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Optimization of the Production of Enzymatic Biodiesel from Residual Babassu Oil (*Orbignya* sp.) via RSM

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Abstract: Residual oil from babassu (*Orbignya* sp.), a low-cost raw material, was used in the enzymatic esterification for biodiesel production, using lipase B from *Candida antarctica* (Novozym® 435) and ethanol. For the first time in the literature, residual babassu oil and Novozym® 435 are being investigated to obtain biodiesel. In this communication, response surface methodology (RSM) and a central composite design (CCD) were used to optimize the esterification and study the effects of four factors (molar ratio (1:1–1:16, free fatty acids (FFAs) /alcohol), temperature (30–50 °C), biocatalyst content (0.05–0.15 g) and reaction time (2–6 h)) in the conversion into fatty acid ethyl esters. Under optimized conditions (1:18 molar ratio (FFAs/alcohol), 0.14 g of Novozym® 435, 48 °C and 4 h), the conversion into ethyl esters was 96.8%. It was found that after 10 consecutive cycles of esterification under optimal conditions, Novozym® 435 showed a maximum loss of activity of 5.8%, suggesting a very small change in the support/enzyme ratio proved by Fourier Transform Infrared (FTIR) spectroscopy and insignificant changes in the surface of Novozym® 435 proved by scanning electron microscopy (SEM) after the 10 consecutive cycles of esterification.

Keywords: biodiesel; Novozym® 435; residual oil from babassu; esterification; response surface methodology

1. Introduction

Biodiesel may be produced by the esterification of free fatty acids or the transesterification of oils and fats with short-chain alcohols [1]. Remarkable, biodiesel has been reported as one of the options to replace or complement traditional fuels, using natural raw materials or renewable biological elements for its production [2–4]. Indeed, it has several advantages for being a renewable, clean, biodegradable,

non-toxic and low polluting energy source, compared to petroleum diesel [5]. In this regard, seeking to increase the commercial competitiveness of biodiesel in the market, it is expected that alternative oilseeds will expand its application as a raw material in the production of biodiesel [6,7].

For instance, babassu *(Orbignya* sp.) is an oil palm that may be an alternative raw material for the production of biodiesel, as it is a non-edible oil with low cost [8–10]. The quality of the babassu oil depends on the almonds; in fact, broken or scratched almonds, when exposed to light and moist air, become rancid in proportion to the time of exposure [8], resulting in an oil with high content of free fatty acids. The deteriorating state of the oil is indicated by the proportions of free fatty acids present and can significantly limit its use. Thus, this oil cannot be used as a raw material in the production of biodiesel using alkaline catalysts because it has high acidity.

Thus, the production of biodiesel may be carried out through the hydroesterification process, which allows the use of raw material with high concentrations of free fatty acids. The process takes place in two stages, the first of which is the hydrolysis reaction of the glycerides that produce free fatty acids and glycerol. The second stage is the esterification reaction of free fatty acids with a short-chain alcohol for the production of esters and water [9]. In this regard, the literature reports the use of the hydroesterification process for the production of biodiesel, with the hydrolysis stage catalyzed by a lipase and the esterification catalyzed by an acid [11], using commercial lipases in both reactions [12] or even with the hydrolysis stage in subcritical water and use of a lipase in the esterification stage [13].

More and more, the interest in biodiesel production using immobilized lipases has been increasing [14–17], due to the advantages that the immobilized enzymes offer in relation to the conventional processes that use chemical catalysts [18–20]. For example, milder reaction conditions, reduced energy consumption, compatibility with the quality of raw materials and recovery of the biocatalyst [21]. In this scenario, Novozym® 435 (lipase B from *Candida antarctica* immobilized on a macroporous resins) the most commonly used lipase in the biodiesel production (see Table 1) [22–24]. In general, lipases are used in a wide variety of biotransformation through several types of reactions, such as hydrolysis, esterification and transesterification [25]; therefore, they are used for *laboratorial* and *industrial applications* [26–33], such as formulation of drugs, modification of racemic alcohols, acids and esters [34]. Besides, lipases can replace synthetic processes in the industry, such as in the cleaning, hygiene, leather, pharmaceutical and food industries (aroma and flavor) [35], once enzyme exhibits a very high degree of substrate specificity and selectivity [36–38].

To authors' knowledge, up to date, this is the first work reported in the literature that investigates both the production of biodiesel Novozym® 435 as a biocatalyst and the free fatty acids obtained from the residual oil from babassu. The chemoenzymatic route used in this communication, chemical hydrolysis followed enzymatic esterification, proved to be an efficient process for the production of biodiesel. Comparatively, transesterification is a slow process that consumes more energy, generating more costs [39]. During the enzymatic transesterification for the production of biodiesel, one of the products formed is glycerol, which can be deposited on the surface of the enzyme, forming an outer layer preventing contact between the substrate and the enzyme active site, reducing the catalytic activity of the enzyme [1,40].

Table 1. Application of Novozym 435 in the transesterification reaction for biodiesel synthesis.

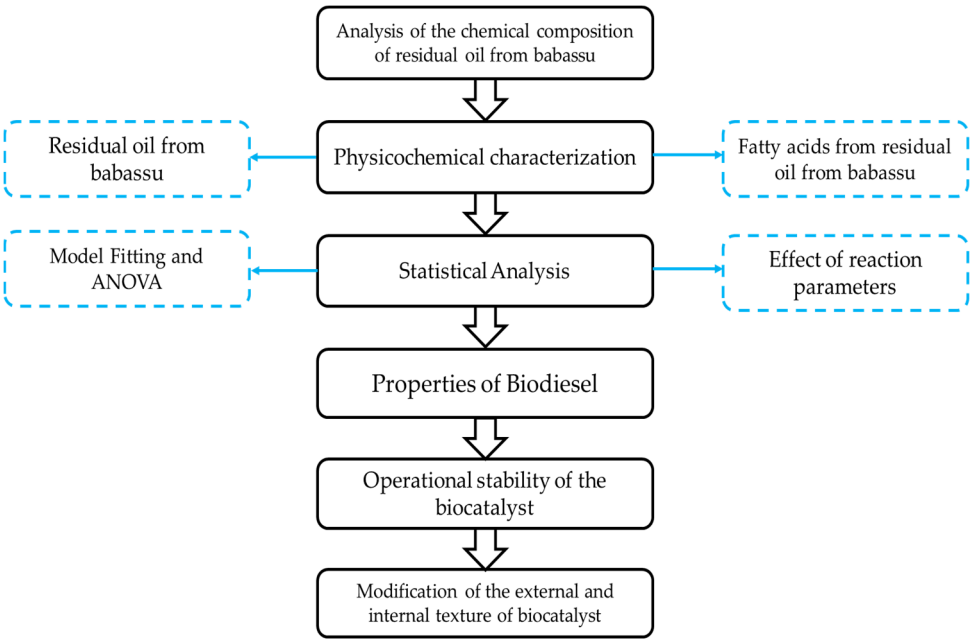
Substrate	Alcohol	Conversion/Experimental Conditions	References
Sweet basil seed oil	Methanol	94.58%/68 h at 47 °C	[41]
Fish oil	Ethanol	52.11%/8 h at 35 °C	[42]
Jatropha oil	Methanol	66.80%/24 h at 40 °C	[43]
Microalgal oil *Botryococcus* sp.	Methanol	88.00%/4 h at 40 °C	[44]
Oils from microalgae	Methanol	19.30%/6 h at 35 °C	[45]
Corn oil	Methanol	91.00%/18 h at 60 °C	[46]
Soybean oil	Methanol	96.00%/4 h at 40 °C	[22]
Palm oil	Methanol	91.00%–92.00%/10, 20, 24 h at 40 °C	[47]
Frying oil	Methanol	89.10%/4 h at 50 °C	[48]

