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Study of enzymatic production of biodiesel using vegetable oils and commercial ethanol

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Biodiesel is a combination of alkyl fatty acid esters generated by a catalyst and supported by acid, basic, or enzymatic processes from vegetable oils to short-chain alcohols such as methanol or ethanol. However, the high costs for raw materials utilising vegetable oil of food grade have made it commercially impracticable to produce this biofuel. Research has therefore expanded with residual oil indicating that biomass from household and industrial waste has been technically feasible. The outcome of this investigation coincides with the enzyme production study of biodiesel utilising residual oil and ethanol, according to this principle. In esterification of commercial oleic acid, the behaviour of commercial immobilised lipase from CAL-B was examined and the variables affecting the process were investigated. Based on the results presented and discussed in this paper, the use of immobilized *Candida antarctica* lipase type B (CAL-B) for biodiesel production is more viable when using acidic substrates, since the best results were achieved with such raw materials and with a reaction rate comparable to esterification with an acid catalyst. The results of this work showed that the enzymatic esterification of commercial oleic acid with ethanol provided a conversion of 87.3% within 60 minutes of reaction, at a temperature of 30 °C, in a stoichiometric proportion and without adsorption of water. Both biocatalysts showed good stability, which produced over 80% conversion and 60 minutes of reaction and could be repeated without substantial loss of activity for at least 10 consecutive occasions.

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KEYWORDS

Vegetable oils; commercial ethyl alcohol; bio diesel; CAL-B.

Introduction

Biodiesel was born basically as a solution to the international oil crisis, but also and mainly because of the environmental problems that our planet is currently facing. The environmental benefits that this new fuel can bring are immense, such as a reduction of up to 78% of carbon dioxide emissions, which is absorbed by the photosynthesis of the oilseeds themselves, a 90% reduction in smoke emissions and a 98% reduction in sulphur emissions in the atmosphere [12]. Among the biodiesel production processes, alkaline alcoholysis with methanol is the most commercially used process. However, despite the low cost of homogeneous chemical catalysts, this production route has some disadvantages such as the non-recovery and reuse of the catalyst, soap production, difficult glycerol recovery, high energy expenditure and the continued dependence on oil when using methanol [6]. However, biotransformation is not yet commercially competitive with conventional catalysis due to the high price of enzymes and their

inactivation by contamination by oil, alcohol or by-products formed during the reaction [5]. Therefore, this study aimed to study the enzymatic production of biodiesel using, primarily, commercial oleic acid and ethanol to evaluate the behavior of lipase in the esterification of acidic substrates and the variables that influence the process. The importance of using commercial oleic acid first is that, in addition to this acid being the most commonly found in waste oils, starting from a pure raw material, the likelihood of enzyme inactivation by residual oil contaminants is reduced, and only the influence of the chosen variables is evaluated, seeking to optimize the process in order to establish the characteristics for industrial applications. Knowing the best conditions for esterification of oleic acid, the behavior of lipase against residual oils and its operational stability after several consecutive reactions were evaluated. We also aimed to compare the behavior of lipase in media with distinct physicochemical characteristics.

According to the literature [9], the enzymatic biotransformation of residual vegetable oil, the object of interest in this paper, presents, in addition to all the advantages the differential that the raw material does not need a pre-treatment, which consists of using it first an acid catalyst to convert the free fatty acids into their respective esters and lower the level of these acids in the oil, and then perform an alkaline trans esterification. Although the enzymatic alcoholysis processes to obtain biodiesel are not yet commercially developed, new results have been reported in articles and patents. The common aspect of these studies is the optimization of reaction conditions, i.e. solvent, temperature, pH, enzyme, among others, in order to establish characteristics for industrial applications. However, both the yield, reaction time and cost of the biocatalyst are still unfavourable when compared with the basic catalysis reaction system. Accordingly, this work aimed to study the enzymatic invention of biodiesel from residual oil and ethanol, verify the influence of process variables and reaction kinetics, seek optimization and cost reduction in obtaining a product with high added value. As the objective of this work was to obtain a biofuel from a residual raw material and, as these residues generally have a high free acid index, the influence of process variables of enzymatic ethanolysis of oleic acid was first studied, and to confirm the feasibility of using lipases in this type of reaction, some experiments were carried out with two residual oils (oleic acid and coconut oil) of different acidity indices.

