

## Article

# Optimization of the Full Hydrolysis of Babassu Oil by Combi-Lipases

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**Abstract:** The concept of combi-lipases is herein explored in the full hydrolysis of babassu oil. The commercially immobilized lipases from *Candida antarctica* (form B) (Novozym<sup>®</sup> 435), *Rhizomucor miehei* (Lipozyme<sup>®</sup> RM-IM), and *Thermomyces lanuginosus* (Lipozyme<sup>®</sup> TL-IM) were evaluated as single and combined biocatalysts by a mixture design with triangular surface. As a result, after evaluating the response desirability profiling for all biocatalysts, the best biocatalyst in the reaction was the combi-lipases composed of 75% of Lipozyme<sup>®</sup> RM-IM, 17% of Novozym<sup>®</sup> 435, and 8% of Lipozyme<sup>®</sup> TL-IM, reaching full hydrolysis (>99%) after 4 h of reaction. Subsequently, such combi-lipases were employed as biocatalysts in the optimization of the reaction in a shorter reaction time (3 h). After optimization by the Taguchi method, full hydrolysis (>99%) was reached under optimized reaction conditions (9 wt.% of biocatalyst content, 1:2 (oil/water), 40 °C, and 180 rpm). Under such conditions, the combi-lipases maintained 70% of their initial activity after 10 reaction cycles. The antimicrobial activity against some of the most common environmental bacteria of the obtained free fatty acids (FFAs) was also evaluated. The FFAs inhibited more than 90% of the growth of *S. aureus*, *E. coli*, and *P. aeruginosus* when using 10 mg FFAs/mL.

**Keywords:** combi-lipases; babassu oil; optimization; antimicrobial activity



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## 1. Introduction

The hydrolysis of vegetable oils is a reaction in which water molecules break the ester bonds in triacylglycerols, the main components of vegetable oils, releasing free fatty acids (FFAs) and glycerol [1–5]. The hydrolysis of vegetable oils may be conducted by chemical [6–8] and biological [9,10] catalysts or even by (bio)catalyst-free processes [11–13]. For instance, the hydrolysis of vegetable oils catalyzed by strong acids (e.g., sulfuric acid or hydrochloric acid) poses the advantage of using residual vegetable oils, which are usually highly acidic feedstocks, without forming soap [14–16]. Nevertheless, it also has considerable disadvantages, such as drastic reactive conditions, requiring high temperatures, along with the corrosion of equipment caused by using strong acids, increasing process costs and necessitating specific safety measures [7,14]. Additionally, acid hydrolysis may produce undesired by-products, excluding its application in industries that require highly pure

FFAs [17,18]. The disposal of acidic wastes also poses an environmental challenge, as they must be neutralized before disposal to prevent alteration of the environment [9,10,19].

In this context, the biological hydrolysis of vegetable oils by enzymes (e.g., lipases) stands out due to its milder reaction conditions (i.e., temperature, pressure, and pH), thus reducing costs and environmental impacts, and preventing FFA unspecific modifications [20–25]. In comparison to chemical hydrolysis, enzymatic hydrolysis of vegetable oils provides greater control over the composition of the released FFAs, thereby being regarded as an eco-friendly alternative to produce bioactive compounds [26–30]. Lipases stand out as the most widely used biological catalysts for this purpose due to their ability to catalyze the hydrolysis of insoluble vegetable oils [31–35]. However, the substrate specificity of lipases may present some limitations when dealing with vegetable oils, as they are heterogeneous substrates [36–40]. Due to this enzyme specificity, some specific triglycerides (for example, the main ones in the composition of a specific oil) in the oil may be rapidly hydrolyzed by a particular enzyme (that is, it is a good substrate for this enzyme), while some others (perhaps a minority in a particular oil) may not be recognized by this enzyme or even be recognized but hydrolyzed with very low efficiency (becoming an inhibitor, or at least a very bad substrate). This can result in incomplete or ineffective hydrolytic processes using only one enzyme [41–44]. To overcome such limitations, the combination of several lipases (known as combi-lipases) with different substrate specificities may be employed to fully hydrolyze vegetable oils [45,46]. Beyond the hydrolysis of vegetable oils, combi-lipases have also been applied in the production of biodiesel [47–49], structured lipids [50–52] and structured phenolic lipids [53]. Herein, we explored the performance of single and combined commercially immobilized lipases in the hydrolysis of babassu oil. The lipases from *Candida antarctica* B (CALB), *Rhizomucor miehei* (RML), and *Thermomyces lanuginosus* (TLL) were chosen as model lipases as they are among the most widely employed biocatalysts in the hydrolysis of vegetable oils [19,54–60].

Babassu is a palm tree native to South America (it may be mainly found in the Northeastern and Northern regions of Brazil) [61,62]. Cultivation of the babassu palm tree has a positive social and ecological impact by generating income for families and, thus, preventing rural exodus and increasing the fertility of the soil [62,63]. According to the Brazilian Institute for Geography and Statistics, the average production of babassu oil was around 26,000 tons in 2023 in Brazil [64]. Even though babassu oil may be used for cooking, it is mainly composed of saturated fatty acids, so its use as an edible oil is restricted due to potential health issues. Therefore, babassu oil has been used as a feedstock for other applications (e.g., production of biodiesel, biolubricant, cosmetics, and pharmaceuticals) rather than in the food industry [65–69]. It is mainly composed of lauric acid (approximately 50%), which poses antimicrobial activity against several bacteria, fungi, and viruses [70–74]. Regarding its antibacterial activity, lauric acid is a lipophilic and hydrophilic saturated medium-chain fatty acid; thus, the amphiphilic nature of such bioactive compound allows for attacking the structure of the cell membranes, ultimately releasing cytoplasmic fluid and decreasing the cell activity of the bacteria [75,76]. In this study, the antimicrobial activity of the FFAs from babassu oil was evaluated considering three of the most common environmental bacteria (*S. aureus*, *E. coli*, and *P. aeruginosus*).

The Improvement of the hydrolysis of vegetable oils goes beyond the selection of the proper biocatalyst; some important reaction parameters must be evaluated (e.g., biocatalyst content, oil/water molar ratio, reactor temperature, and stirring). As such, experimental methodologies aiming to mitigate drawbacks related to longer and/or high-cost (bio)chemical processes often observed in the academic and industrial sectors have been under continuous improvement [77,78]. Through the statistical Design of Experiments (DoE), enhanced knowledge about some reaction factors may be obtained, thereby leading

engineers and/or scientists to better responses even in low-resource scenarios [79,80]. The Response Surface Methodology (RSM) is an effective DoE strategy widely employed to determine the influence of multi-factors, under pre-selected criteria, that can maximize or minimize a desired target (e.g., conversion and yield) [81]. Additionally, the mixture designs with triangular surfaces, a particular type of RSM, may be used to control the best proportion of components in a blend, thus offering multiple possibilities for parameter proportions that may be successfully applied to specific experimental outcomes [81]. Another methodology broadly applied in science and industry is the Taguchi design, which is also world-famous for achieving high-quality experimental results at a low cost [82–84]. The Taguchi method differs from the classical approaches by being focused on the variability of parameters and how it can interact with the main result, establishing low-cost experiment design and maintaining a high-quality output [85,86]. Developed by Dr. G. Taguchi, such robust DoE based on orthogonal arrays is used to reduce the variance in experiments by controlling the signal-to-noise (S/N) ratio, optimizing the design of factors to achieve the desired mean for the output variable [84,87]. S/N ratios are generally used to determine the level of each reaction factor that can optimize the response [87]. Therefore, the Taguchi orthogonal arrays are suitable strategies to rapidly evaluate the influence of some independent variables on a desired dependent variable, with reduced time, labor, energy, and reagents [82,88]. In this study, we employed the standard L9 orthogonal array, which analyzes four independent variables at three levels each in nine runs [82,88].