Remarkably, cost reduction in biodiesel production has been a major challenge for more than 20 years [49]. In this sense, efforts have been made to enable the use of a wide range of raw materials, to optimize the processes of production and to investigate more efficient catalysts. As a result, low-cost raw materials from non-edible resources have been investigated for the production of biodiesel [50], once the cost of the raw material is one factors that directly affects the economic viability of biodiesel production are. In fact, the cost of the raw material is one of the main and most important parameters in the production of biodiesel, as it corresponds to 80% of the total production cost [11]. According to Baskar and Aiswarya (2016) [51], the raw material and the type of catalyst are the most important factors for an efficient production of biodiesel. In this regard, heterogeneous catalysts have been used as an effective substitute for reactions using homogeneous catalysts [52]. As a matter of fact, heterogeneous catalysts such as immobilized enzymes are used as a new method in the purification and production of biodiesel, in the quest to obtain greater conversions, to reuse the catalyst in consecutive cycles and to produce products with high added value and to achieve higher yields, when compared to the processes catalyzed by chemicals catalysts [53,54].

Thus, the aim of this work is to present a route for industrial production of biodiesel, using as raw material the residual babassu oil, obtained from the extraction of damaged almonds, a waste that is discarded in the environment, adding value to this product; furthermore, it seeks to analyze the influence of temperature, molar ratio (FFAs/alcohol), biocatalyst content (Novozym® 435) and time in the production of biodiesel.

2. Results and Discussion

Scheme 1 was designed to facilitate the understanding of the results and discussions.



Scheme 1. Presentation of results and discussions.

2.1. Analysis of the Chemical Composition of Residual Oil from Babassu

The analysis of the chemical composition of the residual oil from babassu was performed by gas chromatography (GC). The results with the free fatty acids present in the residual oil are shown in the chromatogram presented in Figure 1.

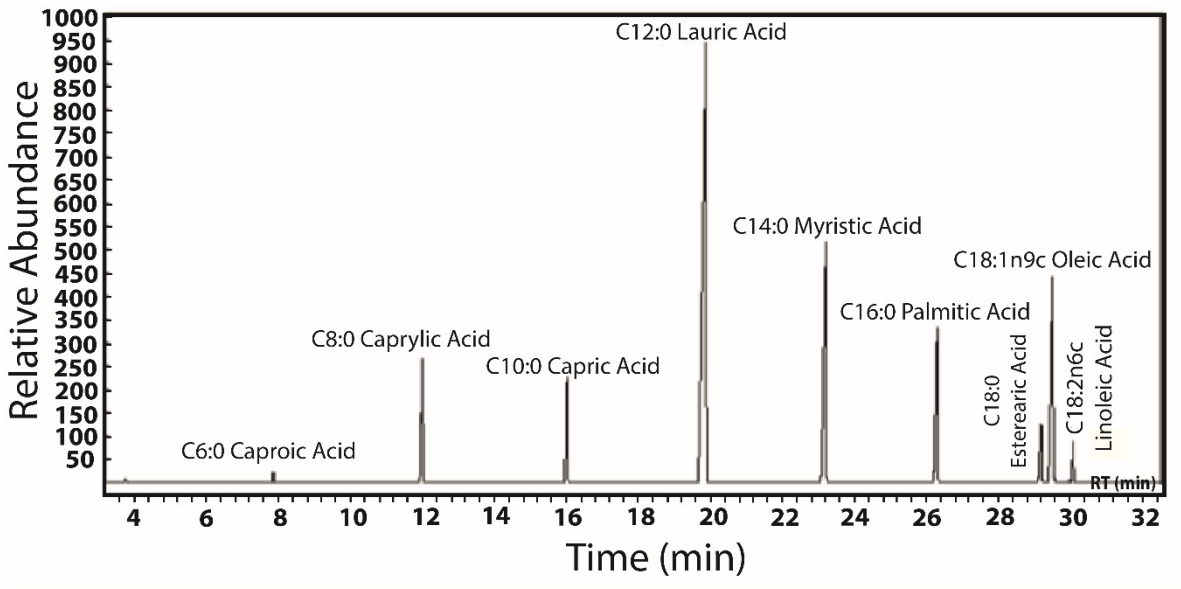


Figure 1. Chromatogram obtained by GC for the determination of the free fatty acids in the residual oil from babassu.

The residual babassu oil chromatogram reveals a well-defined peak of lauric acid (C12:0), whose retention time (rt) is 19.68 min. The GC analysis identified others expressive peaks in the chemical composition of the residual babassu oil: caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c). Melo et al. (2019) [55], analyzed the fatty acid composition of babassu oil, the results confirming the high levels of lauric acid in babassu oil (47.40%). The difference in melting point did not influence the content of lauric acid. The results confirmed the high content of saturated fatty acids of babassu oil; this is mainly due to the high content of lauric acid. As can be seen in Table 2, lauric acid had a content of 41.55% in the composition of residual babassu oil.

Table 2. Percentages of free fatty acids in the chemical composition of residual oil from babassu.

