Research Article

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# Enzymatic transesterification of soybean ethanolic miscella for biodiesel production

**Naiane Sangaletti,[a,c,*] Mara Cea,[b] Marisa Aparecida Bismara Regitano-d'Arce,[c] Thaís Maria Ferreira de Souza Vieira[c] and Rodrigo Navia[b,d]**

## Abstract

**BACKGROUND: Biodiesel production is not economically competitive with petroleum diesel particularly when using virgin and refined vegetable oils. Rich-in-oil miscella obtained from the extraction of soybean oil with ethanol may be a promising feedstock for taking off the refining process, simultaneously introducing an environmental friendly step by replacing hexane by ethanol as a renewable solvent in the oil extraction process. The aim of this study was to investigate the production of biodiesel from the oil–ethanol miscella by direct transesterification using Novozym®435 as catalyst and ethanol as acyl acceptor; simultaneously optimizing the process by response surface methodology and enzyme reuse.**

**RESULTS: The best experimental conditions indicated by the empirical model were temperature 40 °C, oil:ethanol molar ratio 1:4.5 and catalyst concentration 9.5% for 24 h, reaching 85.4% fatty acid ethylic esters (FAEE) yield. Tert-butanol used as co-solvent increased the ethyl esters yield from 18%, keeping a high FAEE yield (over 70%) for more than 3 cycles of enzyme reuse.**

**CONCLUSIONS: Rich miscella has great potential as a low cost feedstock for biodiesel production when Novozym®435 is used as catalyst, simultaneously introducing an environmentally friendly step by using the renewable solvent ethanol in the extraction and transesterification.**


**Keywords:** alternative feedstock; ethyl ester; Novozym®435; ethanol; environmentally friendly

## INTRODUCTION

The rapid depletion of non-renewable fossil fuels has accelerated the development of new environment friendly energy sources. In this context, biodiesel offers advantages such as sustainability and neutral greenhouse gas emissions; it is biodegradable and a renewable source of energy.[1]

Several raw materials have been used to produce biodiesel including soybean, rapeseed, peanut, coconut, palm, and rice oils, among other sources of vegetable oils and animal fats.[2] However, biodiesel production is still not economically competitive with diesel, due to the use of refined edible oils that increase the product's costs.[3] The use of waste cooking oils,[4–6] microalgae,[7] non-edible oils from papaya seed (*Carica papaya*) and rambutam (*Nephelium lappaceum*),[8] sttilingia[9] and *Jatropha curcas*[10] has been investigated to minimize this cost. A friendly alternative that could diminish biodiesel production costs is to perform the oil extraction process using ethanol as solvent. Vegetable oils are usually obtained by using the petroleum-derived-solvent hexane as extraction solvent. In this sense, the possible replacement of hexane by a renewable solvent such as bioethanol may be the first step to positively contribute to the development of a novel and environmentally friendly technology to produce biodiesel. This configuration would allow elimination of the solvent distillation and recovery steps from the extracted miscella (oil + solvent), which are replaced by a cooling (down to 30 °C) period, allowing the formation of two phases, one rich-in-oil miscella (rich miscella) and one rich-in-ethanol miscella (poor miscella) that is recycled on subsequent extractions. In addition, this process also promotes an oil pre-refining stage, with partial removal of phospholipids and free fatty acids (FFA). The rich miscella could contain up to 91% oil and up to 8% ethanol, also containing about 0.4% FFA. Furthermore, the remaining ethanol can be used as acyl acceptor in the reaction. Therefore, the use of this rich miscella for direct transesterification instead of using hexane to extract the oil and a further refining process of soybean oil for biodiesel production

* *Correspondence to: Dr Naiane Sangaletti, Departament of Agro-food Industry, Food and Nutrition, College of Agriculture "Luiz de Queiroz" (ESALQ), University of São Paulo. Avenida Pádua Dias 11, P.O. Box 9, Piracicaba 13418–900, São Paulo, Brazil. E-mail: naisangaletti@yahoo.com.br*

a *Center of Nuclear Energy in Agriculture (CENA), University of São Paulo, Av. Centenário 303, PO Box 96, Piracicaba 13416-000, São Paulo, Brazil*

b *Scientific and Technological Bioresources Nucleus, University of La Frontera, PO Box 54-D, Temuco, Chile*

c *Laboratory of Oils and Fats, Department of Agro-food Industry, Food and Nutrition, College of Agriculture "Luiz de Queiroz" (ESALQ), University of São Paulo, Avenida Pádua Dias 11, PO Box 9, Piracicaba 13418-900, São Paulo, Brazil*

d *Department of Chemical Engineering, University of La Frontera, PO Box 54-D, Temuco, Chile*

may be a promising alternative to avoid the high oil refining costs, simultaneously introducing an environment friendly process.

Transesterification reactions are conventionally carried out using alkaline or acid homogeneous catalysis. Unlike the conventional chemical catalysts used for synthesis of biodiesel, biocatalytic routes permit the transesterification of a wide variety of oily raw materials with high FFA content. Moreover, separation and purification of enzymatically produced biodiesel is easier because of the absence of by-products like soap.[11] Novozym®435, an immobilized lipase, is, to date, the most widely investigated lipase for the transesterification process due to its robustness, broad specificity[11,12] and the relatively high esters yield observed, reaching in some cases 100% efficiency.[4–6,13–17] However, esters yield depends on feedstock and reaction conditions including time, stirring speed,[12] oil:ethanol molar ratio, enzyme concentration and temperature.