Materials and methods

Materials

Three types of lipid material were used for the enzymatic alcoholysis reaction to obtain ethyl esters: Commercial oleic acid (COA) for synthesis (Sigma-Aldrich, 99%), residual coconut oil from Virgin Coconut Oil (VCO) and residual cotton oil obtained from Gokul refoil Vivaan®. Commercial ethyl alcohol (Lab Chem, 99.8% purity) was also used as substrate in all experiments. As catalyst, a commercial immobilized lipase of *Candida antarctica* type B (CAL-B) was purchased from Aumgene Biosciences pvt. Ltd., India, whose hydrolytic activity was 491.98 U/g. This derivative consists of CAL-B lipase immobilized in chitosan, activated with glycidol, ethylenediamine and glutaraldehyde, whose excess moisture was removed by drying with hexane in the presence of 3% butyric acid. The conditions of immobilization, activation and drying of this derivative were the conditions optimized at 6 mg of protein/g of support were immobilized, showing a hydrolytic activity of 369.36 U/g.

Determination of the hydrolytic activity of immobilized enzymes

The determination of the hydrolytic activity of the enzyme was described by Rattanaphra,

[11]. The change in absorbance at a wavelength of 410 nm was monitored for 7.5 minutes, removing approximately 2 mL of the supernatant every 1.5 minutes and being read immediately in the spectrophotometer. A unit of activity (U) was defined as the amount of enzyme needed to hydrolyze 1 mmol of pNPB per minute, and activities were expressed in U/g.

Physical-chemical characterization of raw material and biodiesel

Esters obtained from the alcoholysis of a lipid raw material can only be considered as biodiesel if they meet certain requirements, that is, if they meet the specifications regulated by the National Petroleum Agency (ANP). Next, the analyses carried out in the characterization of the raw material and the ethyl esters obtained by enzymatic synthesis are described. Acidity Index, Density, Moisture Content, Iodine Index, Free, Combined and Total Glycerine were calculated according to the standard procedure (FAO, 1986)

Chromatographic characterization of raw material

The chemical fatty acid composition of oleic acid and residual vegetable oils was determined by transesterifying the raw material with methanol, and identifying the purified methyl esters resulting from the process by gas chromatography. The equipment used was a Thermo Fischer gas chromatograph coupled to a mass spectrometer, with a Column Capillary RTX-5MS adsorber column. Column dimensions were 15 m long, 0.25 mm internal diameter and 0.25 µm liquid film thickness. The column flow was 0.5 mL/min, the detector temperature was 200 °C, the injector temperature was 250 °C and the oven temperature started at 50 °C, remaining at this temperature for 2 minutes when it increased by 4 °C/min until reaching 250 °C. The carrier gas was helium gas and the injected sample volume was 1 µL.

To carry out the transesterification reaction of the substrates, the procedure described by Bart, [2] was adopted, with some modifications. 1 g of oleic acid/vegetable oil was measured in a flat-bottomed flask. 25 mL of saponification reagent was added, stirred vigorously and allowed to heat under reflux for 30 minutes. The flask was then cooled and 25 mL of the esterification reagent was added, stirred vigorously and allowed to heat under reflux for a further 30 minutes. After this time, the flask was cooled and its contents transferred to a separating funnel with the aid of 25 mL of petroleum ether. The formation of 3 phases was observed. The lower two phases were discarded and 20 ml of brine was added to the upper phase. This time, the formation of two phases was observed. The lower phase was discarded and the upper phase (ethyl esters) was transferred to a beaker with sodium sulphate (to remove water residues). Sodium sulphate was filtered, collecting the solution in another beaker. It was allowed to evaporate at room temperature overnight, and only then was the resulting solution injected into the chromatograph.

Enzymatic alcoholysis of oleic acid and residual vegetable oils using lipase as a catalyst

The lipid raw material used in all experiments did not undergo any previous treatment, but a simple filtration to remove particulate matter when using residual oils.

The experiments were carried out in 250 mL Erlenmeyer flasks closed with a glass lid. The amount of acid/oil was kept fixed at 10 g in all experiments, also the amount of lipase was fixed at 5% w/w, based on the measured acid/oil mass. The quantities of ethanol were pre-established by planning experiments. The range of study of variables sought to cover most studies in the literature regarding the enzymatic alcoholysis reaction of vegetable oils.

After calculating the amounts of alcohol, the Erlenmeyer's, containing the substrates and the catalyst, were placed on a rotary shaker, with digital temperature and agitation control (TE - 420 incubator), at 200 rpm. After the reaction time, also defined by a factorial experimental design, the samples were filtered for recovery and subsequent reuse of the enzyme and placed in a separation funnel for phase separation, i.e. water decantation when using oleic acid and coconut oil and glycerine decantation when using cottonseed oil. The upper phase, rich in ethyl esters, was separated and analyzed. In Figure 1 the flowchart of the experimental procedure is represented.