Fatty Acid	Nome	Formula	Molecular Weight (g/mol)	Percentage (%)
C6:0	Caproic Acid	$C_6H_{12}O_2$	116.15	0.80
C8:0	Caprylic Acid	$C_8H_{16}O_2$	144.21	9.64
C10:0	Capric Acid	$C_{10}H_{20}O_2$	172.26	6.85
C12:0	Lauric Acid	$C_{12}H_{24}O_2$	200.31	41.55
C14:0	Myristic Acid	$C_{14}H_{28}O_2$	228.37	14.01
C16:0	Palmitic Acid	$C_{16}H_{32}O_2$	256.40	6.96
C18:0	Stearic Acid	$CH_3(CH_2)_{16}COOH$	284.48	2.66
C18:1n9c	Oleic Acid	$C_{18}H_{34}O_2$	282.47	11.04
C18:2n6c	Linoleic Acid	$C_{18}H_{32}O_2$	280.44	1.76
Others	-	-	-	4.73

2.2. *Physicochemical Characterization of the Residual Babassu oil and Free Fatty Acids*

Table 3 shows the results of the physicochemical characterization of the residual babassu oil and the free fatty acids obtained after the chemical hydrolysis. The data obtained were compared with the values determined by Codex Alimentarius (International Food Standards) [56] and with results found in the literature for babassu oil and soybean oil, which is the most used raw material for biodiesel production in Brazil [57].

Table 3. Results of the physicochemical characterization of residual babassu oil.

Analytical Parameter	Technical Norm	Residual Babassu Oil	Babassu Oil [58]	Soy Oil [57,58]		CODEX [56]
Acidity Level (mgKOH/g)	Adolfo Lutz Institute [59]	4.85[1] 174.44[2]	-	0.41	0.28	0.50
Saponification Index (mgKOH/g)	Adolfo Lutz Institute [59]	271.94	-	-	-	245–256
Oxidative Stability (hours)	EN 14.214 [60]	1.30	-	-	7.16	-
Kinematic Viscosity at 40 °C (mm^2/s)	ASTM D445-18 [61]	3.96 7.78[2]	2.95	3.18	3.15	1.44–1.45
Density at 20 °C (g/cm^3)	NBR 14.065 [62]	0.92 0.87[2]	0.92	0.92	0.92	0.914–0.917

[1]Residual oil, [2]Hydrolyzed oil. Technical standards used: Adolfo Lutz Institute, European Standards (EN 14.214), American Society for Testing and Materials (ASTM D445-18), Brazilian Standard NBR 14.065.

Based on Table 3, the value of the acidity index obtained in the analyzes for the residual babassu oil showed a high value in comparison to the value determined by Codex Alimentarius and the values reported in the literature, which confirms that it is a residual oil. The acidity index deals with the conservation status of oils; due to factors such as exposure to light and heating, the breakdown of triglycerides is enhanced, causing the formation of free fatty acids accompanied by the formation of rancid [63]. Through the acidity index, it was possible to verify the efficient formation of free fatty acids from the saponification reaction followed by acid hydrolysis, since the acid value increased from 4.85 mgKOH/g to 174.44 mgKOH/g. As the present study deals with an esterification reaction using a lipase as biocatalyst, acidity is not a problem. According to Babaki et al. (2017) [21], in the case of a biodiesel production using enzymatic catalysts, the formation of soaps does not occur, which favors the increase in the reaction yield and avoids the necessity of washing step.

The saponification index aims to establish the degree of deterioration of oils and fats; its main function is to verify adulterations by low quality oils, the lower the molecular weight of fatty acids, the higher their saponification rate [64]. In the studies carried out by (Singh et al. 2019) [58], the authors determined the values for the saponification index of some oilseed plants most used in the production of biodiesel. In their studies, oil from canola showed a value of 111.61 mgKOH/g, oil from sunflower 129.54 mgKOH/g, oil from soybean of 109.17 mgKOH/g. In the present study, the saponification value showed a relatively high value of 271.94 mgKOH/g, which means that it contains more saturated fatty acids, thus allowing the saponification followed by acid hydrolysis to be successful.

Oxidative stability is a property that depends on the proportions of saturated and unsaturated fatty acids present in the oils used in the biodiesel production; the high degree of unsaturation favors the oxidation and degradation of oilseeds [65]. Oxidative stability can be defined as the time required to reach the point where the degree of oxidation increases abruptly [66]. Oxidation of lipids is a degradative process, which can lead to loss of oil stability. This process can be favored and intensified by the action of light, which acts as a catalyst [67]. Babassu is rich in lauric acid, a saturated compound that does not favor oxidative rancidity. In this study, the oxidative stability of the babassu residual oil was 1.30 h, which is much lower than that found by Melo et al., (2019) [55], which was 33.69 h. This is due to the fact that the raw material used in this study is a residual oil, favoring the activity of oxidizing agents.

Kinematic viscosity is related to the length of the carbon chain and the degree of saturation of the fatty acids present in oils and fats [63]. Saturated fatty acids have a linear conformation, allowing a more effective interaction between molecules, while unsaturated fatty acids present folds in the carbon chain for each double bond, causing molecular interactions with low efficiency and reducing their kinematic viscosity [68]. It is possible to observe that the value of the kinematic viscosity of the residual oil (3.96 mm^2/s) is greater than that of the fatty acids produced (7.78 mm^2/s), this fact may be

linked to the quality of the raw material since it had both saturated and unsaturated fatty acids, in addition to oxidizing agents favoring their degradation. After the saponification reaction followed by acid hydrolysis, it was possible to obtain saturated fatty acids, increasing the kinematic viscosity [68]. Both results showed high values compared to literature, shown in Table 3.

The density values obtained were (0.92 g/cm^3) for the residual babassu oil and (0.87 g/cm^3) for the free fatty acids. Density is a measure used in the characterization of oils and fats in the liquid state and that varies inversely with the average molecular weight of fatty acids or with temperature and directly with the degree of unsaturation [69]. As the fatty acids obtained were mostly saturated, their value was lower, compared to the residual oil, which was also composed of unsaturated fatty acids [70].

The values of the physicochemical properties of residual babassu oil and free fatty acids are very close to the oils studied in the literature by Singh et al. (2019) [58] and Mihaela (2013) [57] (Table 3), so that babassu residual oil could be further explored as a raw material in the production of biodiesel.

2.3. Statistical Analysis

2.3.1. Model Fitting and Analysis of Variance (ANOVA)

Table 4 presents the conditions proposed by the experimental design to determine the temperature, time, molar ratio (FFAs/alcohol) and biocatalyst content for the production of biodiesel using the free fatty acids from residual babassu oil, ethanol and Novozym® 435 as biocatalyst. In total, 27 experimental tests were performed (24 tests at factorial points, 3 with repetitions at the central point). According to the author Elkelawy et al. 2020 [71], repetitions at the central point are necessary, as they allow the estimation of pure experimental errors and verify the fit of the model.

Table 4. Complete experimental design with 18 factorial points, 6 axial points (2 axial points on the axis of design variable) and 3 replications at the central point, totaling 27 experiments, with the result for the conversion into esters for the esterification of residual babassu oil.

