydrolysis was determined after 4 h. The second-order polynomial equation for the variables is as follows:

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

Where Y is the response variable, $\beta_0$ the constant, $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are the coefficients for the linear, quadratic, and for the interaction effects, respectively, and $X_i$ and $X_j$ the coded levels of variables $x_i$ and $x_j$. The above quadratic equation was used to plot surfaces for all variables.

2.2.4. 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), were determined using the Student's t-test; the second order model equation significance was determined using the Fisher's F-test. The variance explained by model was given by the multiple determination coefficients, $R^2$. For each variable, the quadratic models were represented as contour plots (2D).

2.2.5. Enzyme reuse

After the hydrolysis reaction, the immobilized enzymes were separated from the reaction medium by vacuum filtration using a sintered glass funnel. The biocatalyst was washed 3 times with 5 volumes of n-hexane and the solvent was eliminated by incubation for 24 h at 25 °C.

## 3. Results and Discussion

3.1. Selection of the best *combi-lipase* biocatalyst for soybean oil hydrolysis

Combination of different enzymes is mainly used for cascade or sequential reactions, however, in the present work, is being proposed the design of a *combi-lipase* biocatalyst strategy for the simultaneous hydrolysis of a mixture of different substrates. In Figure 1 is shown the independent hydrolytic activities of the 3 selected immobilized lipases. TLL and RML, both 1,3-regio specific lipases,[21, 26] were more efficient, showing similar activities, whereas CALB, a non-specific lipase,[28] presented a slightly lower activity.

Thus, in order to find the best combination of these enzymes, it was performed a 3-factor simplex-centroid design to found the ideal *combi-lipase* biocatalyst for the hydrolysis of soybean oil. The results obtained for the mixtures design are shown in Table 1, and graphically represented in Figure 2. The lowest conversions were obtained using CALB alone, whereas the highest conversions were observed when higher amounts of RML were used. Mixtures of RML and CALB improved the activity, but this behavior was not observed for mixtures of TLL and CALB, or for TLL and RML. Thus, it is possible to propose that the best *combi-lipase* biocatalyst (among that studied) for hydrolysis of soybean oil is the combination of 80 % of RML and 20 % of CALB.

When used as a single enzyme, both TLL and RML produced the highest activities, their combination, however, did not improve the hydrolysis rate, probably because their similar substrate specificities. However, when CALB, the enzyme showing the lowest activity when used alone, combined in a mixture with RML, the resulting *combi*-biocatalyst improved the conversion rate by 50 %, when compared to the use of RML alone. In a previous report,[15] the mixture of 65 % of TLL and 35 % of RML was found to be the more effective biocatalysts for soybean oil hydrolysis. Under the optimal reaction conditions for the mixture, it was obtained around 70 % of hydrolysis in 4 h in that work. The differences might be explained by the diverse TLL preparations, with TLL covalently immobilized on Lewatit support activated with aldehyde groups,[15] whereas in the present study it was used the commercial TLL form (Lipozyme TL-IM), which is immobilized by adsorption in an anion exchange matrix.[19, 20] These differences regarding nature of support and immobilization protocols are known to greatly affect the enzymes activities.[29-32]

In the next experiments, the hydrolysis of soybean oil was optimized using the *combi-lipase* biocatalyst composed of 80 % of RML and 20 % of CALB.

3.2. Hydrolysis optimization

3.2.1. Model fitting and ANOVA

A CCD was carried out to evaluate the reaction temperature, *combi-lipase* biocatalyst content, and substrate molar ratio (water:soybean oil), and the results are presented in Table 3. The highest hydrolysis conversion was 57.95 % obtained for treatment 8 (54 ºC; 21 % of enzyme relative to oil mass; 10.2 water:soybean oil molar ratio). The experimental data have been adjusted to the proposed model in equation (1) and the second-order polynomial model to hydrolysis reaction is presented in equation (2).

$$Y = 49.49 + 6.21X_1 - 3.55X_1^2 + 0.94X_2 - 2.76X_2^2 + 1.79X_3 + 0.51X_3^2 \quad (2)$$

Where Y is the percentage of conversion for hydrolysis reaction, and $X_1$, $X_2$, and $X_3$, are the coded values of temperature, *combi-lipase* biocatalyst content, and substrate molar ratio, respectively.