The response time for high performance mainly varies with the concentration of Novozym®435 lipase and the oil:alcohol molar ratio.[6,10,11,16,18] Methanol and ethanol are short-chain alcohols that, in excess, can cause the complete inactivation of lipases.[12] When these alcohols are adsorbed by the immobilized enzyme, the entry of triglycerides is blocked stopping the reaction.[19] However, constant stirring can reduce alcohol droplets, or even cause its complete dilution, reducing its contact with the enzymes. Thus, enzymes are protected from inactivation and transesterification in one-step is guaranteed.[12]

Preservation and reuse of the enzyme are extremely important due to its high cost. Washes with solvents such as acetone,[20] tert-butanol[19] and n-hexane[21] have been successfully applied to recover immobilized lipases by removing impurities. In addition, the use of organic solvents together with the substrate (oil:alcohol) helps substrate solubilization and increases enzyme stability.[12] The presence of hydrophilic and hydrophobic substances in the substrate can improve the enzyme performance eliminating negative effects caused by insolubility and adsorption of methanol and glycerol in the support during transesterification reaction.[22,23] Liu *et al.* found that enzymatic activity of immobilized *Pseudomonas cepae* lipase treated with organic solvent depends not only on hydrophobicity but also on the functional groups involved.[24] Indeed, branched structures, which favor enzyme stability compared with straight structures, are also related to enzyme conformation. Tert-butanol, due to its branched structure, stands out among the solvents for substrate solubility and efficient stabilization of lipases in transesterification and esterification reactions.[5,9,14,15,25]

The objective of this study was to explore the rich miscella as alternative raw material for biodiesel production by enzymatic transesterification. The experimental conditions time, oil:ethanol molar ratio, lipase concentration and temperature were studied. Response surface methodology was applied to optimize the process. In addition, recovery and reuse of Novozym®435 was also evaluated. Tert-butanol and isopropanol were used as co-solvent in the reaction in order to extend the stability of the enzyme after successive cycles of use.

## MATERIAL AND METHODS

### Material

Flaked fresh soybean provided by Cargill S/A and ADM S/A was used to obtain the soybean oil-rich miscella. The catalyst Novozym®435, donated by Novozyme S/A (Bagsvaerd, Denmark) was used to produce biodiesel. Methyl heptadecanoate (Sigma-Aldrich, USA) was used as internal standard in gas chromatography assays. All other reagents including ethanol, isopropanol and tert-butanol were of analytical grade.

### Extraction and characterization of rich-in-oil phase from soybean

The extraction of oil from flaked fresh soybean was performed with ethanol 99% using a soybean:ethanol ratio of 1:2 w/v, at 78 °C for 1 h in an intermittent extractor, with mechanical stirrer at 200 rpm. The mixture obtained was kept at room temperature to cool down and separated in three phases: gum phase, rich-in-oil phase (rich miscella) and rich-in-alcohol phase (poor miscella). The rich-in-oil miscella was characterized for its oil content according to Hara and Radin,[26] ethanol content was removed by distillation and measurement for digital densimeter model DMA-48 Anton Paar; unsaponifiable material was determined using the AOCS Ca 6b-53[27] procedure; water content was determined based on the ASTM D6304 norm,[28] free fatty acids were determined using the method AOCS Ca 5a-40,[27] while peroxide value was determined using AOCS Cd 8b-90[27] and phospholipids using ASTM D4951.[28]

### Biodiesel production by transesterification with Novozym®435

To maximize biodiesel production, an optimization following two steps was performed: (1) testing reaction time (kinetics) and (2) determining the best conditions to achieve maximum yield in ethyl esters using response surface methodology (RSM).

All assays were performed in 4 mL glass vials containing 1.12 g of rich miscella (equivalent to 1 g of soybean oil), incubated in a orbital shaker at 200 rpm at controlled temperature. Time, temperature, oil:ethanol molar ratio and concentration of Novozym®435 lipase in the transesterification reaction were evaluated throughout the study.

#### *Kinetic studies*

Assays were carried out taking samples each 2 h until 48 h under the experimental conditions described in the next section. All experiments were done in triplicate. The reaction time was selected by fitting the experimental data to a first-order exponential function.

#### *Experimental design and statistical analysis*

After determining the reaction time to reach the maximum ethyl esters yield, a series of experiments were carried out using a $2^3$ factorial experimental design with quadruplicate at the central

**Table 1.** Variables and levels used in the response surface methodology of the enzymatic transesterification of rich miscella

| | | Levels | | | | |
|---|---|---|---|---|---|---|
| Independent variables | Symbols | −1.68 | −1 | 0 | +1 | +1.68 |
| Oil-to-ethanol ratio(mol mol$^{-1}$) | $MR_e$ | 1:3.0 | 1:3.6 | 1:4.5 | 1:5.4 | 1:6.0 |
| Temperature (°C) | $T_e$ | 30 | 34 | 40 | 46 | 50 |
| Catalyst - Novozym®435[a] | $C_e$ | 4.0 | 6.2 | 9.5 | 12.8 | 15.0 |

[a] Relative to the mass of oil content in the miscella (wt%).



  

point and response surface methodology (RSM). The range levels and study variables are presented in Table 1.