Run	Temperature (°C)	Time (hours)	Molar Ratio (FFAs/alcohol)	Biocatalyst Content (grams)	Conversion (%)
1	30	2	1:1	0.05	70.42 ± 0.04
2	30	2	1:1	0.15	65.19 ± 0.03
3	30	2	1:15	0.05	85.94 ± 0.04
4	30	2	1:15	0.15	91.11 ± 0.05
5	30	6	1:1	0.05	71.22 ± 0.04
6	30	6	1:1	0.15	70.69 ± 0.04
7	30	6	1:15	0.05	91.10 ± 0.05
8	30	6	1:15	0.15	91.07 ± 0.05
9	50	2	1:1	0.05	67.88 ± 0.03
10	50	2	1:1	0.15	68.61 ± 0.03
11	50	2	1:15	0.05	89.92 ± 0.04
12	50	2	1:15	0.15	91.10 ± 0.05
13	50	6	1:1	0.05	68.90 ± 0.03
14	50	6	1:1	0.15	68.07 ± 0.03
15	50	6	1:15	0.05	90.49 ± 0.05
16	50	6	1:15	0.15	91.12 ± 0.05
17	30	4	1:8	0.10	86.07 ± 0.04
18	50	4	1:8	0.10	85.97 ± 0.04
19	40	2	1:8	0.10	88.63 ± 0.04
20	40	6	1:8	0.10	84.81 ± 0.04
21	40	4	1:1	0.10	69.77 ± 0.03
22	40	4	1:15	0.10	91.08 ± 0.05
23	40	4	1:8	0.05	91.07 ± 0.05
24	40	4	1:8	0.15	89.85 ± 0.04
25 (C)	40	4	1:8	0.10	89.79 ± 0.04
26 (C)	40	4	1:8	0.10	89.84 ± 0.04
27 (C)	40	4	1:8	0.10	89.92 ± 0.04

Based on Table 4, it is possible to observe that the highest conversion to fatty acid ethyl esters was found in test 16 with a conversion of 91.12%, at a temperature of 50 °C, for 6 h, in the molar ratio of 1:15 (FFAs/alcohol) and in the biocatalyst content of 0.15 g. Through the software Statistica® 10 software (Statsoft, Tulsa, OK, USA), it was possible to determine the optimized conditions for the synthesis of biodiesel (0.14 g of biocatalyst, molar ratio of 1:18 (FFAs/alcohol), temperature of 48 °C in 4 h), obtaining a yield theoretical of 95.87%. The validation of the proposed model was performed by chromatographic analysis of ethyl esters, following the standard EN 14,103 [72] with some modifications, the value obtained was 96.84%. The Resolution 51/2016 of the Brazilian National Petroleum Agency states that to be considered biodiesel the conversion to esters must present a minimum value of 96.5%. Based on the chromatographic analysis, biodiesel was produced in the present study.

In Table 5, the ANOVA results are presented to adjust the response surface model of second order, using the mean square method. According to equation 2, the coefficients of the response surface model were analyzed. To test the statistical significance of the model, the F-test for ANOVA was used [73]. A very low P (<0.0001) of the model in the F-test proves that the regression is statistically significant at the 95% confidence level. The calculated F of 58.98 was considerably higher than the tabulated F of 2.82, which confirms the significance of the model. To determine the quality of the model, the coefficient of determination (R^2) was evaluated. The obtained R^2 was 0.99, which indicates that 99% of the experimental data are compatible with the data provided by the model. The adjusted R^2 was 0.97, a high value, stating the significance of the model.

Table 5. Analysis of variance (ANOVA) for the empirical model.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	Fcal	Probability (P) > F
Regression	2568.10	14	183.44	58.98	0.0001
Residual error	37.32	12	3.11	-	-
Lack of fit	37.31	10	3.73	867.68	0.001152
Pure error	0.009	2	0.00	-	-
Total	2605.41	26	100.21	-	-

The model presented an R^2 value above 99%, confirming an excellent relationship between the theoretical values predicted by the model and the results obtained experimentally. This correlation is given by the second order polynomial regression equation between the conversion of esters and the process variables: temperature (°C), molar ratio (FFAs: alcohol), biocatalyst content and time, which is given below:

$$\begin{aligned} Y = {} & 32.3289 + 1.8085X_1 - 0.0221X_1^2 + 4.7503X_2 - 0.3768X_2^2 - 0.3234X_1X_2 \\ & + 0.0066X_1X_3 + 0.0029X_1X_4 - 0.0047X_2X_3 - 0.0163X_2X_4 + 9.1230 \end{aligned} \quad (1)$$

In which, Y is the conversion for the esterification reaction; Temperature (°C) (X_1), Time (hours) (X_2), Molar ratio (FFAs: alcohol) (X_3), Biocatalyst content (% m/m) (X_4), are the parameters.

2.3.2. Effect of Reaction Parameters

In the analysis of the Pareto chart, shown in Figure 2, it visually tells us if an independent variable will produce or no effect on the dependent variable; it also shows if an interaction occurs between independent variables will result in a significant planning response. With the Pareto chart, it is possible to observe that the independent variables linear temperature (°C) and linear biocatalyst content (g) do not influence the response variable, which is the conversion into esters. In the chart, it is possible to observe positive and negative values; this implies that the independent variables that present influence under the response in a positive way, the higher the value, the higher the percentage in esters conversion. And for the variables that present a negative influence, the higher the negative value, the lower the percentage in esters conversion [74,75].

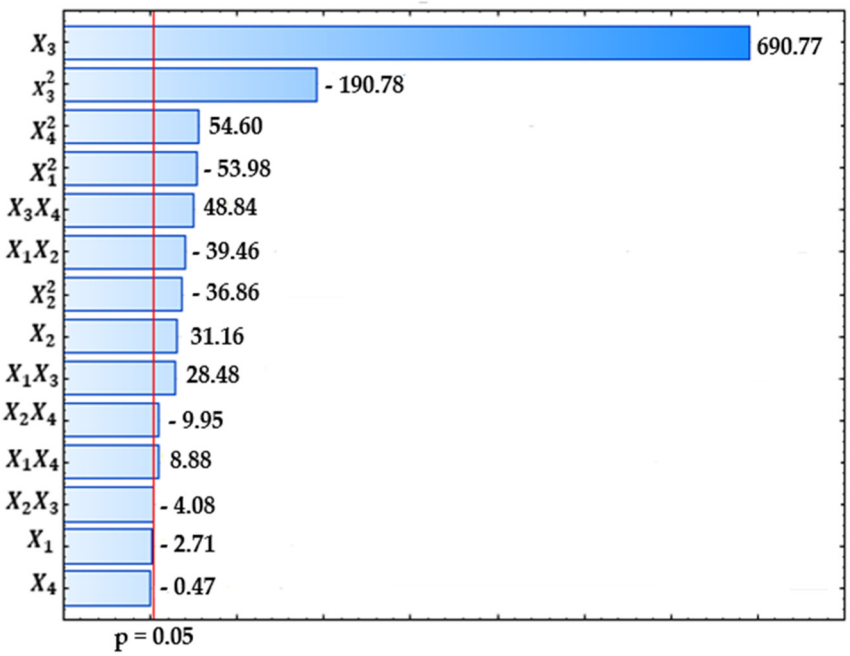


Figure 2. Pareto chart indicating the effect of the estimated values for the studied variables in the enzymatic esterification of the babassu free fatty acids. (X_1) temperature (°C), (X_2) time (hours), (X_3) molar ratio (FFAs: alcohol), (X_4) biocatalyst content (% m/m).