The computed F-value (3.01) was statistically significant (p=0.017). The goodness of the model was checked by the determination coefficient ($R^2$=0.77) and correlation coefficient

(R=0.88) 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 linear effects of the variables on the hydrolysis rate were: temperature, 12.43; *combi*-biocatalyst, 1.88; substrate molar ratio, 3.58. All 3 variables presented positive effects, meaning that changing the variable level from -1 to 1 the response was increased. Temperature was the variable showing the highest effect, while the amount of biocatalyst was the lowest. Comparing the experiments where the only change in reaction conditions was the reaction temperature from (36 or 54 ºC; 1 - 5, 2 - 6, 3 - 7, 4 - 8), it can be observed that the hydrolysis rate increased almost 1.5-fold along with the temperature. Increasing temperature improves the enzymatic activity because of higher solubility of oil and its mobility on the porous support. The interactions between variables and their effects on hydrolysis rate are presented in the series of contour plots depicted in Figure 3, which were generated from the predicted model. Figure 3a clearly shows the positive effect of temperature, the optimal being around 54 ºC, whereas the best amount of *combi-lipase* biocatalyst was close to the central value. The latter was the variable presenting the lowest effect of all. The interactions between substrate molar ratio with the amount of biocatalyst (Figure 3b), and with temperature (Figure 3c) strongly suggest that increasing the water content positively affects the hydrolysis rate. Water, which is a substrate of this reaction, is an important factor to keep the enzyme activity and stability. Temperature and amount of biocatalyst showed a V-shaped behavior where at lower substrate molar ratio level, their effects were more pronounced, and at higher water levels the range of temperature and biocatalyst content to obtain the maximal hydrolysis was wider.

3.2.3. Optimal conditions for hydrolysis and model validation

The optimal conditions for the hydrolysis reaction catalyzed using the mixture of RML and CALB (80 % RML and 20 % CALB) were found to be 53 ºC, 16 % of *combi-lipase* biocatalyst relative to oil mass, and a molar ratio of 12:1 water:soybean oil. Under these conditions the theoretical value for the hydrolysis rate of the reaction predicted by the model after 4 h is 57.9 %. Experimental validation of the proposed model was conducted under optimized conditions with four repetitions and the average hydrolysis rate obtained was 60.4 ± 3.2 %, showing an excellent correlation between experimental results and the statistically predicted by the model.

3.3. Time course of soybean oil hydrolysis

The comparison of soybean oil hydrolysis carried out using combi-lipase (80% RML and 20 % CALB) or the specific lipases used alone (TLL, RML and CALB), is presented in Figure 4. In these experiments, the reactions were performed under the optimal conditions defined by the CCD, thus the performances of the individual lipases were slightly better than those represented in Figure 1. The *combi-lipase* biocatalyst was significantly better than individual application of lipases, being 30 % higher than TLL, 35 % higher than RML and 40 % higher than CALB, suggesting that RML and CALB have indeed different specificities regarding the fatty acids forming the glycerides. The results for the *combi-lipase* biocatalysts were also better than for other lipases. Sharma et al.[8] reported the hydrolysis of cod liver oil by *Candida cylindracea* lipase. The authors obtained 26.9 % of FFA yield in their most suitable conditions after 1 h, while we reached to 35 % in 1 h. Yigitoglu and Temoçin[33] performed the hydrolysis of different vegetable oils catalyzed by lipase from *Candida rugosa* immobilized on glutaraldehyde-activated polyester fibers, obtaining as maximum less than 45 mg of fatty acids after 5 h, while in this work it was reached to around 3500 mg in 5 h.

Moreover, these authors stated that the different degree of hydrolysis for each oil is due to impurities or the physical structure of the oil. Nevertheless, as discussed before, it is important to bear in mind that vegetable oils are a mixture of complex substrates formed by triglycerides, and as it was demonstrated, the difference in the hydrolysis degree may be mainly due to the specificity of each lipase to each fatty acid. Rathod and Pandit[34] in the hydrolysis of different vegetable oils (castor, olive and coconut oils) catalyzed by lipolase, soluble preparation of *T. lanuginosus*, obtained as maximum yield less than 50 % after 12 h. Additionally, these authors concluded that as higher the unsaturation degree of the oil as higher the degree of hydrolysis, which reinforce our idea that the lipase specificity is the main point to be observed in hydrolysis reaction and that mixture of lipases as the *combi-lipase* biocatalysts will be better than individual lipases.