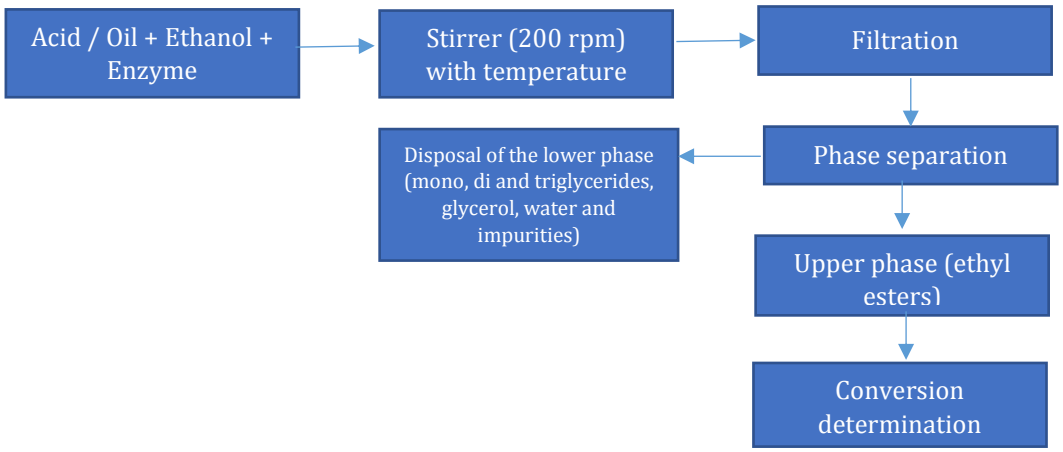

FIGURE 1 Flowchart of the experimental procedure for the enzymatic alcoholysis reaction of oleic acid and residual vegetable oil

The variables chosen to be studied varied according to the starting raw material. When using oleic acid, the influence of the acid:alcohol molar ratio, the temperature of the reaction system and the concentration of water were studied for a reaction time of 8 hours. When the starting raw material was cottonseed oil, the chosen variables were the oil:alcohol molar ratio, temperature and reaction time, as this is a transesterification reaction, since cottonseed oil presented a low acidity index, the literature indicates high reaction times. To verify the effect of the variables on the reaction conversion, as well as to find the conditions that maximized the synthesis of esters, a factorial experimental design with 2 levels and 3 variables was carried out. Actual values of independent variables for Factorial Design 2^3 used to optimize the conditions of enzymatic esterification of oleic acid using a commercial CAL-B as catalyst. Acid:ethanol molar ratio [R] (1:1-1:6), Temperature [TR](30 °C -50 °C) and Water concentration [W] (%w/w) 0 – 20 . These intervals were defined so as to cover most studies in the literature. Actual values of independent variables for Factorial Design 2^3 used to optimize the conditions of enzymatic ethanolysis of residual cottonseed oil using a CAL-B lipase as catalyst[Oil:ethanol[R] molar ratio (1:3-1:9), Temperature [TR]30-50, Time [TO] 24-72 hrs].

It is noteworthy that the experiments were performed randomly and with triplicate of the central point. The experimental data were analyzed with the Statistica 6.0 software. Due to the high acidity index of the residual coconut oil, the best experimental condition obtained with oleic acid was chosen to carry out a reaction with this oil and confirm the feasibility of using lipases in that kind of reaction. The experiments carried out with lipase immobilized in chitosan were also carried out under the best conditions obtained in both the alcoholysis of oleic acid and coconut oil to verify the potential use of this derivative prepared with a low- cost support.

Biocatalyst recovery and regeneration

After the reaction, the biocatalyst was vacuum filtered, collecting the biodiesel in a Kitassate and the lipase retained in a porcelain funnel with filter paper. The biocatalyst was then washed three times with hexane p.a. and dried in a vacuum, then reused to verify the loss of activity in the reaction of alcoholysis of the lipid material.

Neutralization of the ethyl esters obtained

After obtaining the ethyl esters with enzymatic catalysis, we observed that the acidity index was not within the limit established by the National Petroleum Agency (ANP). In an attempt to keep the mixture of esters within specification with regard to the acidity index, a neutralization was made with a glycerine sample resulting from the alkaline Trans esterification process. Therefore, according to Ismail [7], the acidity index of biodiesel was divided by 50.6 to obtain the value in meq/g. To this result, 15% was added and the final value corresponded to 1 g of biodiesel. Starting from the amount of biodiesel that wanted to be neutralized, by a simple rule of three, the amount of biodiesel to be neutralized in meq/g was calculated. Then, the free alkalinity of the glycerine phase was calculated, in meq/g, and this value corresponds to 1 g of glycerine. As the amount of biodiesel in meq/g that one wants to neutralize is already known by a simple rule of three, the amount of glycerine in grams corresponding to the amount of biodiesel to be neutralized was calculated. Then, the biodiesel was mixed with the glycerine, leaving it under stirring and heating until reaching a temperature of 120 °C, remaining at this temperature for 30 minutes. Then, the mixture was placed for decanting the glycerine, analysing the acidity of the upper phase, which corresponds to the neutralized esters.