The interaction between the independent variables and the dependent variable is shown by the three-dimensional response of the fatty acid ethyl esters (FAEE) yield in Figure 3, in which several response surfaces are created for the relationship between the effects of two parameters on the FAEE yield while the third invariable.

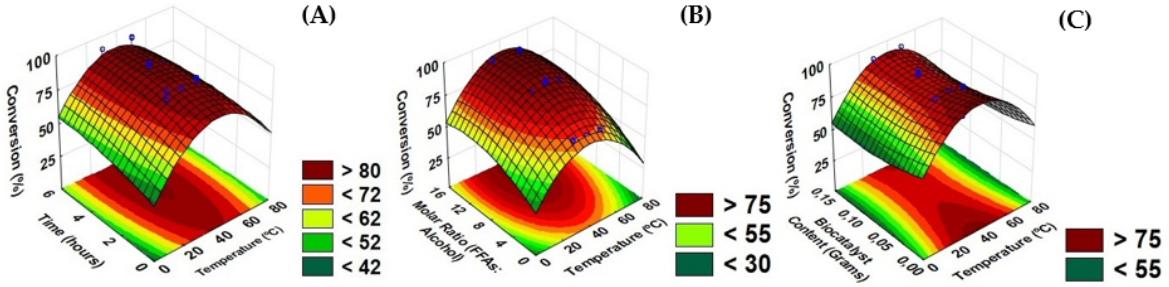


Figure 3. Response surfaces for (**A**) temperature versus time; (**B**) temperature versus molar ratio (fatty acid: alcohol); (**C**) Temperature versus biocatalyst content; reaction medium: babassu free fatty acids 1:1–1:1:15 (free fatty acids/alcohol), biocatalyst mass of 5–15 g. Reactions were carried out for 2–6 h at 30–50 °C at 200 rpm. Further specifications are described in Section 3.

Figure 3A shows the response surface for the analysis of the interaction between time and temperature. Longer reaction times increase the conversion into fatty acid ethyl esters [48]. In the present study, the time varied between 2 and 6 h, but there were no variations in the conversion values in the adopted time interval. This time range adopted in the present work differs from that reported in the literature by Amini et al. (2017) [41], in which time ranging from 12 to 72 h was evaluated, using a 1:12 molar ratio (sweet basil seed oil: methanol), 5% w/w biocatalyst content (Novozym® 435) and a reaction temperature of 40 °C. The yield in ester gradually increased in the interval from 24 to 72 h. In 48 h of reaction, the conversion presented was 89% and at 72 h, the conversion was 92%. It was possible to observe in the present work, in the time of 2 h, using a ratio of 1:15 (free fatty acids/ethanol), with a load of 0.15 g of biocatalyst at a temperature of 30 °C, it was possible to obtain the conversion of 91.11%.

Figure 3B shows a response surface obtained by plotting the biodiesel conversion in relation to the molar ratio (fatty acids/ethanol) and temperature. It is possible to observe that the biodiesel conversion was reduced in a certain molar ratio (fatty acids/ethanol). However, no significant change was observed as the reaction temperature was raised from 30 to 50 °C. High temperatures for heating large reaction volumes mean a cost for any industrial process. Furthermore, at higher temperatures, the enzyme may be denatured, causing the reduction of enzymatic efficiency. Lipases show the activity of more than 90% in the temperature range of 30 to 50 °C [76]. Thus, the temperature was not a parameter that influenced the biodiesel conversion in the present study. The authors Amini et al. (2017) [41], reported in their work that a significant change was observed in the biodiesel conversion in a temperature range from 30 to 40 °C. In the range from 40 to 60 °C, there was no significant change. While in the range from 60 to 70 °C there was a drop-in conversion, which may be linked to the denaturation of the enzyme during the process. Temperature was also a parameter studied by the authors Nguyen et al., 2017 [77], the range adopted was 20 to 40 °C, the authors observed that there was no relevant change in the biodiesel conversion catalyzed by Novozym® 435 using fat from black soldier fly (*Hermetica illucens*) in the presence of methanol. It is possible to observe that the temperature range adopted in the present work and the temperature ranges reported in the literature did not influence the biodiesel conversion.

The molar ratio is one of the main parameters in the biodiesel production. It is important to note that the conversion is influenced by the substrate properties and the nature of the catalyst. For the complete conversion of ethanol and free fatty acids or oil to fatty acid ethyl ester, a stoichiometric ratio of three moles of alcohol is required for each mole of the substrate, which may vary in different systems [41]. The molar ratio (free fatty acids/ethanol) was one of the parameters that most influenced the biodiesel conversion in the present work. It was observed that the conversion to ethyl esters was reduced in the mixture of 1:1 (free fatty acids/ethanol) and that the value increased significantly in the 1:15 mixture (free fatty acids/ethanol), Figure 3B. In other studies, reported in the literature using methanol, excess methanol caused the inactivation of Novozym® 435. The authors Nayak and Vyas, (2019) [78] showed that the enzyme was deactivated at a 1:15 molar ratio (papaya oil/methanol) reducing the conversion to methyl esters; however, in the proportion of 1:3 and 1:9, the reaction rate was directly favored resulting in better biodiesel yields. A similar result was observed that in the proportion range between 1:3 (sweet basil seed oil/methanol) and 1:12 (sweet basil seed oil/methanol) there was a gradual increase in conversion to methyl ester and between 1:12 (sweet basil seed oil/methanol) and 1:15 (sweet basil seed oil/methanol), a reduction was observed [41]. Even with an excess of 1:15 (fatty acids/ethanol), the biocatalyst continued to present its catalytic activity, as it did not suffer from inhibition of ethanol, unlike methanol, which exerts a greater inhibition in enzymatic activity [79].