### 3.4. Enzyme reuse

The industrial applications of biocatalysts require enzymes stabilities in the reaction medium, allowing several batches reactions. Therefore, the *combi-lipase* biocatalyst was submitted to several hydrolyses batches under the optimal conditions in order to check the viability of a repeated process. In between each batch, it was performed a wash with n-hexane because it has been reported in other works[35, 36] that this solvent is very effective to remove any kind of substrate or product remaining after biocatalyst separation, consequently improving the biocatalyst reusability. The results for the repeated batches are presented in Figure 5, showing that it was possible to use the *combi-lipase* biocatalyst for at least 15 batches keeping over 90 % of its initial activity, suggesting that both enzymes retained their activities. It is important to remark that in this case both biocatalysts has to present operational stability. In other works, Lee et al.[14] reported a decrease of 20 % of the initial activity of the mixture of *R. oryzae* and *C. rugosa* lipases in biodiesel synthesis after 5 uses. For individual enzymes, lipase from *C. rugosa* immobilized on membranes showed a decrease of 12.5 % after 5 cycles used in the hydrolysis of olive oil,[7] and when immobilized on polyester fibers a decrease of 75 % after 10 batches.[33]

## 4. Conclusion

It was proposed a new approach for enzymatic reactions catalyzed by lipases involving complex substrates like vegetable oils. A *combi*-lipase biocatalyst improved the reaction rate when compared to each lipase alone. For the hydrolysis of soybean oil, the best *combi-lipase* biocatalyst is composed by 80 % of RML and 20 % of CALB. TLL, even being the more active lipase, did not improve the properties of the *combi-lipase* biocatalyst. The possibility of using a collection of a biocatalyst from the same lipase with changed properties may be a next step in this research to evaluate the real impact that it may have in the design of these reactions. This new concept may be a very useful technology for food industries in the hydrolysis of vegetable oils.

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**Figure legends**

Figure 1: Time course of hydrolysis of soybean oil catalyzed by (■) TLL, (○) RML, and (▲) CALB. Reaction conditions: substrate molar ratio, 3:1 water:soybean oil; biocatalyst content, 10 % by oil mass; 40 ºC.

Figure 2: Triangular surface for the mixture design. Reaction conditions: substrate molar ratio, 3:1 water:soybean oil; biocatalyst content, 10 % by oil mass; 40 ºC; 4 h.

Figure 3: Contour plots for conversion of hydrolysis of soybean oil. (a) Temperature versus biocatalyst content; (b) Biocatalyst content versus substrate molar ratio; (c) Temperature versus substrate molar ratio. In each figure, the missing variable was fixed at the central point.

Figure 4: Time course of hydrolysis of soybean oil catalyzed by (■) TLL, (○) RML, (▲) CALB, and (×) combi-lipase biocatalyst. Reaction conditions: substrate molar ratio, 12:1 water:soybean oil; enzyme content, 16 % by oil mass; 53 ºC.

Figure 5: Enzyme stability over repeated batches of hydrolysis of soybean oil catalyzed by the combi-lipase biocatalyst.