That way, in this study, the enzymatic hydrolysis of babassu oil was evaluated employing commercially immobilized CALB (Novozym 435), RML (Lipozyme RM-IM), and TLL (Lipozyme TL-IM). These lipase biocatalysts were evaluated as single biocatalysts or combi-lipases, with combinations determined by a mixture design (3-factors simplex-centroid design with interior points) under standard conditions. The best biocatalyst formulation was then determined by triangular surface methodology. After selecting the best biocatalyst, some reaction conditions (biocatalyst content, substrates ratio (oil/water), reactor temperature, and stirring rate) were optimized by an L9 orthogonal array of the Taguchi method. Then, the operational stability of the best biocatalyst was determined under optimized reaction conditions. Finally, the activity of the produced FFAs against common bacteria was determined.

## 2. Results and Discussion

### 2.1. Evaluating the Different Biocatalysts in the Hydrolysis of Babassu Oil

TL-IM, RM-IM, and Novozym 435 were employed individually or in combination as biocatalysts in the hydrolysis of babassu oil under fixed reaction conditions (5 wt.% of biocatalysts content, 1:2 (oil/water, *w/w*), 40 °C, 180 rpm, and 1–4 h), as depicted in Table 1. The set of reaction factors employed in the evaluation of the best biocatalyst will be used as the intermediate level of the orthogonal array to determine the best reaction conditions for hydrolyzing babassu oil, as depicted in the following section.

According to Table 1, the best individual or combi-biocatalyst after 1 h of reaction was 100% of RML-IM, reaching up to 58% of hydrolysis yield, followed by the combi-lipases composed of 16.67% of TLL-IM, 66.67% of RML and 16.67% of Novozym 435 with a hydrolysis yield of 52.85%. This behavior was also maintained after 2 h of reaction; however, it changed after 3 h of reaction. After this time, the best biocatalyst formulation was the combi-lipases composed of 50% of RML-IM and 50% of Novozym 435, followed by the combi-lipases composed of 16.67% of TLL-IM, 66.67% of RML and 16.67% of Novozym 435 and, finally, the 100% RML-IM biocatalyst. Nevertheless, it was not possible to reach full hydrolysis yield for any of the biocatalysts (alone or in combination) in the first 3 h of reaction. Indeed, it was only possible to reach approximately full hydrolysis (>99%) of

babassu oil after 4 h of reaction employing either the 50% of RML-IM and 50% of Novozym 435 combi-lipases or 16.67% of TLL-IM, 66.67% of RML, and 16.67% of Novozym 435. The 100% RML-IM biocatalyst, which was the best one in the first hour of reaction, reached only 85% of the hydrolysis yield.

**Table 1.** 3-factors simplex-centroid design with interior points. Reaction conditions: 5 wt.% of biocatalysts content, 1:2 (oil/ water, *w/w*), 40 °C, 180 rpm, and 1–4 h. Novozym 435: commercially immobilized lipase B from *Candida antarctica*; RML-IM: commercially immobilized lipase from *Rhizomucor miehei*; TLL-IM: commercially immobilized lipase from *Thermomyces lanuginosus*.

| Run | TLL-IM | RML-IM | Novozym 435 | Hydrolysis (%) |            |            |            |
|-----|--------|--------|-------------|----------------|------------|------------|------------|
|     |        |        |             | 1 h            | 2 h        | 3 h        | 4 h        |
| 1   | 100.00 | 0.00   | 0.00        | 10.2 ± 0.2     | 11.1 ± 0.2 | 19.8 ± 0.4 | 24.5 ± 0.5 |
| 2   | 0.00   | 100.00 | 0.00        | 58.1 ± 2.4     | 70.8 ± 3.1 | 78.2 ± 3.5 | 85.0 ± 3.7 |
| 3   | 0.00   | 0.00   | 100.00      | 20.9 ± 0.4     | 27.3 ± 0.5 | 32.8 ± 0.7 | 43.5 ± 1.4 |
| 4   | 50.00  | 50.00  | 0.00        | 42.5 ± 1.2     | 58.9 ± 2.5 | 68.3 ± 2.7 | 73.4 ± 3.4 |
| 5   | 50.00  | 0.00   | 50.00       | 21.1 ± 0.4     | 33.5 ± 0.6 | 39.1 ± 0.9 | 45.1 ± 1.4 |
| 6   | 0.00   | 50.00  | 50.00       | 38.8 ± 0.8     | 62.5 ± 2.6 | 84.1 ± 3.6 | 99.6 ± 4.4 |
| 7   | 33.33  | 33.33  | 33.33       | 43.0 ± 1.2     | 59.5 ± 2.5 | 72.3 ± 3.1 | 85.2 ± 3.7 |
| 8   | 66.67  | 16.67  | 16.67       | 25.9 ± 0.5     | 43.0 ± 1.3 | 54.2 ± 2.3 | 66.6 ± 2.5 |
| 9   | 16.67  | 66.67  | 16.67       | 52.8 ± 2.2     | 71.0 ± 3.1 | 82.1 ± 3.6 | 99.9 ± 4.5 |
| 10  | 16.67  | 16.67  | 66.67       | 36.2 ± 0.8     | 60.6 ± 2.5 | 71.2 ± 3.1 | 93.4 ± 4.1 |

In any case, to determine the best biocatalyst to hydrolyze babassu oil among those evaluated herein (Table 1), based on the fitted triangular surfaces of Figure 1, a response desirability profiling analysis was conducted (Figure 2), which is a statistic tool used to analyze the multi-response optimization of an input variable (the biocatalyst composition, in this study) with a desirability score ranging from 0 to 1 or, in this study, from 0 to 100% of hydrolysis yield. As such, in this study, the desirability profiling target is to reach the highest score, which is 1 or in the terms of this study, 100% hydrolysis yield. In Figure 2a–d, the best concentration (x-axis) of any immobilized lipase under study (Novozym 435, RML-IM, and TLL-IM) in the final biocatalyst (combi-lipase or not) is determined by the peak of the highest hydrolysis yield (y-axis).

As a result, as depicted in Figure 2a, 100% of RML-IM was selected as the best biocatalyst after 1 h of reaction, as in the analysis of Table 1. Indeed, the hydrolysis yield after 1 h of reaction (50%) was achieved using a biocatalyst composed of 100% RML-IM, whereas the concentration of Novozym 435 and TLL-IM in this biocatalyst should be 0% to reach such a hydrolysis yield. The same approach was employed to determine the best biocatalyst (combi-lipase or not) in the following hours of reaction. For instance, after 2 h of reaction, the best biocatalyst determined by the response desirability profiling analysis was slightly different from the one determined by the analysis of Table 1. Accordingly, as depicted in Figure 2b, the best biocatalyst was the combi-lipase composed of 75% RML-IM + 16% of Novozym 435 and 9% of TLL-IM. After 3 h of reaction, 67% RML-IM + 33% of Novozym 435 was the best biocatalyst, as depicted in Figure 2c, along with 75% RML-IM + 17% of Novozym 435 and 8% of TLL-IM (after 4 h of hydrolysis), as depicted in Figure 2d. Therefore, the 75% RML-IM + 17% of Novozym 435 and 8% of TLL-IM combi-lipase was selected as the best biocatalyst for further optimization studies as it was the only one to reach full hydrolysis yield of babassu oil.