Multiple regression analysis and RSM were employed to predict the ideal conditions for highest ethyl esters yield from rich miscella. The analysis featured 18 values distributed into 8 points for each apex 4 center points and 6 axial points. Each test was conducted in duplicate.

All assays were performed in random order, according to the procedure described by Barros Neto *et al.*[29] The data were adjusted to a second-order polynomial equation (Y). This equation describes the relationship between the response variable (fatty acid ethyl esters) with the independent variables ($MR_e$, $T_e$, $C_e$).

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

where:

$Y$ = fatty acid ethylic esters (%) – (FAEE)

$\beta_0$ = regression coefficient of the intercept term

$\beta_i$ = regression coefficient of the linear term

$\beta_{ii}$ = regression coefficient of the quadratic term

$\beta_{ij}$ = regression coefficient of the interaction term

$X_i$ and $X_j$ = coded independent variables

The statistical significance of the experimental data was evaluated and the response surface was generated by software 'Statistica 7'. The statistical model used to describe the responses of the factorial design was formulated in terms of each effect and evaluated based on the coefficient of determination ($R^2$) and the $F$ test.[29] The $F$ test, which tests the significance of the regression and the model lack of fit, was performed by analysis of variance (ANOVA) with confidence level of 95%. Only significant terms ($P \leq 0.05$) were considered in the equation. The ratio between the quadratic averages (QA) the regression and residues ($QA_R/QA_r$) and the lack of fit and pure error ($QA_{lf}/QA_e$) were compared with the $F$ values established by Fisher.[29]

### Catalyst recovery and reuse

In order to verify the possibility of recovering and reusing Novozym®435 after reaction, the enzymes were filtered and placed to react again until a low FAEE conversion was observed. The recovery was carried out by washing Novozym®435 using rich miscella, isopropanol, tert-butanol and 96% ethanol. After reaction, the ester phase was collected to determine the FAEE (%) obtained, to evaluate enzyme reuse in 24 h cycles. The enzymes were filtered and washed three times with 1 mL of solvent followed by three washes with 1 mL of rich miscella to remove the solvent inside the support according to the methods described by Chen and Wu[19] and Ognjanovic *et al.*[16] A control was carried out under the same conditions but without washing. For the recovery assays the experimental data were analyzed by a first-order kinetic model. All experiments were performed in triplicate.

An evaluation of the internal surface of the Novozym®435 microspheres before and after reaction was performed by examination through scanning electron microscopy analysis (SEM) using JEOL JSM6380LV equipment.

### Enzymatic transesterification with co-solvents

Transesterification of the rich miscella with lipase Novozym®435 was tested in the presence of isopropanol and tert-butanol. The reaction conditions were determined by RSM. The amount of solvent added to the reaction was determined based on the method described by Li *et al.*[14] Assays were performed in individual vials by adding concentrations of 0.5, 0.75, 1, 2.5, 5, 10 and 100% isopropanol related to the oil mass. Similarly, experiments were conducted employing tert-butanol at concentrations of 0.5, 0.75, 1, 2.5, 5, 10, 12.5, 15 and 100% related to the oil mass. The Tukey test was applied to evaluate the difference between the averages with a significance level of 95% in the conversion to ethyl esters.

After choosing the solvent and its optimal doses, assays were conducted to determine the stability of Novozym®435. The enzymes, after each 24 h cycle, were filtered and returned immediately to a new reaction. The tests were repeated until a noticeable stability in the conversion curve was observed.

### Ethyl esters analysis

The ethyl esters yield was determined by gas chromatography and mass spectrometry according to the method described by the Comité Européen de Normalisation (EN14103).[30]

## RESULTS AND DISCUSSION

The rich miscella presented the following characteristics: 90 wt% soybean oil, 7.6 wt% ethanol (oil:ethanol molar ratio 1:1.6), unsaponifiable material 1%, water 0.3%, free fatty acids 0.4%, peroxide value 10 meq $kg^{-1}$ of miscella and phospholipids 0.57%. This demonstrates that rich miscella is appropriate as feedstock for transesterification reaction without the need for subsequent refining processes.

### Kinetics

Researchers have shown that the relationship between time and catalyst concentration is inversely proportional, where an increment of enzyme concentration reduces the maximum conversion time.[6,10,16,18] Figure 1 shows the enzymatic reaction progress curve during the transesterification of rich miscella. The reaction was monitored periodically over a period of 48 h. A first-order kinetic model was applied to establish the time required to reach maximum yield of FAEE. The experimental data were fitted to the empirical exponential function $y = a(1 - e^{-kx})$, where $y$ = fatty acid ethyl ester (FAEE) (%), $x$ = reaction time (h), $a$ is correlated with the long time triacylglycerols (TAG) concentration and $k$ the rate constant. The high determination coefficient, $R^2 = 0.99$, guaranteed excellent fit between experimental data and the first-order equation. The values obtained for the constants $a$ and $k$ were 84.4568% TAG and 0.1138 $h^{-1}$, respectively.

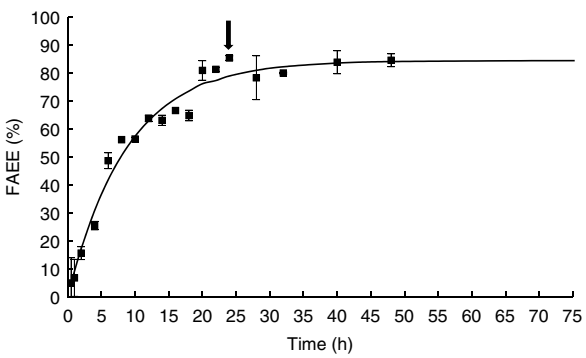


**Figure 1.** Fatty acid ethyl esters (FAEE) conversion kinetics of rich miscella using Novozym®435 as catalyst. The arrow indicates time 24 h.