Table 1: Experiments performed in the mixture design

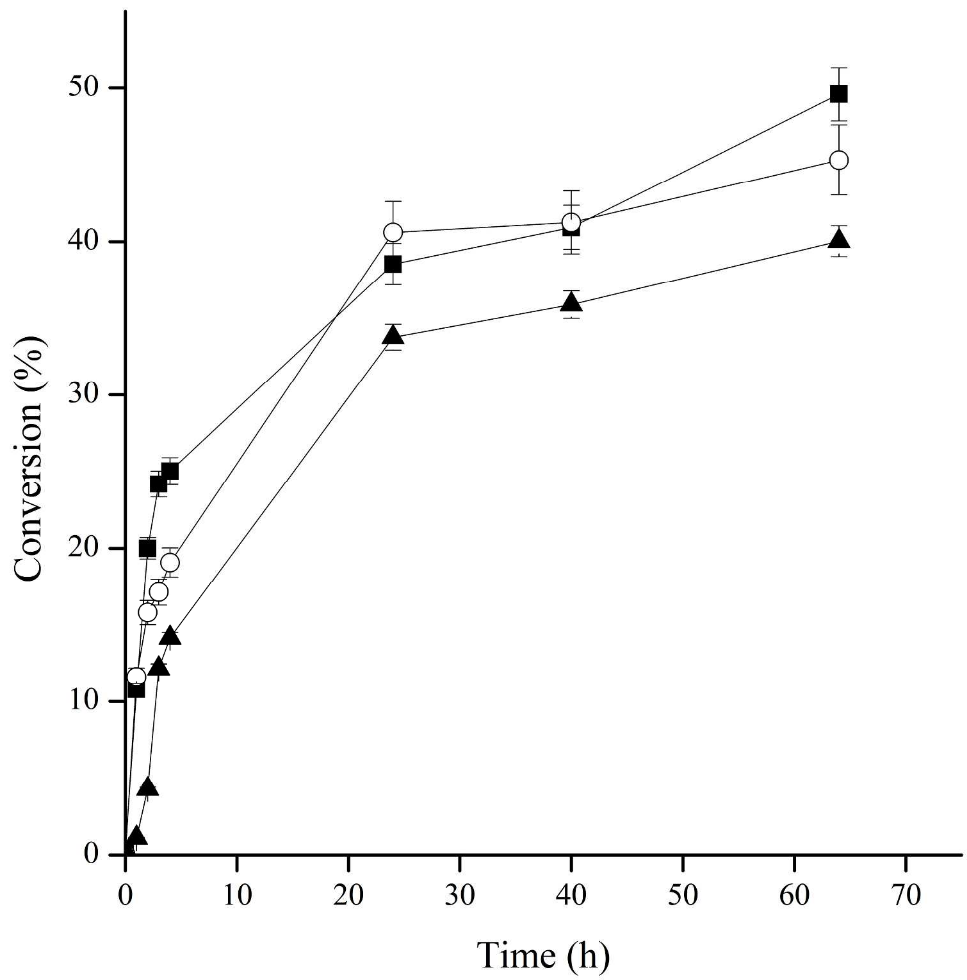
| Experiment | TLL | RML | CALB | Conversion (%) |
|---|---|---|---|---|
| 1 | 1.000 | 0.000 | 0.000 | 21.40 |
| 2 | 0.000 | 1.000 | 0.000 | 30.30 |
| 3 | 0.000 | 0.000 | 1.000 | 4.61 |
| 4 | 0.500 | 0.500 | 0.000 | 21.79 |
| 5 | 0.500 | 0.000 | 0.500 | 13.45 |
| 6 | 0.000 | 0.500 | 0.500 | 23.87 |
| 7 | 0.333 | 0.333 | 0.333 | 22.44 |
| 8 | 0.667 | 0.167 | 0.167 | 24.42 |
| 9 | 0.167 | 0.667 | 0.167 | 25.04 |
| 10 | 0.167 | 0.167 | 0.667 | 24.68 |

Table 2: Process variables and their levels used in the CCD

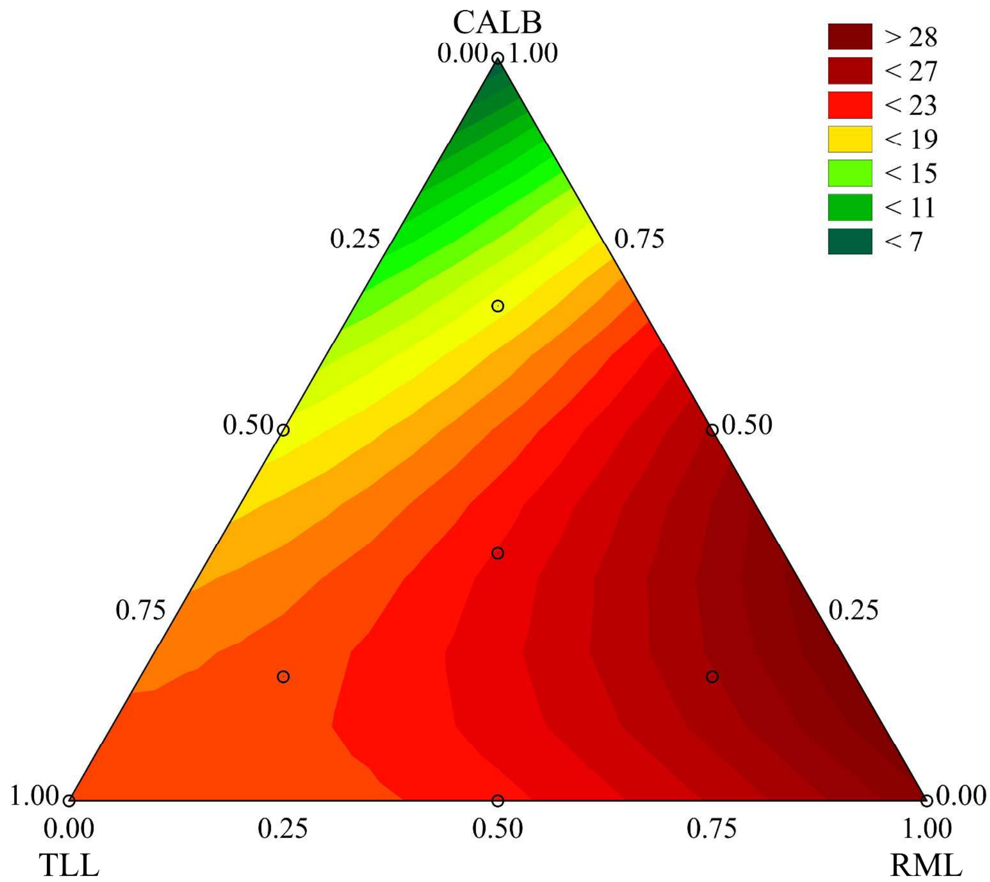
| Variables | Name | Coded Levels | | | | |
|---|---|---|---|---|---|---|
| | | -1.68 | -1 | 0 | 1 | 1.68 |
| $X_1$ | Temperature (ºC) | 30 | 36 | 45 | 54 | 60 |
| $X_2$ | Biocatalyst Content (% relative to the oil mass) | 5 | 9 | 15 | 21 | 25 |
| $X_3$ | Substrate Molar Ratio (water: soybean oil) | 3 | 4.8 | 7.5 | 10.2 | 12 |