Combi-lipases have been successfully used in other oil hydrolysis research. For example, RM-IM, Novozym 435, and TL-IM were employed as biocatalysts (alone or combined) in the hydrolysis of soybean oil [45]. Prior to reaction condition optimization, the best biocatalyst (80% of RM-IM and 20% of Novozym 435) was determined by a mixture

design with triangular surfaces, reaching no more than 30% of conversion after 4 h of reaction at 40 °C and 200 rpm for a substrate molar ratio of 1:3 (oil/water) and 10 wt.% of biocatalyst content [45]. In another instance, the same biocatalysts were employed (alone or combined) in the hydrolysis of virgin coconut oil and, prior to reaction condition optimization, it was possible to reach hydrolysis yields not higher than 35% after 1 h of reaction under ultrasonic treatment (37 kHz and 300 W) and 30 °C employing a substrate molar ratio of 1:2 molar ratio (oil/water) and a biocatalyst content of 10 wt.%, which was composed of RML-IM (75%) and Novozym 435 (25%) [44]. Coconut oil and babassu oil have similar FFA profiles, which may justify the similar best biocatalyst for both reactions; however, the combi-lipase (8% TL-IM + 17% Novozym 435 + 75% RM-IM) proposed herein exhibited better performance and lower energy demand than previous examples.

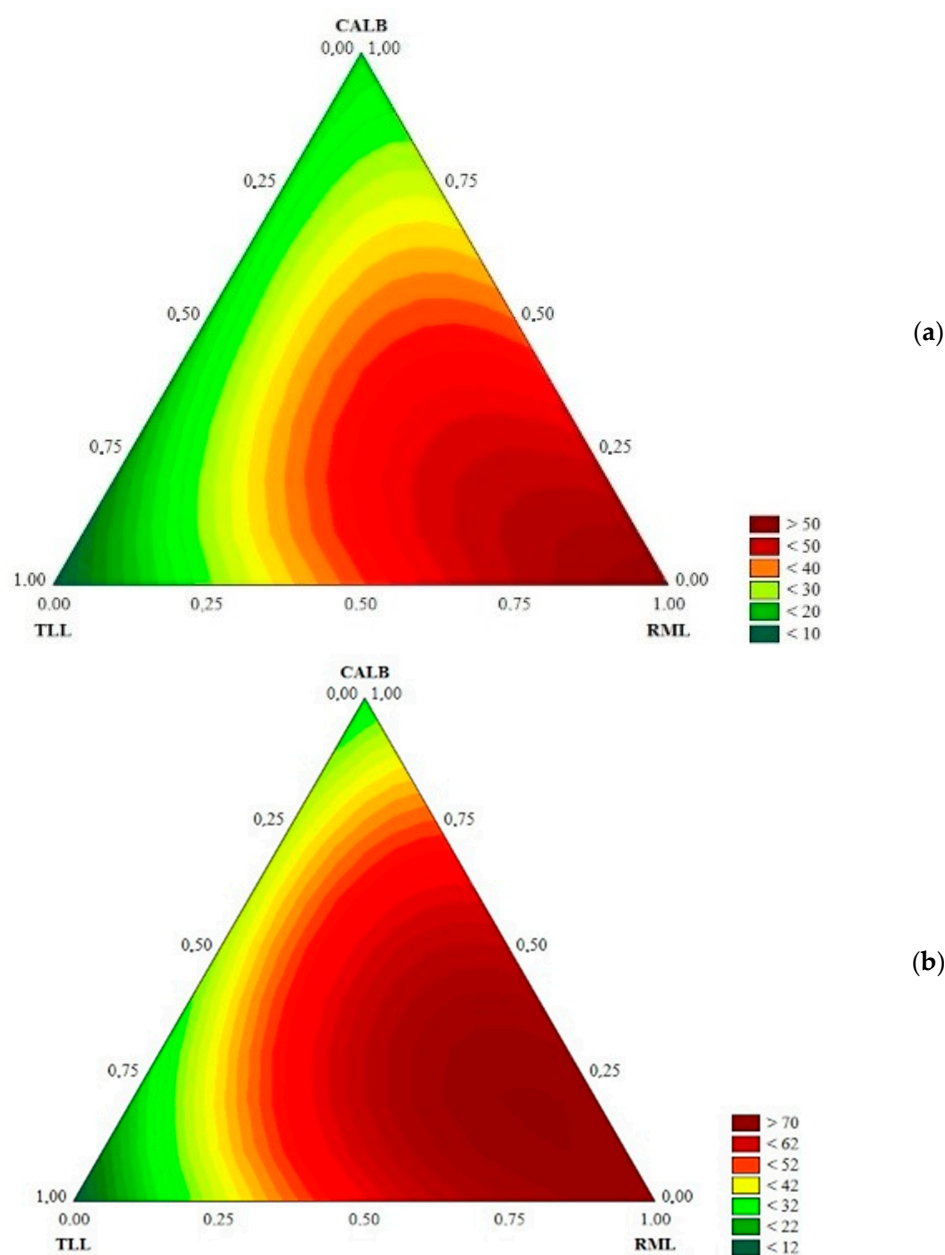


Figure 1. Cont.