To analyze the free alkalinity of the glycerine phase, approximately 2 g of the sample was measured and mixed with 25 mL of ethyl alcohol neutralized with 0.25 N sodium hydroxide, using a 1% phenolphthalein solution in alcohol as an indicator. Then, the mixture was titrated with 0.1 N hydrochloric acid and the free alkalinity was calculated according the procedure developed by FAO 1986.

Results and discussion

Physical-chemical characterization of raw material

Table 1 shows the result of the physicochemical characterization of the lipid raw materials used in this work. As can be seen in Table 1, oleic acid and residual coconut oil have a high acidity index, which makes their use in alcoholic reactions with a basic catalyst unfeasible. However, cottonseed oil had a low acidity index.

TABLE 1 Physicochemical characterization of lipid raw materials used in alcoholysis reactions and ethyl esters

PROPERTIES	lipid raw materials used in alcoholysis reactions			ethyl esters	
	Oleic acid	Residual coconut oil	Residual cotton oil	Ethyl esters	ANP limit
Acidity index (mg of KOH/g)	191.7	195.1	1.78	25.4	0.5
Density at 20 °C (Kg/cm³)	893.40	917.36	918.74	879.96	850-900
Iodine index (g/100 g)	92.50	7.21	105.08	6.74	**
Moisture content (%)	0.07	0.25	0.07	0.05	0.05
Free glycerin (%)	ND	0.03	0.07	0.015	0.02
Total glycerine (%)	ND	3.17	8.41	0.204	0.25

It is also observed that oleic acid and residual cottonseed oil had a high iodine index, indicating the strong presence of unsaturated compounds, i.e. palmitoleic acid, linoleic acid and oleic acid, which is confirmed by the chromatographic analysis, as described in topic 3.2. The presence of unsaturations is not recommended because double and triple bonds are extremely reactive and cause both the oil and the resulting biodiesel to have a low resistance to oxidation and a greater tendency to form "gum" deposits in engines [10]. The residual coconut oil, on the other hand, had a much lower iodine index, which suggests the low presence of unsaturated compounds, also confirmed by chromatographic analysis.

Chromatographic characterization of raw material

Table 2 lists the percentages of fatty acids obtained by gas chromatography for oleic acid and residual vegetable oils. Based on the chemical composition, the average molar mass of oleic acid is 282.47 g/mol. Based on the chemical composition and considering that the residual coconut oil, due to its high acidity index, is mainly constituted by free fatty acids and not by triglycerides, the average molar mass of this oil is 217.60 g/mol.

Figure 2 shows the residual coconut oil chromatogram with the most representative peaks and their respective retention times. The most intense peak, whose retention time is 21.32 minutes, refers to lauric acid (dodecanoic acid), present in greater quantity in the oil sample. Based on Table 2, the average molar mass of residual cottonseed oil is 827.01 g/mol. Figure 3 shows the residual cottonseed oil chromatogram with the most representative peaks and their respective retention times. The most intense peak, whose retention time is 11.99 minutes, refers to linoleic acid, present in greater quantity in the oil sample.

TABLE 2 Percentages of fatty acids for oleic acid; Percentages of fatty acids for residual coconut oil and Fatty acid percentages for residual cottonseed oil

Fatty acid	Composition (%)
Percentages of fatty acids for oleic acid	
Myristic acid	0.8585
Palmitoleic acid	2.0402
Palmitic acid	2.5755
Cis oleic acid	95.344
Percentages of fatty acids for residual coconut oil.	
Caprylic acid	2.6664
Capric acid	3.7774
Lauric acid	52.1362
Myristic acid	22.624
Palmitic acid	10.1404
Linoleic acid	0.5151
Cis oleic acid	0.6565
Trans oleic acid	5.555
Stearic acid	2.8987
Fatty acid percentages for residual cottonseed oil.	
Myristic acid	0.4848
Palmitoleic acid	0.2222
Palmitic acid	21.614
Linoleic acid	47.9245
Cis oleic acid	23.0381
Trans oleic acid	4.7066
Stearic acid	3.0098

Figure 2 shows the oleic acid chromatogram. A well-defined peak is observed for oleic acid, with a retention time of 12.2 minutes. However, other peaks were detected, recording the presence of other fatty acids, in smaller amounts, indicating that the acidity shown in Table 3.1 is not related only to oleic acid.