For the biodiesel production, the lipase content generally considered is 2 to 10% in relation to the weight of the oil. Increasing the enzyme load causes a greater conversion to ethyl esters [48,80]. Figure 3C shows the interaction between the biocatalyst contend and the temperature. As well as the temperature, the biocatalyst content (0.05–0.15 g) did not have a significant change in the conversion; however, the greatest conversions were achieved when offered a 0.15 g biocatalyst content. Nayak and Vyas, (2019) [78] reported that the increase in the amount of catalyst from 0.5% to 1% by weight, improved the yield in methyl esters. Though, when a higher load of biocatalyst (1%–1.5% by weight) was offered in a molar ratio, with proportions 1:3 to 1:15 (papaya oil/methanol), the yield decreased due to the formation of a gel and the increase in viscosity in the mixture [81]. Yu et al. (2010) [22], studied the influence of increased biocatalyst content at a 1:1 molar ratio (soy oil/methanol) in the transesterification process using Novozym® 435. They observed that as a content of biocatalyst was varied from 0% to 10% in relation to the weight of the oil, the yield in methyl esters was also increased, the best load considered was 6%, before this value, the yield of the methyl ester showed a sharp increase; after this value, the yield was considerably slower. Based on Figure 3C, it is possible to conclude that there was less interaction between the biocatalyst content and temperature in the conversion of biodiesel [82].

2.4. Properties of Biodiesel

Table 6 presents the properties of biodiesel produced by free fatty acids from residual babassu oil. The values were compared with the literature and European standards EN 14,214 [60] and ASTM D6751 [83]. As noted in the table, most properties were similar to those found in the literature. Besides, almost all properties of biodiesel, density (820 kg/m^3), viscosity (1.57 mm^3/s), ester content (96.84%) and the acid value (<0.8 mg KOH/g) met the European standard EN 14,214 and the ASTM specification, only the density that did not meet the standard.

Table 6. Biodiesel properties of residual oil from babassu obtained enzymatically.

Analytical Parameter	Technical Norm Adopted	Biodiesel Babassu	Reference [58]	ASTM D6751 [83]	EN 14,214 [60]
Acidity Level (mgKOH/g)	Adolfo Lutz Institute [59]	0.41	0.425	0.5 maximum	0.5 maximum
Ester content (%)	EN 14,103 [72]	96.8	nr[a]	nr[a]	96.5
Kinematic Viscosity at 40 °C (mm^2/s)	ASTM D445-18 [61]	1.6	4.2	1.9–6.0	3.5–5.0
Density at 20 °C (kg/m^3)	ASTM D1480 [84]	820	872	<880	860–900

[a] nr = none reported.

The density is one of the significant characteristics of the fuel. It is used to know the necessary quantity of fuel supplied by the injection systems for the exact combustion of the fuel to occur [85]. The fuel that has a high density has more mass when compared to a fuel with a lower density. As a result, the amount of energy and the fuel/air ratio are affected by the density inside the combustion chamber. The density value can be influenced by several factors, for example, type of raw material, ethyl ester profile and the biodiesel production process [86]. Viscosity is an important specification to define the flow capacity of the fuel [87]. When the viscosity has a high value, an inadequate atomization is created in the fuel, which causes the deposition of dirt and reduces the thermal efficiency [58]. Among the properties of biodiesel, the lowest acid value and the highest ester content in biodiesel propose that the lipase-catalyzed process is a promising alternative to the chemically catalyzed process [58]. The density and viscosity of babassu biodiesel obtained are lower than those reported by [58]; this is because these properties are negatively influenced by the reduction in the length of the carbon chain of the oil used as raw material and the structure of the ethyl ester of individual fatty acids [88,89].

2.5. Operational Stability of the Biocatalyst

Figure 4 shows the conversion in ethyl esters produced after different cycles of reuse using Novozym® 435, under optimal conditions of reactions. It is possible to verify that at the end of 10 consecutive cycles, the enzyme maintained 91% of catalytic activity.

The results obtained are in agreement with those presented by other authors. Márin-Suárez et al. (2019) [42], investigated the reuse of Novozym® 435, lipase from *Rhizomucor miehei* (RML) and lipase from *Thermomyces lanuginosus* (TLL), with different enzymes concentrations, in the transesterification of residual fish oil for the production of biodiesel. Novozym® 435 lost only 16% of its initial activity after the second re-use and, after that, the ethyl esters production was kept constant for 10 cycles of reuse, regardless of the mass of enzyme used. On the other hand, RML and TLL lost more than 75% of their activity after the first reuse and almost 90% after the second cycle, to 12.5 and 25% in mass of enzyme. However, both lipases showed the same behavior after reuse, they showed a rapid deactivation after the first cycle, probably caused due to alcohol inhibition and glycerol adsorption (the co-product of transesterification reaction) by lipase support [90]. As glycerol is insoluble in hexane, it is probably not able to remove the glycerol layer in the case of more hydrophilic supports [91,92].

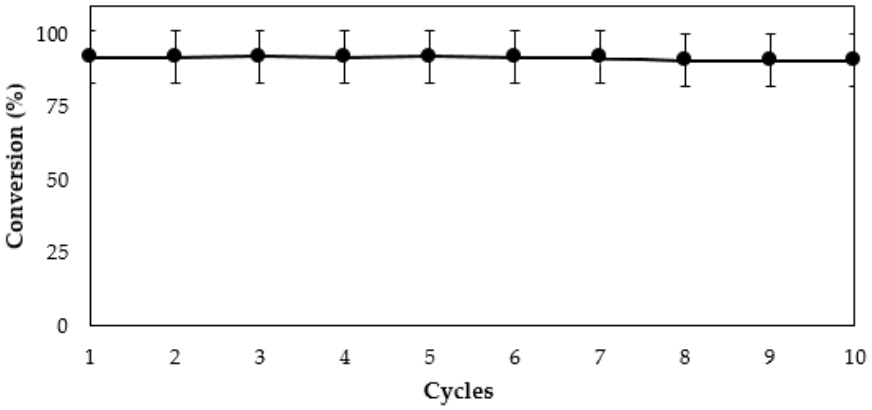


Figure 4. Cycles of activity of Novozym® 435 in the enzymatic esterification of babassu free fatty acids at 48 °C, molar ratio 1:18 (FFAs/alcohol), biocatalyst content 0.14 g and 4 h of reaction.