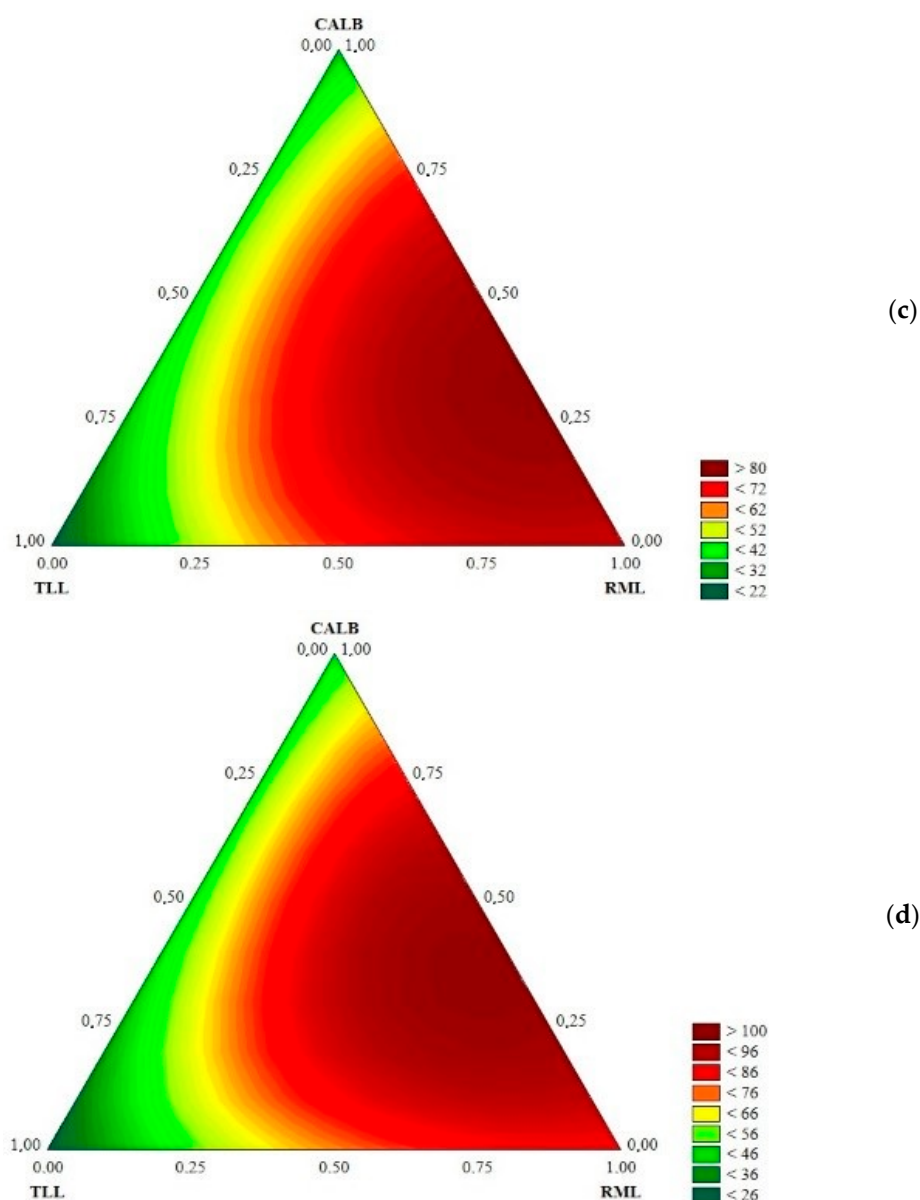


**Figure 1.** Fitted triangular surface for the 3-factor simplex-centroid design with interior points. Reaction conditions: 5 wt.% of biocatalysts content, 1:2 (oil/water,  $w/w$ ), 40 °C, 180 rpm, and 1 h (a), 2 h (b), 3 h (c), and 4 h (d). CALB (Novozym 345): Commercially immobilized lipase B from *Candida antarctica*; RML (Lipozyme RM-IM): Commercially immobilized lipase from *Rhizomucor miehei*; TLL (Lipozyme TL IM): Commercially immobilized lipase from *Thermomyces lanuginosus*.

These results were apparently surprising, as using the initial reaction rates, RML-IM almost triplicated Novozym 435 and was over five-fold more efficient than TLL-IM. However, considering that each enzyme may have different efficiency versus different glycerides and that some can even be inhibitors of the main enzyme, it is possible to predict that the effect of using mixtures of lipases will be visualized when the reaction advances, as in this situation, the percentage of bad substrate or even inhibitor components of the glycerides will increase. This way, the percentage of substrate modification used to select the optimal biocatalyst is a key point in the process. Too low conversions can hide the minority presence of components that can have a negative effect on the enzyme performance, and too high modification levels do not permit perceiving differences between the different biocatalyst formulations. The use of several reaction times can help to perform a much better selection of the optimal biocatalyst formulation. Several factors may explain

the different performance of a determined biocatalyst performance specificity, selectivity, inhibition and/or denaturation [36].

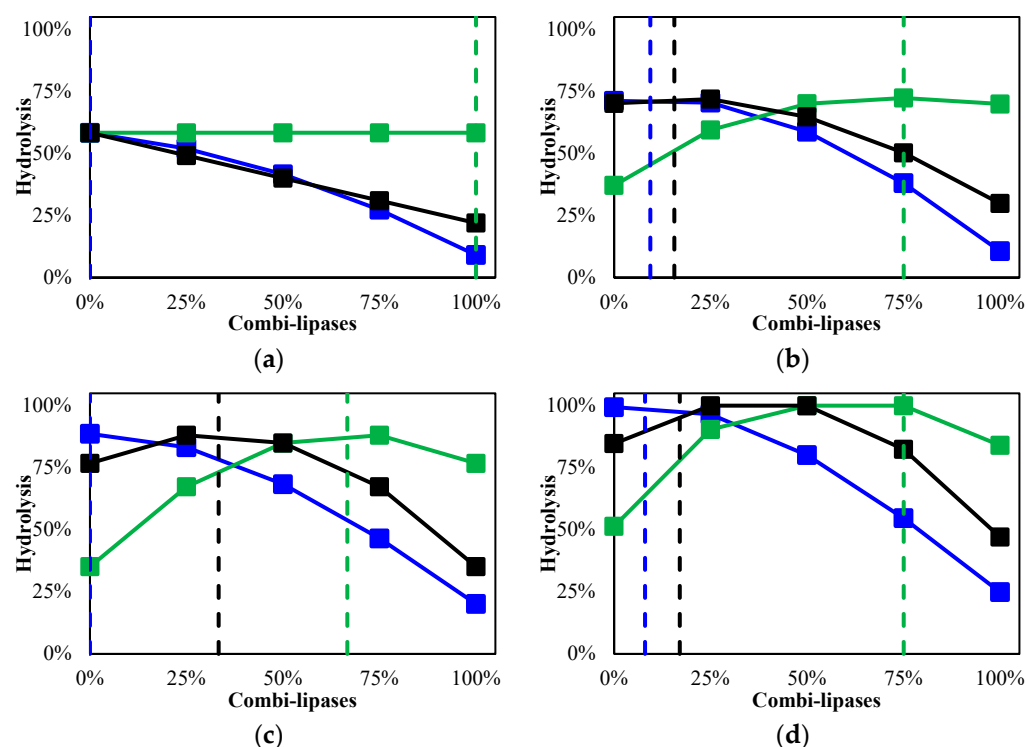


**Figure 2.** Response desirability profiling for Novozym 435, RML-IM and TLL-IM. Reaction conditions: 5 wt.% of biocatalysts content, 1:2 (oil/water,  $w/w$ ), 40 °C, 180 rpm and 1 h (a), 2 h (b), 3 h (c) and 4 h (d). Novozym 435: Commercially immobilized lipase B from *Candida antarctica* (black line); RML-IM (Lipozyme RM-IL): Commercially immobilized lipase from *Rhizomucor miehei* (green line); TLL (Lipozyme TL-IM): Commercially immobilized lipase from *Thermomyces lanuginosus* (blue line).

## 2.2. Determining the Best Reaction Conditions

The selected biocatalyst (8% TL-IM + 17% Novozym 435 + 75% RM-IM) permitted to fully hydrolyze babassu oil after 4 h of reaction, under fixed reaction conditions (5 wt.% of biocatalysts content, 1:2 (oil/water,  $w/w$ ), 40 °C, 180 rpm). However, there may be interest in further reducing the reaction time and understanding the influence of some reaction factors (biocatalyst content, substrate ratio (oil/water), reactor temperature, and stirring). For this purpose, a L9 orthogonal array by Taguchi method was performed employing the 8% TL-IM + 17% Novozym 435 + 75% RM-IM combi-lipases as biocatalysts, as depicted in Table 2.