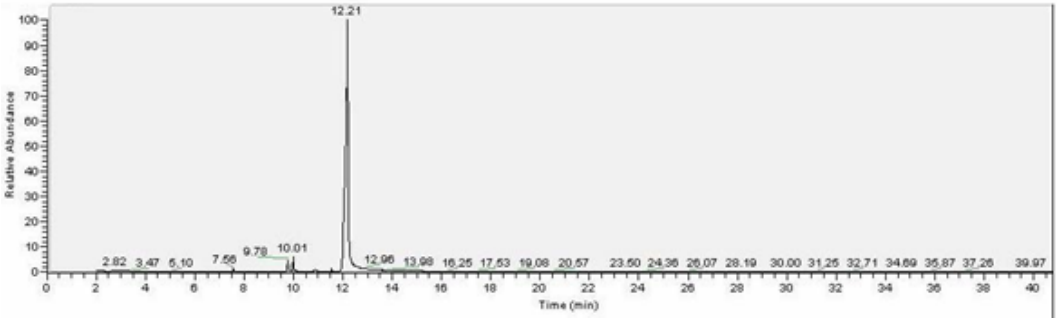

FIGURE 2 Oleic acid chromatogram

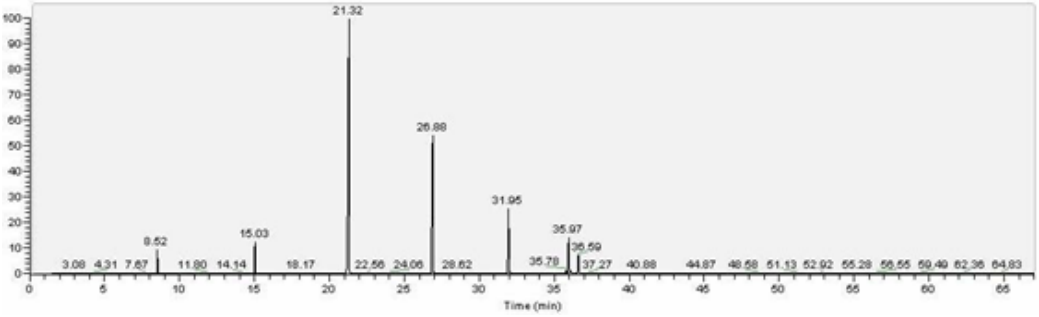

FIGURE 3 Chromatogram of residual coconut oil.

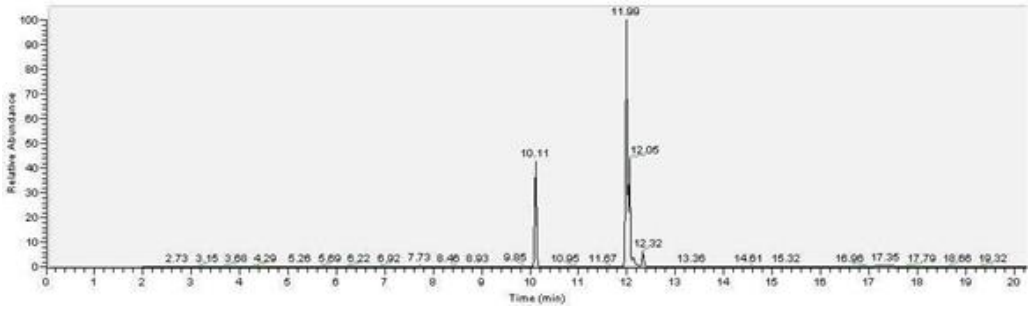

FIGURE 4 Chromatogram of residual cottonseed oil.

Enzymatic alcohololysis of oleic acid using commercial lipase from cal-b as a catalyst

Table 3 demonstrates the results obtained in the factorial experimental design for the enzymatic esterification of commercial oleic acid using commercial lipase as catalyst. The reaction time was kept fixed at eight hours for all experiments, since, in preliminary tests, higher conversions to esters were observed in this time, which was later confirmed when studying the reaction kinetics. Based on these values, the experimental error was approximately 0.68%, showing a good reproducibility of the experiments. Observing Table 3, it can be seen that the highest biodiesel conversion was 88.36% under conditions of lower temperature (30 °C), higher acid:alcohol molar ratio (1:6) and lower water concentration (W = 0%).

However, as will be explained later, the temperature and the concentration of water added to the reaction medium had a non-significant and negative effect, respectively, on the conversion to esters and, despite the positive effect of the molar ratio, a stoichiometric excess was not necessary to obtain reasonable conversions.