2.6. Modification of the External and Internal Texture of Biocatalyst

The Novozym® 435 is produced from immobilization of the lipase B from *Candida antarctica*, adsorbed on a macroporous resin composed by poly (methyl methacrylate acid) (PMMA), crosslinked with divinylbenzene (DVB). It is reported that after the esterification reaction may occur the enzyme desorption [93]. Thus, FTIR spectra were obtained for the biocatalyst before (Figure 5a) and after (Figure 5b) the esterification reaction of free fatty acids, in order to obtain information about its structure. For the Novozym® 435 samples, broadbands in the range of 3600–3100 cm^{-1} correspond to O–H and N–H stretching. The vibrational modes of asymmetric and symmetric stretching methyl groups were observed at 2950 and 2875 cm^{-1}, respectively. The band centered in 1732 cm^{-1} can be related to the stretching vibration of carbonyl groups ν(C=O) of the PMMA [94]. The bands at 1452 and 1512 cm^{-1} confirm the presence of DVB in the structure of the biocatalyst since it can be associated with the bond stretching between carbon atoms in the aromatic ring [95]. Moreover, the bands observed from 869 to 682 cm^{-1} are characteristic of the C–H out-of-plane bending of alkyl-substituted benzene. The presence of the lipase B from *Candida antarctica* can be confirmed by bands at 1654 and 1539 cm^{-1} that correspond to the Amide I and Amide II [96]. These same bands also were observed in the spectrum obtained after the esterification reaction. However, it is important to analyze not only the profile and the association of the bands. The ratio between the bands at 1732 cm^{-1} and 1654 cm^{-1} can be used to verify if there is any charge in the biocatalyst related to the enzyme or support. For the Novozym® 435 (Figure 5a), this ratio (3.663) is greater than catalyst after reaction (Figure 5b) (2.678), suggesting a small change in the support/enzyme ratio after the reaction.

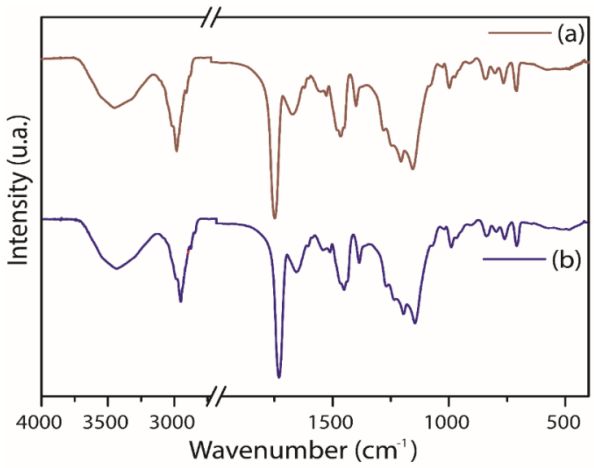


Figure 5. Fourier Transform Infrared (FTIR) spectrum of the Novozym® 435 before (**a**) and after (**b**) the esterification reaction.

SEM images were used to evaluate the morphology and textural properties of the biocatalyst before (Figure 6a,b) and after (Figure 6c,d) the esterification reaction. The biocatalyst showed a spherical morphology with a diameter ranging from 600 to 700 µm. Additionally, it was observed for both samples a low roughness on their surface. In order to evaluate the dispersion of elements in the surface of the material, Energy Dispersive X-ray Spectroscopy EDS images were also obtained (inset Figure 6b,d). The dispersion of elements in the samples is very similar (Table 7), not being observed any significant change in the surface of the biocatalyst after the esterification reaction.

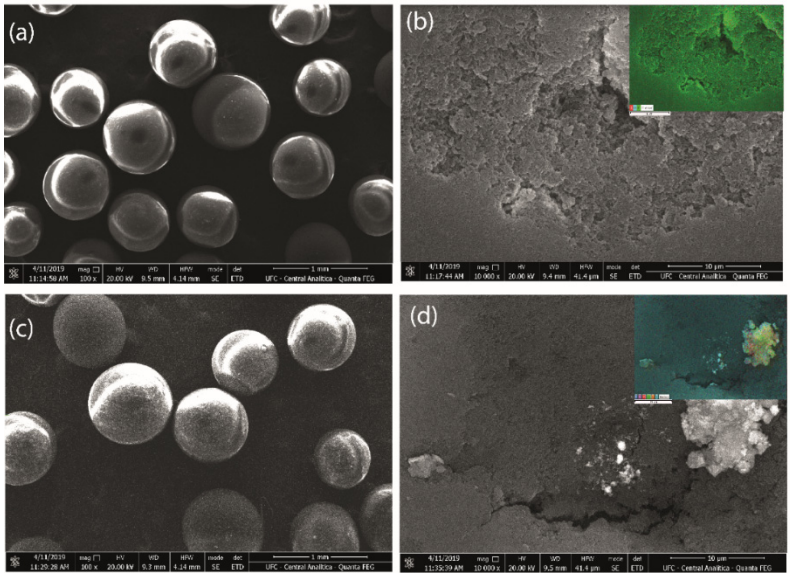


Figure 6. Scanning electron microscopy (SEM) and mapping obtained of Novozym® 435 before (**a**,**b**) and after (**c**,**d**) the esterification reaction.

Table 7. Percentage in weight of the elements on the surface of the Novozym® 435 before and after the esterification reaction.

Map Sum Spectrum (%)		
Elements	**Novozym® 435 before Reaction**	**Novozym® 435 after Reaction**
C	84.9	83.3
O	15.0	14.9
S	0.2	0.2
Al	-	0.2
Ca	-	1.2
Si	-	0.1

FTIR is one of the most powerful techniques of molecular analyses for material characterization. This technique has high-sensibility and allows the identification of many functional groups. Thus, since Novozym® 435 is formed by immobilization of the lipase B from *Candida antarctica* adsorbed on a macroporous resin composed by poly(methyl methacrylate acid) (PMMA) crosslinked with divinylbenzene (DVB), it is possible to use the FTIR as a tool to the identification of subtle change in this materials. Besides, this technique is based on the analyses of a certain amount of materials, usually in the milligram range. SEM and elementary analysis from EDS spectrum are usually obtained from the upper few micrometers of the sample, i.e., it is not a bulk analysis technique. However, these techniques provide information on a small area of the sample, since the measurement is being carried on the surface of a single particle or in a small set of particles.

3. Materials and Methods

All experiments were carried out at least in duplicate. The results are presented as the average of these values and the standard deviation (generally below 10%).

3.1. Materials

The commercial lipase (E.C. 3.1.1.3) Novozym® 435 (10,000 PLU/g) was purchased by Sigma-Aldrich Brasil Ltda (São Paulo, São Paulo, Brazil). The alcohol used was ethanol (P.A. 99.96%) from Dinâmica (São Paulo, São Paulo, Brazil) and the refined residual babassu oil was purchased at a local market (Redenção, Ceará, Brazil). The reagents used in the characterization of the raw material were all of the analytical grades.

3.2. Physicochemical Characterization of Residual Babassu Oil

Table 8 presents the analytical parameters and technical standards used for the physicochemical characterization of residual babassu oil.

Table 8. Analytical parameters and technical standards used for the physicochemical characterization of residual babassu oil.