It should be considered that the higher the substrate ratio, the higher the concentration of oil. The amount of biocatalyst in our experimental design is defined by the volume of oil in the reaction, so as to keep the oil-to-biocatalysts ratio. This means that not always a higher percentage of hydrolysis is related to a higher enzyme activity, as we are comparing different substrate concentrations. However, in the optimization, we have decided that the reaction yield is the key. The highest hydrolysis yield (>99%) was reached in run #9 at the lowest stirring level (120 rpm), intermediate temperature level (40 °C), and the highest biocatalyst content (9 wt.%), and substrates ratio (1:3) ( $v/v$ , oil/water)) levels, thereby suggesting that biocatalyst contents and substrates ratio higher than the evaluated in this study could result in higher hydrolysis yield. Nevertheless, it was already possible to reach full hydrolysis under (>99%) under the above reaction conditions, even after only 3 h of reaction. The use of higher biocatalyst content and substrate ratio (water) would increase the OPEX (Operational Expenditure) of the process. Moreover, a higher amount of biocatalyst

could complicate the real implementation of the process (increasing the problems of the filtration and biocatalyst washing step). Furthermore, a higher amount of water will mean a decrease in the substrate concentration (and, that way, product concentration). That way, we decide to fix these conditions as the optimal ones for the reaction. Moreover, employing the “larger-is-better” function, the highest hydrolysis yield of babassu oil would be reached under a similar reaction set (9 wt.% of biocatalyst content and 40 °C) but using a lower substrate ratio level—1:2 (oil/water) instead of 1:3 (oil/water)—and higher stirring level—180 rpm instead of 120 rpm. Such results suggest that full hydrolysis may be reached either using 1:2 or 1:3 substrate ratios; however, for a lower substrate ratio level (1:2, oil/water), a higher stirring level (180 rpm) is required, and, for a higher substrate ratio level (1:3, oil/water), a lower stirring level (120 rpm) is enough.

**Table 2.** L9 orthogonal array to optimize the hydrolysis of babassu oil by the combi-lipase (8% TL-IM + 17% Novozym 435 + 75% RM-IM). Reaction conditions: 1–9 wt.% of biocatalyst content, 1:1–1:3 substrate ratio (oil/water, *w/w*), 30–50 °C, 120–240 rpm and 3 h. Novozym 435: Commercially immobilized lipase B from *Candida antarctica*; RML-IM (Lipozyme RM-IL): Commercially immobilized lipase from *Rhizomucor miehei*; TLL (Lipozyme TL-IM): Commercially immobilized lipase from *Thermomyces lanuginosus*.

| Run | Biocatalyst Content (wt.%) | Substrates Ratio (Oil/Water) | Temperature (°C) | Stirring (rpm) | Hydrolysis (%) |
|-----|----------------------------|------------------------------|------------------|----------------|----------------|
| 1   | 1                          | 1:1                          | 30               | 120            | 29.8 ± 0.5     |
| 2   | 1                          | 1:2                          | 40               | 180            | 75.7 ± 3.3     |
| 3   | 1                          | 1:3                          | 50               | 240            | 46.3 ± 1.3     |
| 4   | 5                          | 1:1                          | 40               | 240            | 88.6 ± 3.9     |
| 5   | 5                          | 1:2                          | 50               | 120            | 63.9 ± 2.4     |
| 6   | 5                          | 1:3                          | 30               | 180            | 45.3 ± 1.3     |
| 7   | 9                          | 1:1                          | 50               | 180            | 70.8 ± 3.3     |
| 8   | 9                          | 1:2                          | 30               | 240            | 52.2 ± 1.6     |
| 9   | 9                          | 1:3                          | 40               | 120            | 99.5 ± 4.6     |

Herein, to make full use of the Taguchi design approach and reduce water consumption as much as possible, the level of the factors proposed by Equation (3) was selected as the optimal reaction conditions to fully hydrolyze babassu oil after 3 h of reaction employing the combi-lipase composed by 8% TL-IM + 17% Novozym 435 + 75% RM-IM as optimal biocatalyst.

In other studies, the optimal reaction conditions of a pre-determined combination of TL-IM/RM-IM (65%/35%) were studied for the hydrolysis and transesterification of soybean oil at 30 °C, 200 rpm and 10 h of reaction [89]. According to the authors, 25 wt.% of biocatalyst content and 1:3 oil/water were the parameters found to reach a yield of  $95.7 \pm 3.2\%$  for hydrolysis. Even though a smaller amount of catalyst was employed in our study (9 wt.% of biocatalyst content), a higher reaction yield was obtained (>99%) in a shorter reaction time—3 h instead of 10 h. In another interesting approach, a TL-IM-catalyzed hydrolysis of waste cooking oil was conducted, obtaining almost 90% of hydrolysis yield after 9 h of reaction at 30 °C and 300 rpm and using a substrate ratio of 1:4 (oil/water, *w/w*), 3 g of biocatalyst (enzyme/reaction medium ratio of 1:100 *w/v*) [90]. As seen in the studies above, a higher oil/water ratio (1:3 and 1:4 instead of 1:2, that is, lower concentrations of products were obtained) and stirring rates (200 rpm and 300 rpm instead of 180 rpm) were chosen as the optimal conditions for the hydrolysis of vegetable oil. Nevertheless, specifically in our case, a lower substrate ratio level of 1:2 (oil/water) was sufficient to promote good contact between phases and make reaction kinetics favorable, resulting in a high hydrolysis yield of >99%.

Regarding the influence of the evaluated reaction factors on the hydrolysis of babassu oil, the reactor temperature had the most pronounced influence, as depicted in Figure 3. Accordingly, increasing the reactor temperature from the lowest level (30 °C) to the intermediate level (40 °C) resulted in an increase of the S/N ratio and, ultimately, of the hydrolysis yield, so that 40 °C was selected as the optimal temperature. Such an increase in the hydrolysis yield may be attributed to the reduction of the viscosity of babassu oil and the concomitant increase in its solubility in water at higher temperatures (although this will remain very low), which may have minimized mass transfer limitations and, thus, facilitated substrate availability [91]. Furthermore, higher temperatures may enhance the molecular mobility of the substrates, accelerating the catalytic turnover rate [92,93]. Nevertheless, temperatures exceeding the highest level (50 °C) may have negatively affected the activity of the lipases of the combi-lipases, potentially due to structural enzyme denaturation [94,95]. This limitation underscores the need for a careful balance between optimizing reaction yields and preserving enzymatic activity. The highest influence of the reactor temperature on the hydrolysis of babassu oil was also determined by the highest delta value (Table 3) and lowest *p*-value (Table A1).