Table 3: Experimental design and results of the CCD

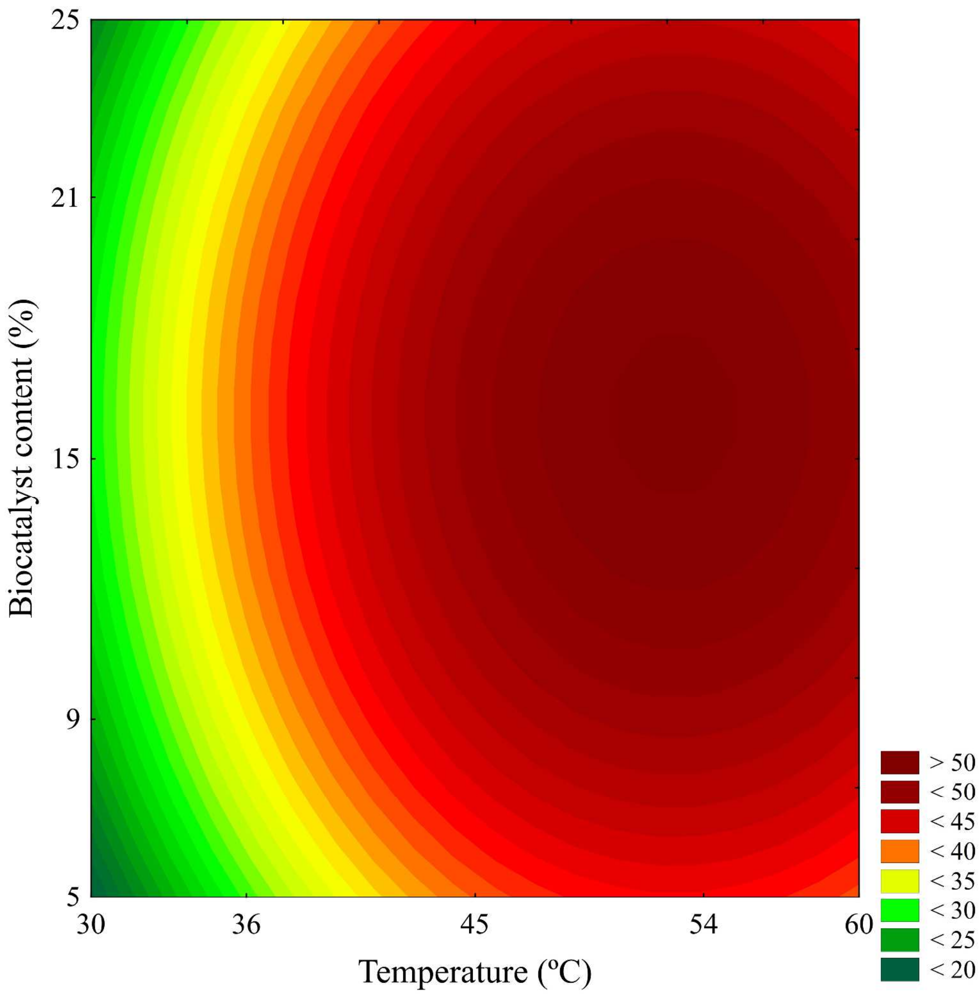
| Treatment | $X_1$ | $X_2$ | $X_3$ | Hydrolysis Conversion (%) |
|---|---|---|---|---|
| 1 | -1 | -1 | -1 | 33.57 |
| 2 | -1 | -1 | 1 | 40.03 |
| 3 | -1 | 1 | -1 | 41.96 |
| 4 | -1 | 1 | 1 | 35.23 |
| 5 | 1 | -1 | -1 | 55.01 |
| 6 | 1 | -1 | 1 | 51.88 |
| 7 | 1 | 1 | -1 | 49.70 |
| 8 | 1 | 1 | 1 | 57.95 |
| 9 | -1.68 | 0 | 0 | 30.35 |
| 10 | 1.68 | 0 | 0 | 42.92 |
| 11 | 0 | -1.68 | 0 | 36.36 |
| 12 | 0 | 1.68 | 0 | 41.41 |
| 13 | 0 | 0 | -1.68 | 42.31 |
| 14 | 0 | 0 | 1.68 | 53.96 |
| 15 (C) | 0 | 0 | 0 | 49.40 |
| 16 (C) | 0 | 0 | 0 | 48.94 |
| 17 (C) | 0 | 0 | 0 | 49.49 |
| 18 (C) | 0 | 0 | 0 | 51.14 |