  

According to regression of the experimental data, the calculated effect on 99% of the value of the limit function $y$ ($x \rightarrow \infty$) was 83.75% of FAEE at 42 h, reaching an asymptotic value after 72 h with 84.46% of FAEE (Fig. 1). The experimental data shows an apparent equilibrium after 24 h of reaction, reaching a FAEE (%) yield of 85.4% which differs by 6% from the value predicted (78.95%) by the first-order kinetic model.

### Optimization of enzymatic ethanolysis conditions

The ethanolic rich miscella obtained from the extraction of soybean, was subjected to transesterification reaction varying the concentration of Novozym®435, reaction temperature and oil:ethanol molar ratio (Table 2). The assay number 16 showed the highest FAEE yield (86%) at 50 °C. However, only 2% more FAEE than assays 10 and 11 developed at 40 °C was obtained, suggesting that the input of energy needed to elevate the temperature from 40 to 50 °C to increase FAEE yield might not be necessary.

Table 3 shows the estimated effects of the different variables on the ethyl esters production where the reaction temperature and concentration of the catalyst were the most important variables affecting FAEE yield, which increased when the temperature and the concentration of the catalyst increased. Lipases usually present intervals of optimal temperatures between 30 and 60 °C in reaction medium. Furthermore, increasing their concentration can accelerate the reaction.[31] High yields of alkyl esters have been reached in transesterification reactions of wastes frying oil, *Jatropha* oil and sunflower oil, when Novozym®435 was used at a temperature of around 45 °C and a catalyst concentration above 13%.[6,10]

In this research the FAEE yield decreased when the enzyme concentration was increased to 15%, which can be related to poor homogenization of the system, which reduces the contact between the catalyst surface and substrate (Table 2). In fact, it has been demonstrated by De Paola *et al.* that for high enzyme loading, a higher stirring speed is needed to obtain best performance.[32]

**Table 2.** Experimental design conditions and reaction yield for the ethanolysis of rich miscella

| Run | $MR_e$ (oil:ethanol) | $T_e$ (°C) | $C_e$ (wt%) | $FAEE_{observed}$ (%) | $FAEE_{predicted}$ (%) |
|---|---|---|---|---|---|
| 1 | (1:3.6) | 34 | 6.2 | 59.48 | 62.10 |
| 2 | (1:5.4) | 34 | 6.2 | 64.78 | 64.45 |
| 3 | (1:3.6) | 46 | 6.2 | 73.27 | 73.37 |
| 4 | (1:5.4) | 46 | 6.2 | 79.30 | 78.99 |
| 5 | (1:3.6) | 34 | 12.8 | 72.37 | 72.03 |
| 6 | (1:5.4) | 34 | 12.8 | 75.05 | 73.86 |
| 7 | (1:3.6) | 46 | 12.8 | 76.85 | 76.53 |
| 8 | (1:5.4) | 46 | 12.8 | 83.42 | 81.62 |
| 9 | (1:4.5) | 40 | 9.5 | 80.99 | 82.28 |
| 10 | (1:4.5) | 40 | 9.5 | 84.27 | 82.28 |
| 11 | (1:4.5) | 40 | 9.5 | 84.58 | 82.28 |
| 12 | (1:4.5) | 40 | 9.5 | 81.79 | 82.28 |
| 13 | (1:3) | 40 | 9.5 | 68.96 | 68.08 |
| 14 | (1:6) | 40 | 9.5 | 72.76 | 74.33 |
| 15 | (1:4.5) | 30 | 9.5 | 73.11 | 72.75 |
| 16 | (1:4.5) | 50 | 9.5 | 86.63 | 88.74 |
| 17 | (1:4.5) | 40 | 4.0 | 64.45 | 63.05 |
| 18 | (1:4.5) | 40 | 15.0 | 70.83 | 73.61 |

$MR_e$: ratio molar; $T_e$: temperature; $C_e$: catalyst concentration; FAEE: fatty acid ethyl esters.

**Table 3.** Estimation of the effect of explanatory variables on FAEE yield

| | Effect | Standard deviation | Descriptive level ($P$) |
|---|---|---|---|
| Average | 82.27750 | 0.613803 | 0.000000[a] |
| $RM_e$ (L) | 3.72291 | 0.692096 | 0.000126[a] |
| $RM_e$ (Q) | −7.84576 | 0.683106 | 0.000000[a] |
| $T_e$ (L) | 9.51776 | 0.750181 | 0.000000[a] |
| $T_e$ (Q) | −1.08684 | 0.738031 | 0.164646 |
| $C_e$ (L) | 6.28279 | 0.802201 | 0.000003[a] |
| $C_e$ (Q) | −9.88505 | 0.808448 | 0.000000[a] |
| $RM_e$ (L) x $T_e$ (L) | 1.63047 | 0.963537 | 0.114428 |
| $RM_e$ (L) x $C_e$ (L) | −0.25900 | 0.963735 | 0.792343 |
| $T_e$ (L) x $C_e$ (L) | −3.38724 | 0.963398 | 0.003796[a] |

[a] Significant at 95% confidence level ($P \leq 0.05$).
L = linear.
Q = quadratic.