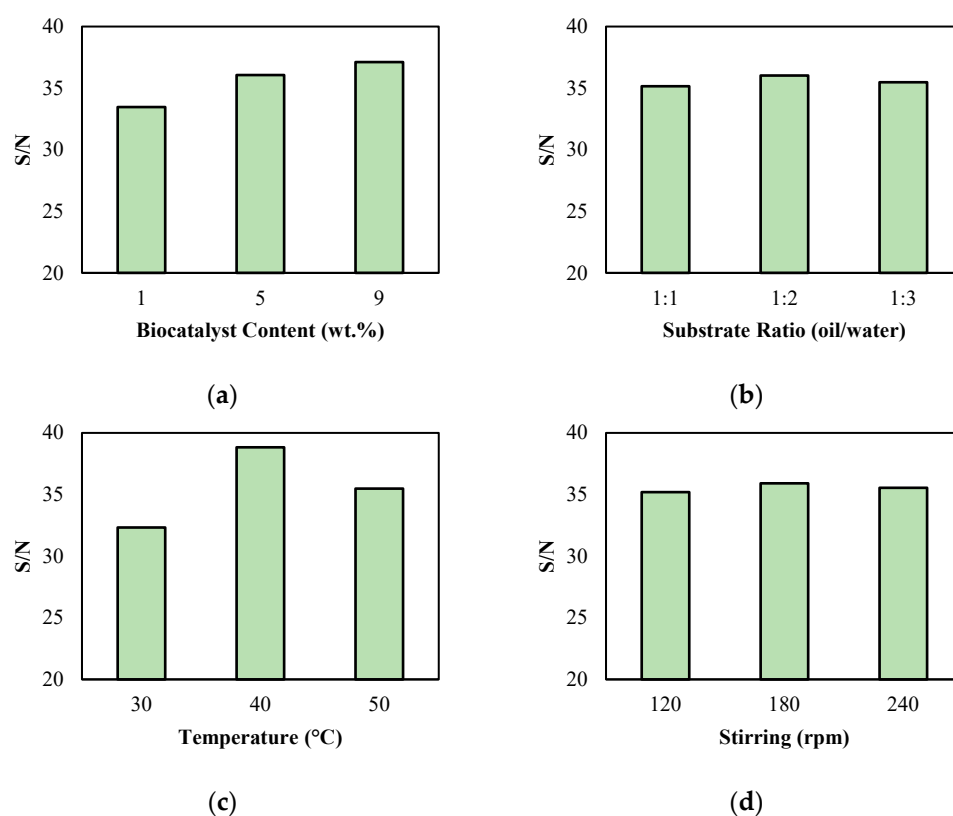


**Figure 3.** Optimization of reaction conditions of the hydrolysis of babassu oil employing the combi-lipase (8% TL-IM + 17% Novozym 435 + 75% RM-IM). Reaction conditions: 1–9 wt.% of biocatalyst content (a), 1:2–1:3 substrate ratio (oil/water, *w/w*) (b), 30–50 °C (c), 120–240 rpm (d) and 3 h. Novozym 435: Commercially immobilized lipase B from *Candida antarctica*; RML-IM (Lipozyme RM-IL): Commercially immobilized lipase from *Rhizomucor miehei*; TLL (Lipozyme TL-IM): Commercially immobilized lipase from *Thermomyces lanuginosus*.

**Table 3.** S/N ratios for biocatalyst content (wt.%), substrate ratio (oil/water,  $w/w$ ), reactor temperature ( $^{\circ}\text{C}$ ), and stirring (rpm) at each level of the free biocatalysts.

| Level   | Biocatalyst Content | Substrate Ratio | Temperature | Stirring |
|---------|---------------------|-----------------|-------------|----------|
| Level 1 | 33.46               | 35.15           | 32.32       | 35.19    |
| Level 2 | 36.06               | 36.01           | 38.83       | 35.91    |
| Level 3 | 37.10               | 35.46           | 35.47       | 35.53    |
| Delta   | 3.64                | 0.86            | 6.50        | 0.72     |
| Ranking | 2                   | 3               | 1           | 4        |

The biocatalyst content had the second most pronounced influence on the hydrolysis of babassu oil, as also depicted in Figure 3. The increase in the biocatalyst content from the lowest level (1 wt.%) to the highest level (9 wt.%) led to an increase in the S/N ratios and, thus, hydrolysis yield. The results confirm the importance of adequate biocatalyst levels, with potential particle aggregation at concentrations exceeding 9 wt.%, thereby impairing mass transfer and reducing the apparent activity of the lipases [96]. The second-highest influence of the biocatalyst content on the hydrolysis of babassu oil was also determined by the highest delta value (Table 3) and lowest  $p$ -value (Table A1).

On the other hand, the substrate ratio (oil/water,  $v/v$ ) and reactor stirring had the least pronounced influence on the hydrolysis of babassu oil, respectively, as depicted in Figure 3, Tables 3 and A1. Although the substrate ratio (oil/water) and reactor stirring were not as critical as the reactor temperature and biocatalyst content, maintaining a minimum stirring of 180 rpm and an oil/water ( $w/w$ ) ratio of 1:2 ensured proper mixing of the reactants. Further increases in stirring or water content led to only marginal increases in the hydrolysis yield. In any case, a proper substrate ratio and reactor stirring may ensure the dispersion of the substrates and immobilized biocatalysts, mitigating diffusional limitations and/or mass transfer problems within the reaction medium and, ultimately, increasing the hydrolysis yield [93,97].

### 2.3. Operational Stability of the Biocatalysts

The operational stability of the most effective biocatalyst (8% TLL-IM + 17% Novozym 435 + 75% RML-IM) for the full hydrolysis of babassu oil, as well as the individual performance of TLL-IM, Novozym 435, and RML-IM, was assessed under optimized reaction conditions (9 wt.% of biocatalyst content, 1:2 (oil/water),  $40^{\circ}\text{C}$  and 180 rpm) for several 3 h cycles. As depicted in Figure 4, the hydrolytic activity of the combi-lipases decreased over each new reaction cycle, reaching 73% of the initial hydrolysis yield after 10 reaction cycles. Only individual TLL-IM exhibited lower stability than the combi-biocatalyst, maintaining only 30% of the initial hydrolysis yield after 10 consecutive cycles of hydrolysis. And this biocatalyst accounted for only 8% of the initial combi-biocatalyst mass and it was the least active one (see Table 1). RML-IM accounts for 75% of the combi-lipases biocatalyst, maintaining approximately 90% of its initial hydrolytic activity after 10 consecutive reaction cycles, and Novozym 435 even increased the initial yield (111% of the initial hydrolysis yield) after 10 consecutive reaction cycles. This clearly shows the important role of the combination of the 3 biocatalysts in the reaction. Although only TLL-IM is seriously inactivated along the reaction, and this individual biocatalyst accounts for an apparently very low percentage of oil hydrolysis, its inactivation led to a general decrease in the combi-biocatalyst performance, which greatly exceeded the expected value of losing the TLL activity. However, it cannot be disregarded that some of the FFAs were produced by the combi-lipases (in this case, by TLL) but not by individual RML-IM or Novozym 435, which can affect the activity of these lipases. Figure 4 shows how, after 3 cycles, the yields from using the combi-lipase at the optimal time were decreased compared to those from

the most stable RML (although by prolonging the time by 2 additional hours, similar yields to the initial ones could be obtained).

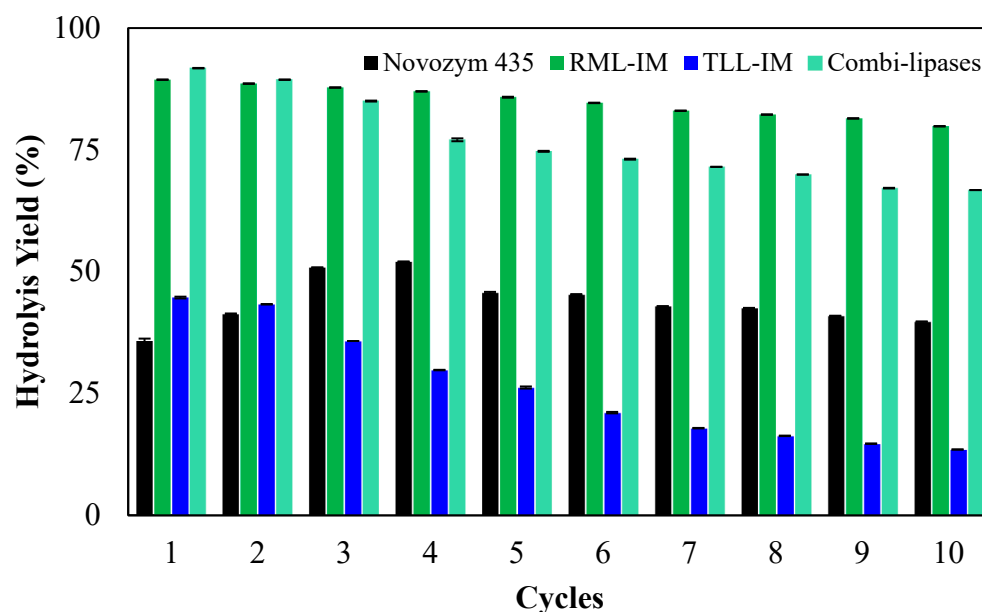


**Figure 4.** Operational stability of the biocatalysts under optimized reaction conditions. Reactions conditions: 9 wt.% of biocatalyst content, 1:2 (oil/water), 40 °C, 180 rpm and 3 h. Combi-lipases: 8% TLL-IM + 17% Novozym 435 + 75% RML-IM; Novozym 435: Commercially immobilized lipase B from *Candida antarctica*; RML-IM (Lipozyme RM-IL): Commercially immobilized lipase from *Rhizomucor miehei*; TLL (Lipozyme TL-IM): Commercially immobilized lipase from *Thermomyces lanuginosus*.