254x254mm (300 x 300 DPI)




1031x1031mm (96 x 96 DPI)

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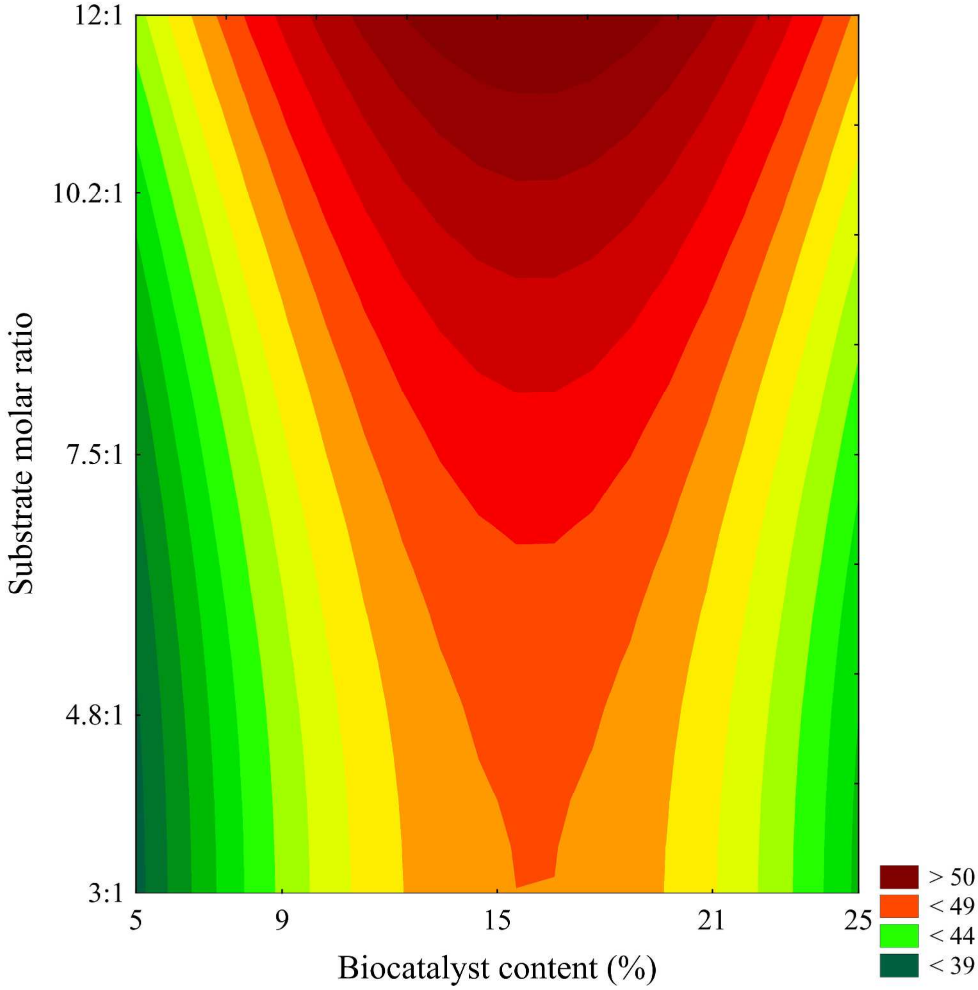