The oil:ethanol molar ratio had greater influence when it was increased to 1:6, which caused a reduction in the esters yield. Organic solvents can affect the stability of hydrogen bonds, and hydrophobic and electrostatic interactions of the proteins in different ways. Protein denaturation can be provoked by excess ethanol due to the rupture of hydrogen bonds, modifying the enzyme conformation and resulting in deactivation.[33]

The quadratic model adjusted after analysis of the effects is represented by the FAEE equation:

$$FAEE = 82.28 + 1.86RM_e + 4.76T_e + 3.14C_e - 3.92RM_e^2 - 4.94C_e^2 - 1.69T_e.C_e$$

The linear terms are represented by $RM_e$, $T_e$ and $C_e$, the quadratic terms by $RM_e{}^2$ and $C_e{}^2$, and the interaction by $T_eC_e$. The positive linear terms indicate an increase in FAEE yield and the negative ones indicate a reduction in FAEE yield when the variable concerned increases. Terms in $T_e{}^2$, $RM_eT_e$ and $RM_eC_e$ were not significant ($P > 0.05$), and were not considered in FAEE equation.

The adjusted quadratic model explained 94.97% ($R^2$) of the total variance of the system. The $R^2$ value and adjusted $R^2$ (92.45%) indicated strong correlation between the observed and the predicted values of the response. The value of $F_{obs}$ ($QA_R/QA_r = 37.74$) for the regression was 15 times higher than the $F_{table(9;18)}$ (2.42) with ($P \leq 0.05$), and the lack of fit was not significant ($P = 0.066$). This indicates that the model is valid and useful for predictive purposes (Table 4).

Based on the regression presented in Table 4 this quadratic model was used to generate the response surface and to calculate the best conditions at which the ethanolysis could be carried out to give optimal conversion. Walking through the response surface, it was attempted to reconcile the higher ethyl esters production with lower energy consumption, as well as low catalyst and ethanol concentration. Thus, the best conditions indicated by the empirical model for the enzymatic transesterification of miscella were $RM_e$ 1:4.5 (level 0), $T_e$ 40 °C (level 0) and $C_e$ 9.5% (level 0). These conditions are likely to provide the best process response, leading to the highest ester yield.

Model validation was obtained by comparison with experimental data used for the RSM fitting and data used for enzyme reuse



  

**Table 4.** Analysis of variance (ANOVA) to assess the quality of the quadratic model

| Variance source | Quadratic summation | Degrees of freedom | Quadratic average | $F_{obs}$ value | *P*-value |
|---|---|---|---|---|---|
| Regression | 1331.27 | 9 | 147.9185 | 37.74 | 0.004[a] |
| Residues | 70.56 | 18 | 3.9198 | | |
| Lack of fit | 36.186 | 5 | 7.2373 | 2.74 | 0.066 |
| Pure error | 34.369 | 13 | 2.6438 | | |
| Total | 1401.822 | 27 | | | |
| $R^2 = 0.9497$ | | | | | |
| Adjusted $R^2 = 0.9245$ | | | | | |

[a] Significant at 5% level.
$F_{Table\ (9;18)} = 2.46$.
$F_{Table\ (5;13)} = 3.03$.

and recovery assays (see section below), which were acquired at the same experimental conditions.

Yields of fatty acids esters (FAE) up to 99% were easily achieved with Novozym®435 catalyst with the oil subjected to a purification step such as washing, filtration, neutralization or degumming. Ognjanovic *et al.* optimized the process with refined sunflower oil and produced 99% FAME.[16] Su *et al.* obtained 96.2% fatty acids esters after washing with Jatropha oil.[10] Maceiras *et al.* converted 89.1% FAME using waste frying oil filtrate.[34] The advantage of using rich-in-soybean oil miscella is that degumming and neutralization processes are unnecessary, which reduces feedstock production time and waste generation. The rich miscella used in this study presented FAEE yields close to that obtained by Hernández-Martín and Otero, where refined soybean oil with 50% lipase Novozym®435 in 7 h reaction with 1:4.5 (oil:ethanol) was used.[11] Therefore, this result suggests that the use of rich miscella as raw material can be an excellent alternative to virgin vegetable oils for biodiesel production.

## REUSE AND RECOVERY OF IMMOBILIZED ENZYMES

Ethyl esters yield was 85.4% in the first cycle of use of lipase Novozym®435, in the second cycle it was 54.1%, in the third cycle 31.7% and in the fourth cycle 18.1%. Yield decreased after each reuse of the lipase Novozym®435, and was decreased by 30% between the first cycle and second cycle. This deactivation has been attributed to the adsorption of glycerol[35,36] and phospholipids[13,37] by the support. Thus, the glycerol adsorbed forms a hydrophilic layer in the support surface which prevents contact between the enzyme and the hydrophobic substrate.[35,37]

In order to avoid enzyme deactivation, isopropanol, tert-butanol, ethanol (96%) and the rich miscella were used as solvents to remove impurities fixed by the immobilized lipase. Effective removal of glycerol, and enzyme activity restoration have been reported after successive washes with hydrophilic solvents such as isopropanol.[37] In addition, the use of alcohols with three or more carbons leads to a regeneration of immobilized enzymes greater than 75% by washing them with tert-butanol and 56% with 2-butanol.[19] In this sense, Maceiras *et al.* reached a constant methyl esters yield during four cycles washing Novozym®435 with 1-butanol.[34]

Figure 2 shows the ethyl esters yield within the experimental time. As shown, experimental data fit well to an empirical exponential equation ($y = ae^{kx}$), where $y$ = relative conversion of ethyl esters (%) and $x$ = reaction time (h). The coefficient $a$ can be correlated with the long time fatty acid ethyl esters concentration and $k$ is the parameter indicating the esters rate conversion by lipase, where, the higher the value of $k$, the higher the enzymatic activity. The values of the regression coefficients of control and all treatments with solvents are presented in Table 5.