In fact, the thermal stability of RML is usually much lower than that of CALB and TLL but only the activity of TLL decreased in a significant way [98–101]. It was recently reported that, under the same inactivation conditions (60 °C and pH 7.0), the thermal stability of TLL immobilized on octyl agarose may be much lower than that of CALB immobilized on the same support, depending on the post-immobilization strategy. Firstly, when the immobilized biocatalysts were coated by polyethylenimine (PEI), the thermal stability of TLL was higher than that of CALB; however, as the post-immobilization goes on by crossing the PEI-coated immobilized biocatalysts with glutaraldehyde (GLU), the thermal stability of CALB surpasses that of TLL [98]. The inactivation of the biocatalyst may be caused by real enzyme inactivation or by the release of the enzymes from the support. The combi-lipases are exposed to higher concentrations of FFAs and a lower pH value, as the yields are higher than those obtained when the enzymes are used in an individual way. The problems generated by the inactivation of TLL biocatalyst made it necessary to prolong the reaction cycles to maintain the initial yields. We solved the problem by adding a fresh TLL biocatalyst equivalent to the inactivated one using data from operational stability achieved using just the TLL biocatalyst, after 10 cycles (no shown results). However, a better solution should be to design a more stable TLL biocatalyst. The use of better biocatalysts with higher stabilization values and avoiding risks of enzyme release should prevent the decrease in the combi-biocatalysts performance, whatever the cause of biocatalyst inactivation, for example, by using hydrophobic heterofunctional biocatalysts [102–105].

#### 2.4. Antimicrobial Activity of the Produced FFAs

The antimicrobial analysis of the FFAs from babassu oil at a concentration of 10 mg mL<sup>−1</sup> demonstrated an inhibition rate of 94.9% (±9.18) against *S. aureus*, 90.8% (±1.25) against *E. coli*, and 99.7% (±0.39) against *P. aeruginosa*, as depicted in Figure 5, and this was not significantly increased using higher concentrations. These findings indicate that the

FFAs from babassu oil, used at 10 mg/mL, exhibited a significant desirable antimicrobial activity (>80%) against the most common environmental bacteria. It was recently reported that concentrations of around 1 mg of babassu oil are enough to inhibit *E. coli* (ATCC 10536) and *P. aeruginosa* (ATCC 9027) and 0.81 mg for *S. aureus* (ATCC 6538) [106]. The oil exhibited activity even after undergoing hydrolysis.

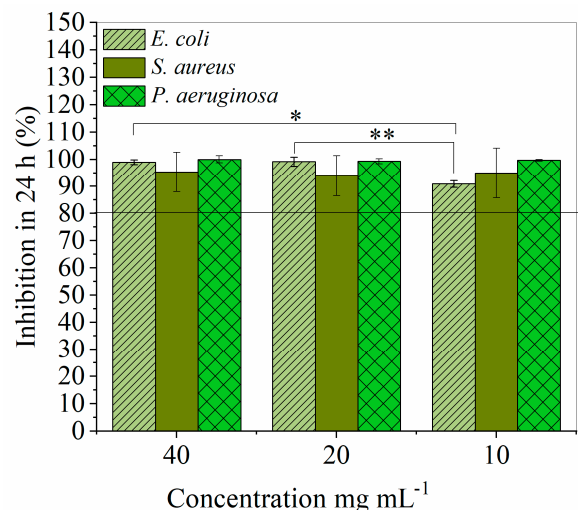


**Figure 5.** Antimicrobial analysis of hydrolyzed babassu oil (40, 20, and 10 mg mL<sup>-1</sup>) against *S. aureus*, *E. coli*, *P. aeruginosa*. The ANOVA test and Dunn's post-test with multiple comparisons were applied. Values with significant differences were considered if  $p < 0.05$ . \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ .

### 3. Materials and Methods

#### 3.1. Materials

The commercially immobilized lipases from *Thermomyces lanuginosus* (Lipozyme<sup>®</sup> TL-IM), *Rhizomucor miehei* (Lipozyme<sup>®</sup> RM-IM), and lipase B from *Candida antarctica* (Novozym<sup>®</sup> 435) were kindly donated by Novozymes Spain (Alcobendas, Spain). Babassu oil was purchased from Sincoplema (Caxias, Maranhão, Brazil). According to our previous study [107], it is mainly composed of caprylic acid (3.79%), capric acid (5.42%), lauric acid (47.75%), myristic acid (16.54%), palmitic acid (8.58%), stearic acid (3.45%), oleic acid (12%), and linoleic acid (2.46%). The software Statistica<sup>®</sup> (Version 14.1.0.8, Statsoft South America, São Caetano do Sul, São Paulo, Brazil) was used to design and analyze the experiments.

#### 3.2. Methods

The experiments were conducted at least in triplicate, with the results being presented as the average of these values (standard deviation typically below 5%).

##### 3.2.1. Experimental Design of the Combi-Lipase Biocatalyst

A mixture design with a triangular surface (3-factor simplex-centroid design with interior points) study was conducted to determine the optimal combi-lipase for the hydrolysis of babassu oil, employing TL-IM, RM-IM, and Novozym 435 or their mixtures as biocatalysts, as shown in Table 1. The hydrolysis was performed under fixed conditions for 1–4 h as follows: 5 wt.% of biocatalysts content, 1:2 (oil, water,  $w/w$ ), 40 °C, 180 rpm. The hydrolysis yield was determined as follows: first, the oil phase containing the FFAs was separated from the aqueous phase through decantation; then, the FFAs-phase (0.5 g) was diluted in 10 mL of ethyl alcohol, followed by the addition of 3 drops of phenolphthalein as

pH titrating reagent and, finally, the mixture was titrated with sodium hydroxide (50 mM). The hydrolysis yield (H%) of the product was calculated using Equation (1) [108,109]:

$$H (\%) = \frac{M_{\text{NaOH}} \times V_{\text{NaOH}} \times 10^{-3} \times MM}{m \times f} \quad (1)$$

In the given equation,  $M_{\text{NaOH}}$  (mol/L) is the molarity of the NaOH solution;  $V_{\text{NaOH}}$  (mL) is the volume of NaOH spent on the titration; MM is the average molecular mass FFA from babassu oil (g/mol); m (g) is the mass of the sample to be analyzed; and f is the babassu oil fraction at the beginning of hydrolysis.