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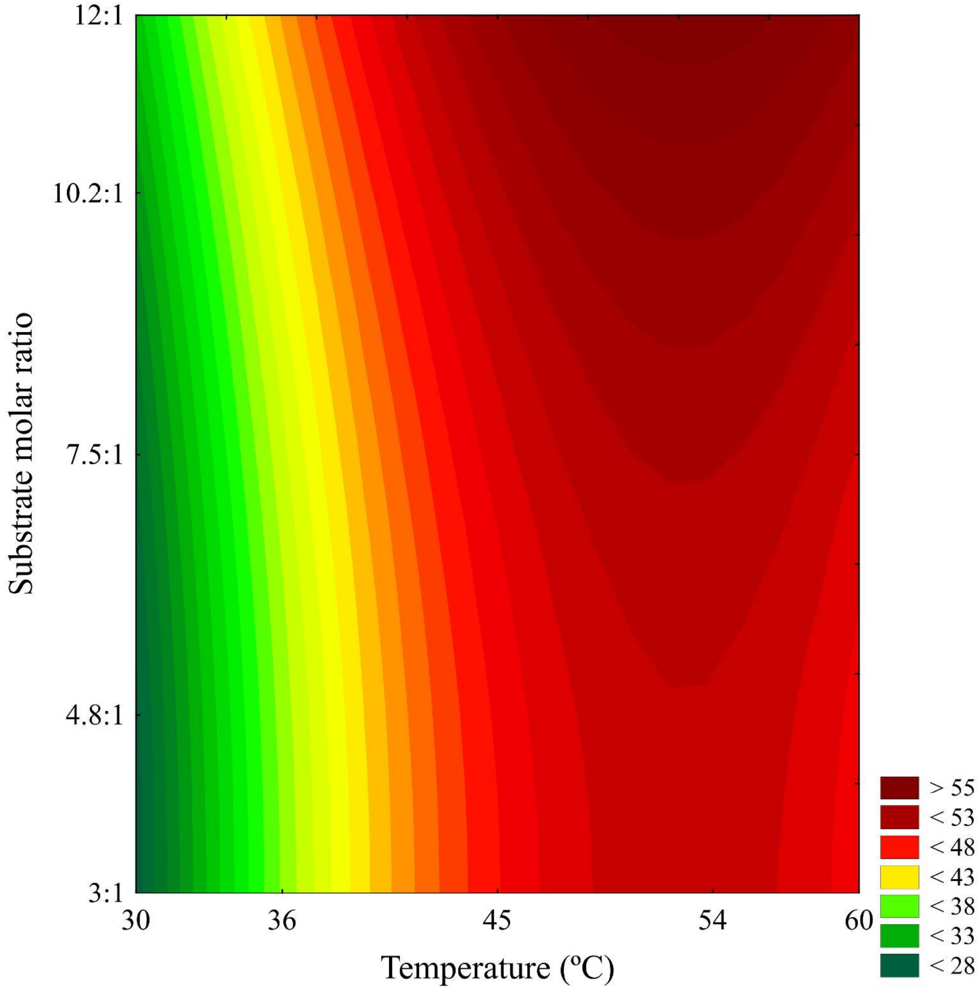


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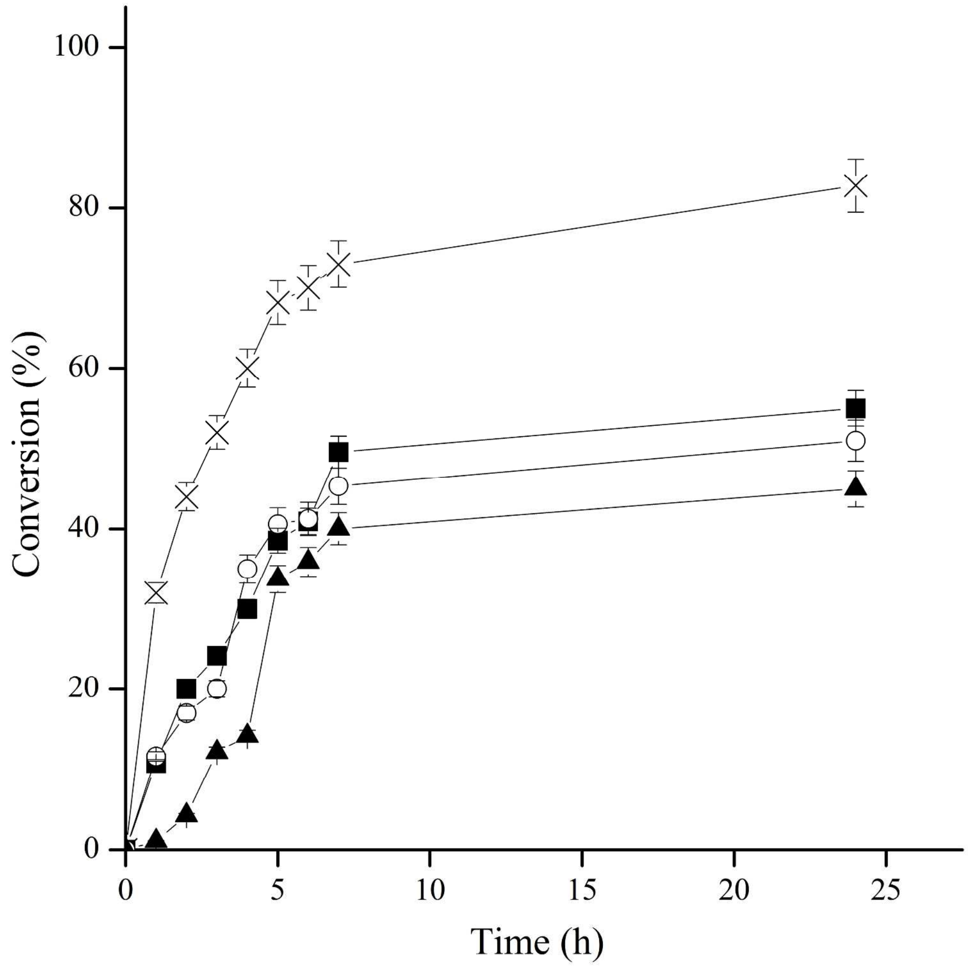