At each reuse (24 h), the lipase activity was reduced. After 96 h (four cycles) FAEE (%) yield decreased as follows: control (78%), rich miscella (79%), ethanol (83%), isopropanol (69%) and tert-butanol (72%) (Fig. 2).

The $k$ values presented in Table 5 show a greater decrease in enzyme activity when ethanol (96%) was used, followed by the rich miscella and finally tert-butanol. The control and the enzyme washed with isopropanol presented the highest values of $k$ compared with the other treatments. A decrease of lipase activity after its first use was detected and this behavior may be related to the irreversible occupation of enzyme active sites by other molecules. In fact, Naranjo *et al.* stated that a reduction of ethyl esters yield after reuse of *Candida antarctica* lipase B was mainly due to the immiscibility between triacylglycerides and short chain alcohols such as methanol and ethanol.[38] Small alcohol droplets adsorb onto the immobilized lipase surface, blocking the entry of acylglycerol. Also, the high oil:ethanol molar ratio when adding ethanol in one step could have led to enzyme deactivation.[4,18]

According to José *et al.* polymethylmethacrylate (PMMA), material used as support for immobilized *Candida antarctica* lipase B (Novozym®435), dissolves in alcohol. The alcohol then diffuses into the catalyst remaining strongly adsorbed.[17] Ethanol changes the texture of the spheres channels, increasing the irregularities of the polymer.

In Fig. 3 electron micrographs of the internal surface of Novozym®435 are presented for catalyst without treatment (control) and the same catalyst after one cycle and washing with solvents and rich miscella. A greater roughening of the surface of the polymeric material and non-uniform internal surface can be seen, compared with the surface before cycling and washing.

After the first reaction cycle modification of the support can be seen (Fig. 3(b)). The surface of the spheres presents irregularities and deformations not only when washed with ethanol, but also with the other solvents (Fig. 3(c) to (f)) and with rich miscella containing 7.6% alcohol in its composition (Fig. 3(c)).

The images obtained (Fig. 3) confirm the existence of deformities that show leaching of support material and loss of lipases, causing a reduction in ethyl esters yield after each reuse.

The immobilized enzymes washed with rich miscella and ethanol suffered the fastest deactivation, probably due to the

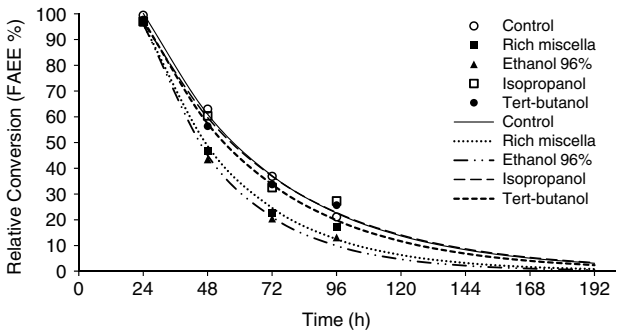


**Figure 2.** Relative conversion of ethyl esters (FAEE) after enzyme washing with different solvents.



  

**Table 5.** Parameters *a* and *k* and the effect calculated for the treatments of Novozyme435® with solvent

| Treatment | Predicted data | | | Experimental data | |
|---|---|---|---|---|---|
| | *a* | *k* | $R^2$ | $x_{exp}$ | $y_{exp}$ |
| Ethanol 96% | 205.00 | −0.03159 | 0.997 | 96 | 13.1 |
| Rich miscella | 189.82 | −0.02840 | 0.996 | 96 | 17.3 |
| Tert-butanol | 165.67 | −0.02212 | 0.999 | 96 | 25.6 |
| Control | 164.67 | −0.02069 | 0.998 | 96 | 21.1 |
| Isopropanol | 156.27 | −0.02005 | 0.987 | 96 | 27.3 |

$x_{exp}$: reaction time (h).
$y_{exp}$: relative conversion of the of ethyl esters FAEE yield (%).