TABLE 3 Results of Factorial Design 2^3 with triplicate of the central point having as variable the response to the conversion obtained after 8 hours of enzymatic esterification of oleic acid using a commercial lipase of CAL-B

Molar Ratio(R)	Temperature (T)	water concentration W (% p/p)	Conversion (%)
01:01	30 °C	0	86.41 ± 0.57
01:06			87.35 ± 0.57
01:01		20	77.60 ± 0.57
01:06			86.60 ± 0.57
01:01	50 °C	0	87.04 ±0.57
01:06			86.70 ± 0.57
01:01		20	76.40 ± 0.57
01:06			81.90 ± 0.57
01:03.5	40 °C	10	85.91 ± 0.57
01:03.5			85.09 ± 0.57
01:03.5			87.24 ± 0.57

Table 3 shows the statistical analysis performed using the Statistica 6.0 software. It can be seen that the variable water concentration and molar ratio between the reagents cross the line of significance level of 95%. Therefore, the acid:alcohol molar ratio and the water concentration had a statistically significant effect on the conversion to esters, within the range studied for each variable.

Table 4 shows the estimated effects of the conducted experimental design. It can be observed that the p values obtained for the analyzed factors are less than 0.05, except for temperature and for the interactions between temperature and molar ratio between the reactants, between temperature and water concentration and for the interaction between the three variables. Therefore, it is confirmed that the only parameter without significant effect within the experimental ranges studied was temperature. Another important observation that can be made from Table 4 is that the water concentration had a smaller effect, indicating that it is the most significant variable in the process. Furthermore, the effect of this variable was negative, which was already expected since the excess of water favours the ester hydrolysis reaction, decreasing the equilibrium conversion. Thus, from the linear regression analysis of the results obtained, it was possible to obtain a polynomial model to describe the conversion into ethyl ester, within the ranges of the studied variables (Table 5).

TABLE 4 Estimate of the main effects and their interactions in the conversion of oleic acid to ethyl esters

Factors	Effects	Standard deviation	P
Average	8447727	0.292	0.000
Temp (°C)	-1.763	0.684	0.123
Molar Ratio	5.998	0.684	0.018
water concentration (%)	-6.073	0.684	0.009
Temp* Molar Ratio	-1.518	0.684	0.528
Temp* water concentration	-0.048	0.684	0.265
Molar Ratio * water concentration	2.693	0.684	0.033
Temp* Molar Ratio * water concentration	-1.773	0.684	0.376

TABLE 5 Shows the analysis of variance (ANOVA) for the response variable (conversion) of the enzymatic alcoholysis reaction of oleic acid using CAL-B lipase as catalyst

Source of Variation	Regression	Error	Total SS
Quadratic Sum SQ	195.82	1.8709	197.69
Degrees of Freedom Df	8 24	2	10
MQ Square Mean	47	0.9354	
F	26.16		

$R^2 = 0.9479$; $F_{8;2;0.05}=19.37$

It is observed that the coefficient of determination (R^2) obtained by the regression is 0.94. The F test can be performed to confirm the fit of the model obtained. According to Robert Kissell [13], for the parameters of the model to have statistical significance, the F value of the regression must be greater than the tabled F value. Comparing the F value of the regression with the tabulated (Tabulated =19.37), there is a significant regression. Therefore, the experimental data are well represented by the adjusted model, that is, the model obtained for the significant variables can be used to predict the conversion to ester, within the studied range. This conclusion is also confirmed by Figure 5, which shows the comparison between the values observed experimentally and those predicted by the adjusted model. The analysis of Figure 3.5 shows little dispersion of the points that are close to the representative line, which proves the good fit of the model.

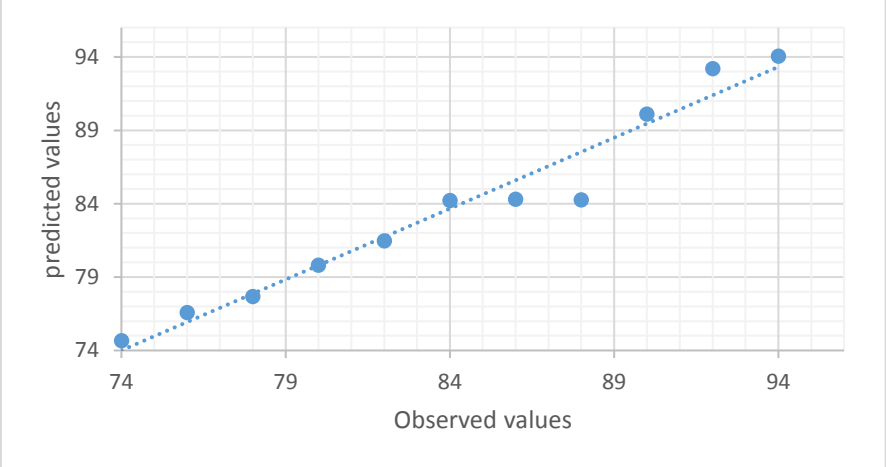

FIGURE 5 values predicted by the model versus experimental values of the conversion to ethyl esters obtained by the experimental design 2^3 with triplicate of the central point of the enzymatic esterification of oleic acid

Temperature effect (TR)