### 3.2.2. Experimental Design of the Reaction Conditions

Some reaction factors of the hydrolysis of babassu oil were optimized by the Taguchi method, employing a standard orthogonal array L9 (“L” represents the Latin square and “9” represents the number of experiments) to determine the best biocatalyst content, substrate ratio (oil/water), reactor temperature and stirring, as depicted in Table 2. The hydrolysis yield was determined as described above. To maximize the hydrolysis yield, the “larger-is-better” function was employed to determine the signal/noise (S/N) ratios for the hydrolysis yield of each assay of the L9 orthogonal array. The S/N ratios for each assay were calculated by Equation (2).

$$\frac{S}{N} = -10 \log \left( \frac{1}{n} \sum_{i=1}^n \frac{1}{y_i^2} \right) \quad (2)$$

In the given equation, y is the hydrolysis yield to the corresponding essay, it is the number of replicates, and n is the number of responses for the combination of factor levels in any specific parametric combination according to Table 2.

The predicted S/N ratio under optimal conditions to achieve the highest conversion was estimated by Equation (3). In which,  $\bar{S}/N$  is the arithmetic mean of all S/N ratios,  $S/N_j$  is the S/N ratio at the optimal point for each factor and n is the number of factors significantly affecting the process.

$$\frac{S}{N_{\text{predicted}}} = \bar{\frac{S}{N}} + \sum_{j=1}^n \left( \frac{s}{N_j} + \bar{\frac{s}{N}} \right) \quad (3)$$

### 3.2.3. Antimicrobial Activity of the Produced FFAs

The antimicrobial activity of the FFAs from babassu oil was analyzed against *E. coli* (ATCC 11303), *P. aeruginosa* (ATCC 27853), and *S. aureus* (ATCC 25923), all from the American Type Culture Collection (ATCC). The methodology used was microdilution methodology in 96-well polystyrene plates standardized [110]. The bacterial strains were inoculated in Mueller Hinton Broth (MHB) and incubated at 35 °C. After 24 h, the bacterial cultures were centrifuged at 9000 rpm and 4 °C for 5 min, with subsequent removal of the supernatant. The bacteria were resuspended in a fresh medium and the inoculum was adjusted to 106 CFU mL<sup>-1</sup>. In a 96-well plate, 100 µL of the FFAs from babassu oil (40, 20, and 10 mg mL<sup>-1</sup>) and 100 µL of the bacterial suspension were added to each well. Only wells with MHB with bacteria were used as positive controls. Dimethyl sulfoxide (DMSO, 1%) was used to improve the solubility of the FFAs from babassu oil. This DMSO concentration was included in the control to ensure that any observed effects were exclusively attributed to the FFAs. After 18 h of incubation, bacterial growth was assessed by measuring absorbance at 620 nm (SpectraMax i3x, Molecular Device, Sunnyvale, CA, USA). The antimicrobial activity was calculated using Equation (4).

$$\text{Antimicrobial activity (\%)} = \left( \frac{F_{\text{sample}} - F_{\text{blank}}}{F_{\text{control}}} \right) \times 100\% \quad (4)$$

The data collected underwent descriptive analysis and an evaluation of normality. Since the samples followed a normal distribution, one-way ANOVA was used for other analyses. Results were reported as mean, with statistical significance defined as  $p < 0.05$ .

#### 4. Conclusions

The results in this paper clearly exemplify the importance of selecting adequate conversion degrees when selecting the best biocatalysts for a specific reaction. This is more important when using heterogeneous substrates (as the substrate molar ratio will change along the process) or when the experimental conditions are changing along the process (e.g., pH is altered during the reaction studied in this paper). At first glance, using initial conditions, in which 50% yield is achieved, may enable a good comparison of the different biocatalyst formulations, making it easy to detect increases or decreases in the yields during optimization. However, these moderate conversions of the whole substrate mixture can mask the negative effects of some of the components of the substrate mixture. We should consider a situation where the oil is composed mainly of triglycerides that are readily hydrolyzed by an enzyme (that we can call “good substrates”) but that a small percentage of the oil is composed of triglycerides that cannot be hydrolyzed by this enzyme (that we can call “bad substrates”). However, they are properly hydrolyzed by a second enzyme, which is less efficient in the hydrolysis of the main triglycerides than the other enzyme. To detect an advantage of the use of a mixture of enzymes, only when the relation of these “bad” substrates versus the other “good” substrates for the main enzyme becomes very high (when a large percentage of the “good substrate” has been consumed), this “bad” substrate may produce a slowdown of the enzyme activity, and the advantages of the use of combi-lipases become evident. Under these circumstances, the use of higher conversion yields (that means longer reaction times and more time consumed in the optimization) can show these effects. In fact, the best solution to better understand the results is the use of several reaction times. In this paper, the use of different reaction times in the combi-biocatalyst optimization has enabled us to detect the importance of using combi-lipases in the hydrolysis of babassu oil. The combi-lipases composed of 75% of RML-IM, 17% of CALB-IM, and 8% of TLL-IM were the best biocatalyst formulation to hydrolyze babassu oil, reaching approximately full hydrolysis (>99%), whereas the individual lipases gave lower maximum yields. However, RML-IM exhibited the best performance if analyzing only 1 h of reaction, but the reaction catalyzed by this biocatalyst reached only 85% of hydrolysis yield after 4 h. These results highlight the potential of combi-lipases over lipases alone in the modification of vegetable oils. Despite reaching approximately full hydrolysis yield in the first step of this study, the reaction was further evaluated to reduce the time of reaction (from 4 h to 3 h); however, higher biocatalyst content was required (from 5 wt.% to 9 wt.%). One may choose to reduce the reaction time by increasing the biocatalyst content, or vice versa. Indeed, the biocatalyst content showed the highest influence on the yield obtained in the hydrolysis of babassu oil. The 75% of RML-IM + 17% of CALB-IM + 8% of TLL-IM combi-lipases exhibited moderate operational stability (more than 70% of the initial hydrolysis yield after 10 reaction cycles), apparently mainly associated with the substantial decrease of TLL-IM activity. This should be further investigated and better lipase combi-biocatalysts built to prevent this enzyme activity inactivation (e.g., improving the immobilization of TLL). The obtained FFAs from babassu oil exhibited high antimicrobial activity over different bacteria, probably due to the high concentration of lauric acid.

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R.F.-L. and R.S.V.; data curation, R.R.C.M. and T.F.d.S.; writing—original draft preparation, R.P.S.S., L.L.A., A.A.O.J., T.F.d.S. and T.G.R.; writing—review and editing, R.F.-L., R.R.C.M., T.G.R. and R.S.V.; visualization, R.F.-L. and R.R.C.M.; supervision, R.F.-L., R.R.C.M. and R.S.V.; project administration, R.F.-L., R.R.C.M. and R.S.V.; funding acquisition, R.F.-L. and R.S.V. All authors have read and agreed to the published version of the manuscript.

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## Appendix A

**Table A1.** ANOVA for L9 orthogonal array ( $\alpha = 0.05$ ). {} indicates that has no (or negligible) effect.

|                     | dF    | SS  | MS    | F      | p-Value |
|---------------------|-------|-----|-------|--------|---------|
| Biocatalyst Content | 21.12 | 2   | 10.56 | 21.97  | 0.007   |
| {Substrate Ratio}   | 1.14  | {2} |       |        |         |
| Temperature         | 63.46 | 2   | 31.73 | 66.015 | 0.001   |
| {Stirring}          | 0.78  | {2} |       |        |         |

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