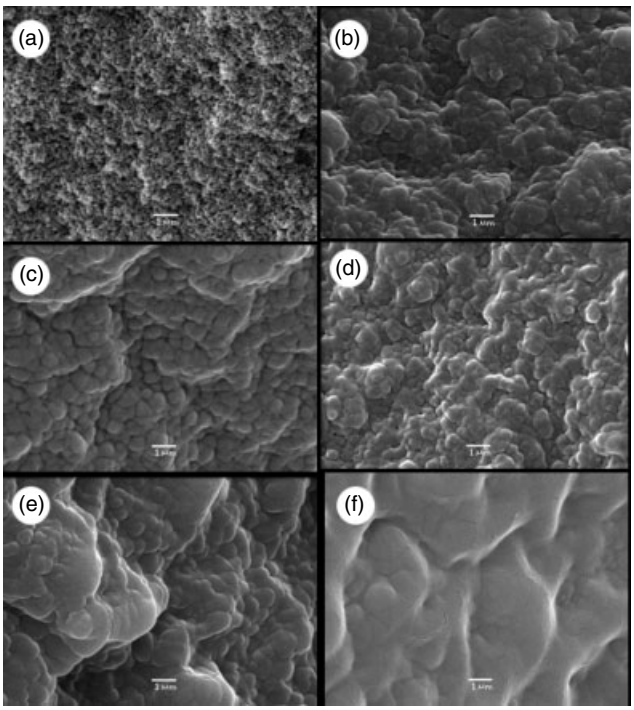


**Figure 3.** Scanning electron micrographies (10 000× magnification) of (a) intact internal surface of Novozym®435, (b) internal surface after one cycle, (c) internal surface after washing with rich miscella, (d) internal surface after washing with ethanol (96%), (e) internal surface after washing with isopropanol and (f) internal surface after washing with tert-butanol.

presence of phospholipids deposited over the catalyst surface and ethanol toxicity, respectively. Chen and Wu,[19] Soumanou and Bornscheur,[20] Rodrigues *et al.*[21] and Azócar *et al.*,[25] obtained satisfactory results after washing the enzymes with different solvents, but their experiment involved the use of purified or refined oils and stepwise addition of alcohol. The presence of phospholipids in the rich miscella may provoke enzyme deactivation and the presence of ≈7.6 wt% ethanol in its composition may have further contributed to this fast deactivation after washing. In addition, it has been stated that the glycerol produced can reduce enzyme activity, even with successive solvent washings and under agitation at 200 rpm.[36–38]

These results suggest that the solvents tested were not able to remove impurities such as phospholipids from the immobilized lipase surface. The use of non-polar solvents such as hexane, heptane or chloroform could remove phospholipids. However, these solvents besides being highly flammable and toxic, do not dilute ethanol or glycerol.[5,23]

### Transesterification employing co-solvent

To reduce the exposure of Novozym®435 to phospholipids and diluted ethanol and glycerol during the reaction, a co-solvent was added to increase the life-time of the catalyst for transesterification of the rich miscella.

The use of co-solvents and solvents in the transesterification reaction has been studied by many researchers,[5,14,15,23,25] and shown to increase the yield of esters, simultaneously increasing the stability and reuse of enzymes.

Alcohols such as methanol, ethanol and even glycerol have low solubility in non-polar solvents such as hexane, heptane and isooctane, presenting log $P > 2$. Tert-butanol and isopropanol are considered moderately polar solvents with log $P = 0.80$ and 0.32, respectively. With these properties, both can promote the solubility of substrate and co-product (glycerol) during esters production, avoiding premature deactivation of the enzyme.[5,14,22]

The effect of tert-butanol (Fig. 4(a)) and isopropanol (Fig. 4(b)) used as co-solvent was evaluated in the transesterification reaction with rich miscella and the immobilized lipase Novozym®435.

It was observed that tert-butanol promoted better conversion to ethyl esters than isopropanol when used as co-solvent. The ethyl esters yield increased by 6% relative to the control when isopropanol was used at a concentration of 0.5%. However, when using tert-butanol (5%), an increase of 18% in ethyl

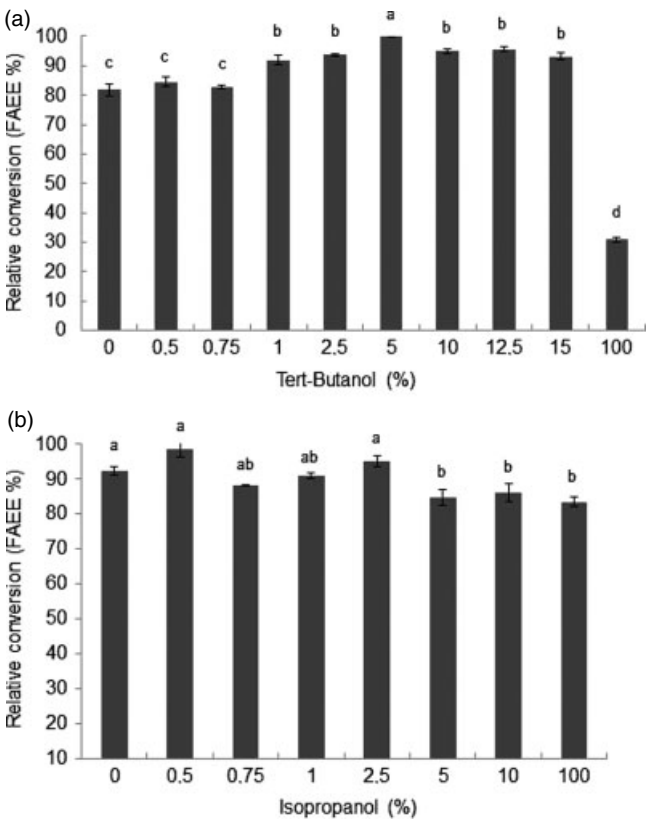


**Figure 4.** Effect of different concentrations of (a) tert-butanol and (b) isopropanol on the transesterification reaction of rich miscella. The letters 'a', 'b', 'c' and 'd' show significant differences ($P \leq 0.05$).