The influence of temperature on the kinetics of the esterification reaction of oleic acid with ethanol using CAL-B lipase as catalyst the biodiesel conversion rate decreases rapidly until the enzyme is likely to be deactivated above 40 °C. In this work, the temperature did not influence the conversion, within the studied range, the reaction kinetics in the lowest water concentration (W = 0%) and in the molar ratios 1:1 and 1:6, respectively. It is clear that despite a slight favouring of the conversion in the first minutes of reaction with increasing temperature, the equilibrium conversion is very similar for both temperatures, and the reaction rate, especially when using acid molar ratio: 1:1 alcohol, it is pretty much the same, reaching balance almost at the same time. From the results, it is observed that for both amounts of temperature, a conversion above 80% is

obtained after 60 minutes of reaction. A conversion above 80% is achieved with a temperature of 50 °C only after 120 minutes of reaction. Therefore, it is not necessary to self-expend energy with the increase in temperature, since it is possible to obtain a good conversion at a temperature close to room (30 °C) and at a speed practically equal to the temperature of 50 °C.

Effect of acid:alcohol molar ratio (R)

The positive effect of the acid on the enzymatic esterification of oleic acid with ethanol using CAL-B as a catalyst is demonstrated by the molar ratio on the enzymatic esterification of oleic acid with ethanol using CAL-B as a catalyst: Although the equilibrium conversions are greater in the higher molar ratio condition, the values are quite close, indicating that no stoichiometric excess is required, making the enzymatic process more advantageous than conventional chemical catalysis in this regard.

These results are in agreement with what has been reported in the literature. Ferdous *et al*. verified the same effect for the molar ratio [4], when studying the influence of operational conditions and limitations of external mass transfer in the synthesis of fatty acid esters using a *Candida antarctica* lipase. These authors studied various molar ratios and found that as the amount of alcohol increased, the conversion to esters progressed more slowly and the equilibrium conversion increased. In this study they verified the existence of a maximum molar ratio in which above this value the initial reaction rate started to decrease, which suggests the existence of an optimal molar ratio.

Effect of water concentration (W)

According to Figure 4, increasing the concentration of water in the reaction medium had a negative effect on the conversion to esters within the range studied, that is, at equilibrium, as the amount of water increased, the conversion of biodiesel decreased. This effect is also clearly which shows the kinetics of the esterification reaction of oleic acid with ethanol using CAL-B as a catalyst at a temperature of 30 °C and an acid:alcohol molar ratio of 1:1. It is known that lipases need a certain amount of water to maintain their active three-dimensional conformation, even when the enzyme is covalently bound to a support, since water interacts with the hydrophilic groups located on the surface of the enzyme leading to conformation open lipase. However, there is a minimum concentration of water in almost anhydrous media in which above this value, the conversion rate decreases significantly, facilitating the hydrolysis of the ester [1].

Enzymatic alcohololysis of oleic acid using lipase from CAL-B type b immobilized in chitosan as a catalyst

Figure 6 shows the comparison of the results obtained in the esterification reaction of oleic acid with ethanol using the chitosan derivative and CAL-B as catalyst. According to Figure 6, it is observed that the equilibrium conversion is practically the same. However, the reaction speed at the beginning is slower when using the chitosan derivative as a catalyst.

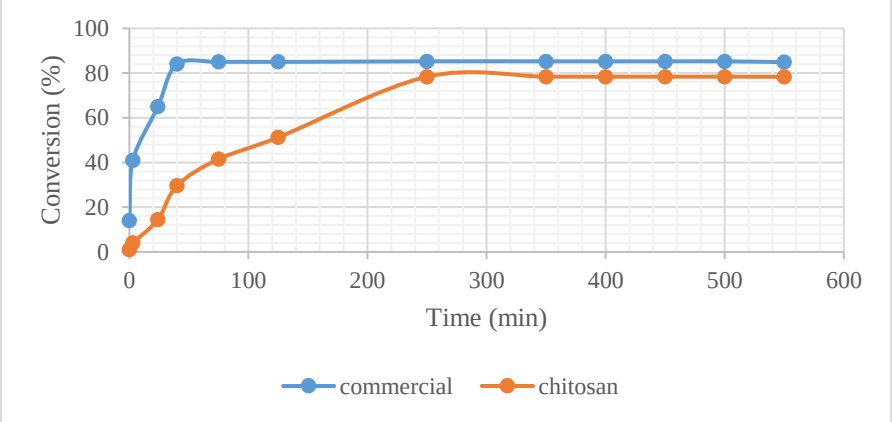

FIGURE 6 Comparison of the behavior of CAL-B Lipase Immobilized in Chitosan with CAL-B in the ethanolysis of oleic acid at a temperature of 30 °C, acid:alcohol molar ratio 1:1 and without addition of water

This observation can be accounted for as follows: First, the amount of immobilized enzyme per gram of support for the chitosan derivative is less than for CAL-B, i.e., the charge of the chitosan derivative is lower, making the reaction speed slower. Another possible explanation is the diffusional problem caused by the activation of the derivative with glutaraldehyde. According to Ferdous *et al*. [4], the low conversions obtained with derivatives treated with glutaraldehyde can be explained by the fact that access to the active site of the lipase was blocked by the chemical bond established between the lipase and chitosan activated during the immobilization process, which may have involved amino acids from the active site, causing steric restrictions, or improper conformational changes, leading to a reduction in enzyme activity.