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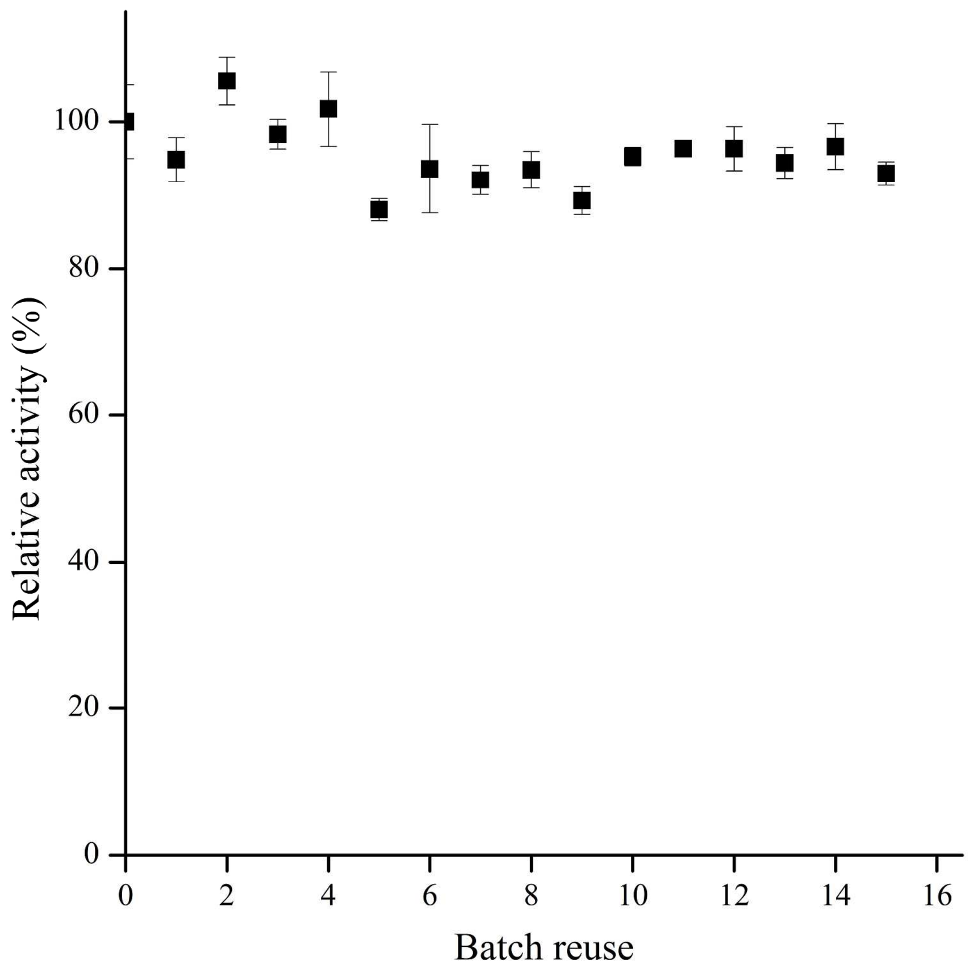


254x254mm (300 x 300 DPI)