Analytical Parameter	Technical Standard
Acidity level (mgKOH/g)	Instituto Adolfo Lutz [59]
Saponification Index (mgKOH/g)	Instituto Adolfo Lutz [59]
Oxidative Stability (hours)	EN14214 2003 [60]
Kinematic Viscosity at 40 °C (mm^2/s)	ASTM D445-18 [61]
Density at 20 °C (g/cm^3)	NBR 14,065 [62]

3.3. Analysis of the Chemical Composition of Residual Babassu Oil

For the preparation of methyl esters from residual babassu oil by transesterification, it was used the experimental procedure described by [59]. To determine the chemical composition of fatty acid methyl esters of residual babassu oil, the analysis was performed by gas chromatography (GC) (Agilent, Santa Clara, CA, USA). The equipment used was the gas chromatograph with flame ionization detector (GC-FID) of the brand VARIAN and model 450-GC, with a capillary column CP-WAX 52. The dimensions of the column were: 30 m length, 0.32 mm internal diameter and 0.25 μm liquid film thickness.

3.4. Production of Free Fatty Acids

For the production of free fatty acids, the experimental procedure described by [97] was used, adjusted for the use of a reflux and heating system rather than an ultrasound system. Thus, using a saponification reaction followed by acid hydrolysis, it was possible to obtain the free fatty acid from the residual babassu oil. The reaction occurred in a system formed by a condenser coupled to a 500 mL round-bottomed reaction flask suspended in a water tank.

3.5. Esterification Reactions

In all esterification reaction experiments, Novozym® 435 was used as the biocatalyst and ethyl alcohol as a solvent of the reaction. The free fatty acids used in the reactions were obtained from the saponification reaction followed by acid hydrolysis of residual babassu oil. The influence of the molar ratio (FFAs/alcohol), biocatalyst content, time and temperature were evaluated for the experiments. The reactions were performed in 10 mL Erlenmeyer flasks, containing the substrates and the biocatalyst, placed in a rotary shaker with digital temperature control and agitation Incubator TE-4200 (TECNAL, Piracicaba, São Paulo, Brazil) at 200 rpm.

3.6. Analysis of the Percentage of Ethyl Esters

For monitoring the percentage of ethyl esters formed by the reaction, the methodology described by [98] and [99] was used, the analytical method of determining the acid index, official methodology [100], was used.

3.7. Design of Experiment

For the accomplishment of the experiments, an experimental design was performed, evaluating the molar ratio (FFAs/alcohol), mass of biocatalyst, temperature and reaction time [101].

In order to determine the optimal conditions for the production of biodiesel, a central composite design (CCD) of 4 variables was carried out using Statistica® 10 software (Statsoft, Tulsa, OK, USA). Table 9 shows the 4 variables, each at 3 levels. The design was constructed of 18 factorial points, 6 axial points (2 axial points on the axis of design variable) and 4 replications at the central point.

Table 9. Numerical and coded values of the variables adopted in the experimental design for the esterification of the free fatty acids of residual babassu oil.

Codes	Variable	Levels		
-	-	−1	0	1
X_1	Temperature (°C)	30	40	50
X_2	Time (hour)	2	4	6
X_3	Molar Ratio Fatty Acid: Alcohol (m/v)	1:1	1:8	1:15
X_4	Biocatalyst Content (%m/m)	5	10	15

The experimental design response variable (Table 9) was adjusted through a second-order polynomial equation presented in Equation (1). The objective is to correlate the independent variables with the response variable. Equation (1) considers the linear, quadratic and interaction effects among the variables. This was used to plot response surfaces for all variables.

The second order polynomial equation for the variables is as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_i X_i X_j + \sum X_{ii} X_1^2 \ + \varepsilon \quad (2)$$

In which, Y is the response variable, β_0 is the constant,β_i, β_{ii}, β_{ij} are the coefficients for linear, quadratic and interaction effects, respectively. X_i and X_j the coded levels of variables X_i and X_j and ε is the pure error. The above quadratic equation was used to plot surfaces for all variables.

3.8. Statistical Analysis Using Response Surface Methodology

The experimental data obtained were analyzed through the response surface methodology using the Statistica® 10 software (Statsoft, Tulsa, OK, USA).

3.9. Gas Chromatography (GC) Analysis

The conversion of the methyl esters was performed according to EN 14,103 norm [72], with some modifications. Shortly, approximately 50 mg of biodiesel was weighed into a 2 mL vial and 1 mL of the methyl nonadecanoate solution (10 mg/mL) was added. One microliter of the sample was withdrawn using a micro-syringe (10 µL) and injected into the gas chromatograph VARIAN-GC 450 (Agilent, Santa Clara, CA, USA) with flame ionization detector, column (DB-WAX)-phase: polyethylene glycol, dimensions 60 m long × 0.32 mm internal diameter × 0.25 µm film thickness.

3.10. Operational Stability of the Biocatalyst

The operational stability of Novozyme® 435 was evaluated under optimal conditions for the production of biodiesel from the free fatty acids of the residual babassu oil. At the end of each cycle, the

biocatalyst was separated from the reaction medium by vacuum filtration using filter paper. Then, the biocatalyst was washed three times with hexane, dried again in vacuo and then taken to the desiccator where it was held for 30 min [102].

3.11. Modification of the External and Internal Texture of Biocatalyst

3.11.1. Scanning Electron Microscopy (SEM)

Micrograph images were obtained by SEM FEG Quanta 450 with EDS. The samples were fixed in a carbon tape and metalized with silver by the Metalizator Quorum QT150ES (Metalizator Quorum QT150ES, Quorum Technologies, Laughton, England). Ten Pascals of pressure was applied in the SEM chamber, with an incident electron beam of 20 kV.

3.11.2. Fourier Transform Infrared Spectroscopy(FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) analysis was carried out in a PerkinElmer 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) used to record spectra in the range between 4000–400 cm^{-1}. Previously, the spheres were dried, grounded to powder and pressed (~10 mg of the sample to 100 mg of KBr) in disk format.

4. Conclusions

To authors' knowledge, up to date, this work reported for the first time in the literature the production of biodiesel from residual babassu oil through enzymatic esterification. Novozym® 435 has been studied as biocatalyst and the reaction parameters have been optimized to maximize conversion to fatty acid ethyl esters. The ideal reaction conditions were determined by RSM and a biodiesel yield of 96.84% was obtained under the optimized production conditions (0.14 g biocatalyst, 1:18 (FFAs/alcohol), 48 °C and 4 h). Under the optimized conditions, Novozym® 435 was used in 10 consecutive cycles of esterification, with a slight reduction in the biodiesel yield of 5.84% after the tenth reuse. The biodiesel properties were analyzed and showed reasonable agreement with the specifications of the European standard EN 14,214 and ASTM D6751, which implies that the free fatty acids of the residual babassu oil may be a promising raw material for the production of biodiesel.

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