Enzymatic alcohololysis of residual coconut oil using CAL-B as a catalyst

This comparison was performed to see if the residual oil did not denature the enzyme; CAL-B behaved similarly in the esterification reaction of residual coconut oil with ethanol, proving the potential use of this lipase to obtain esters from acidic raw materials.

Activity cycles

The conversions obtained after 11 ethanolysis reactions of residual coconut oil using the same enzyme sample for both CAL-B and the chitosan derivative. Both lipases were just washed with hexane and vacuum dried before reuse. The results show the stability of lipases, proving the viability of enzymatic catalysis for the production of biodiesel from substrates with high free acidity, since the high cost of the enzyme is one of the most important factors in enzymatic catalysis, restricting the industrial production of biodiesel by this alternative route. These results are also in agreement with what other authors have observed. Ferdous *et al*. verified the maintenance of the CAL-B activity after ten consecutive cycles [4].

Enzymatic alcohololysis of residual cotton oil using CAL-B as a catalyst

The experimental error was around 2.19 percent based on the data acquired in the design of experiments, indicating high repeatability of the tests. The greatest biodiesel conversion was 82.66% after 72 hours of reaction at the lowest temperature (30 °C) and lowest alcohol:oil molar ratio (3:1). The statistical analysis was carried out on the data using the Statistica 6.0 programme. We can see from the Pareto Diagram that the variables molar ratio, response time, and interactions between ratio and temperature and temperature and time all cross the 95 percent significance level. As a result, the alcohol:oil molar ratio and reaction

time, as well as the interactions between temperature and time and molar ratio and temperature, all had statistically significant effects on ester conversion. Temperature had a detrimental effect, albeit it was not significant, indicating that this enzyme performs best at moderate temperatures. The molar ratio showed a negative impact as well, indicating that excess alcohol may have caused enzyme inactivation. The influence of response time was positive, leading to the conclusion that the longer the reaction time, the higher the conversion. The impacts of the examined variables were similar to the effects of the factors analysed in the experimental design with oleic acid, despite the fact that a considerably longer period was required to obtain good conversions with cottonseed oil. The estimated impacts of the cottonseed oil experimental planning are shown in the results. Except for temperature and the interaction between molar ratio and time, and the interaction between the three variables, the p values obtained for the studied components are less than 0.05. As a result, the only parameter in the experimental ranges tested that had no significant influence was temperature.

Characterization of ethyl esters

As this study aimed at obtaining ethyl esters from a residual raw material, and given the good results achieved with the esterification of acidic residual coconut oil using the chitosan derivative, Table 1 presents the characterization of the esters produced by the alcoholic reaction of coconut oil using a lipase of CAL- B immobilized in chitosan as a catalyst, under the condition of lower temperature (30 °C), lower oil:alcohol molar ratio (1:1) and without addition of water. Comparing the characterization of the ethyl esters obtained with the limits allowed by Resolution 7 of the National Petroleum Agency (ANP) clarifies that the only parameter that was not within the specification was the acidity index. However, after submitting the mixture of ethyl esters to a neutralization with glycerine resulting from the biodiesel production process by transesterification with a basic catalyst, the acidity index reduced to 0.76 mg KOH/g, being very close to the limit required by the ANP.

Conclusion

The use of immobilised *Candida antarctica* lipase type B for biodiesel production is more viable when using acidic substrates, according to the results presented and discussed in this chapter, because the best results were obtained with such raw materials and at a reaction rate comparable to esterification with an acid catalyst.

According to Marchetti, *et al*., the methanolysis of commercial oleic acid with sulfuric acid resulted in a conversion of about 87 percent into methyl ester after 60 minutes of reaction at 90 °C, a 1: 3 acid:alcohol molar ratio, and adsorption of water produced throughout the process [8]. At the temperature of 110 °C and a molar ratio of 1:9, a maximum conversion of 88.18% in methyl ester was achieved in experiments without water adsorption. Meanwhile, the results of this study demonstrate that the enzymatic esterification of commercial oleic acid with ethanol yielded an 87.3% conversion in 60 minutes at 30 °C, in a stoichiometric proportion, and without water adsorption.

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