  

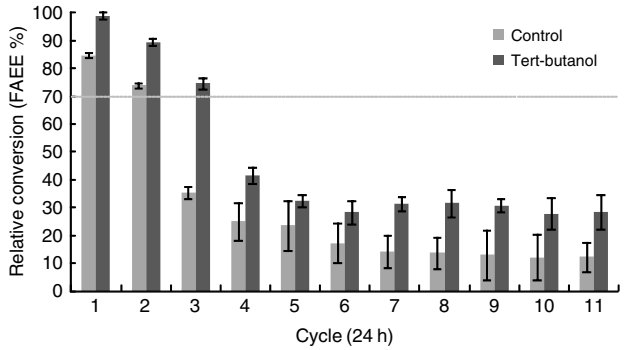


**Figure 5.** FAEE relative conversion (%) after different numbers of reaction cycles. Gray line represent 70% conversion.

esters conversion compared with the control (without tert-butanol) was observed. At concentrations of tert-butanol lower and higher than 5%, a reduction in ethyl esters yield was observed, which differed statistically with significance level of $P \leq 0.05$ (Fig. 4(a)). Reduced concentrations of tert-butanol may lead to incomplete dissolution of alcohol and glycerol in the reaction. High concentrations of tert-butanol might have caused dilution of the reactants present in the reaction system.[14]

Successive tests were performed reusing the immobilized enzymes with tert-butanol as co-solvent (5%). At each cycle (24 h), the enzymes were filtered and immediately placed without treatment in fresh rich miscella (Fig. 5).

The FAEE (%) yield remained >70% for up to three cycles, FAEE yield decreased to 40% after 4 cycles and remained almost constant after 5 cycles (Fig. 5). Li *et al.*[14] reached 30 cycles with fatty acid methyl ester (FAME) over 60% conversion yield and Azócar *et al.*[25] 17 cycles (over 50% FAME) using rapeseed oils with very low phospholipids content and tert-butanol as co-solvent in the transesterification reaction using Novozym®435.

The moderately hydrophilic property of tert-butanol kept glycerol and phospholipids diluted in the substrate, avoiding contact of these impurities with Novozym®435 and extending its use.

A variety of immobilized lipases from specific microorganisms have been applied for biodiesel production. Those immobilized lipases have been prepared via adsorption, covalent bonding, entrapment, encapsulation and cross-linking.[39,40] They include Chitosan-based hydrogels,[41] glutaraldehyde,[40] microporous polymers,[42] textile membrane,[43] niobium oxide,[44] polysiloxane–polyvinyl alcohol hybrid,[44,45] bentonite,[46] celite,[47] activated carbon[38] and polyurethane nanofibers-embedded LiCl,[48] among others.

The lipase B from *Candida antarctica* is one of the most widely used in the industry and is immobilized on methylmethacrylate resin (Novozym®435). However, this hydrophobic support can adsorb polar compounds such as phospholipids, glycerol and ethanol leading to lipase activity loss.[13,16,17,37,49] Additionally, it has been demonstrated that a hydrophobic polymer of polypropylene, named Accurel MP used as support material for lipases immobilization, can avoid glycerol adsorption, however, this catalyst needs to be further investigated regarding its economic feasibility at industrial scale.[49]

According to Fjerbaek *et al.*, the longer the reuse of the same enzyme, the higher the productivity that can be obtained for a given batch of enzyme, thereby lowering the biodiesel production costs.[50] Thus, productivity calculations (equation[50] below) of the amount of ethylic ester produced per amount of enzyme was used to evaluate the productive potential when using tert-butanol and solvent-free systems.

$$\text{Productivity (kg FAEE/kg enzyme)} = \frac{\text{FAEE Yield (\%)} \times \text{N (number of reuse)} \times 100}{\text{Enzyme Conc. (wt\%)}}$$

Assuming 0.95 kg oil yields, 1.0 kg biodiesel and 0.1 kg glycerol, the calculation was performed considering FAEE yields >70% and 9.5 wt% enzyme (Fig. 5). For the solvent-free system the enzyme was used in two cycles of reaction (N = 2), while for the co-solvent system three cycles (N = 3) were considered.

Productivity using tert-butanol as co-solvent was 27.6 kg FAEE $kg^{-1}$ enzyme, corresponding to 1.6 times higher than that of the solvent-free system (16.7 kg FAEE $kg^{-1}$ enzyme) (Fig. 6). This fact demonstrates that productivity was higher when tert-butanol was used as co-solvent for biodiesel production with rich miscella. Tert-butanol can be easily recovered and reused in further reactions.

Sotoft *et al.* carried out process simulation and economical evaluation of an enzymatic biodiesel production plant that used high quality rapeseed oil and methanol in solvent free and co-solvent production processes.[51] This simulation demonstrated that the use of a co-solvent together with enzymes is not economically viable because of the extra high energy input required for co-solvent distillation.

However, rich miscella could be directly transesterified by enzymatic catalysis and ethanol (renewable source), being a cheaper feedstock than other edible oils by eliminating the refining process and reducing the generating wastes. In addition, it seems that enzymes could be replaced every three cycles when using tert-butanol as co-solvent, which can lead to a reduction in production costs.

## CONCLUSION

The rich miscella studied can be subject to direct transesterification with ethanol using the enzymatic catalyst Novozym®435. Through response surface methodology, the best experimental conditions for ethyl esters production were $T_e$ 40 °C, $RM_e$ 1:4.5 and $C_e$ 9.5% for 24 h, reaching a maximum ethyl esters conversion yield of

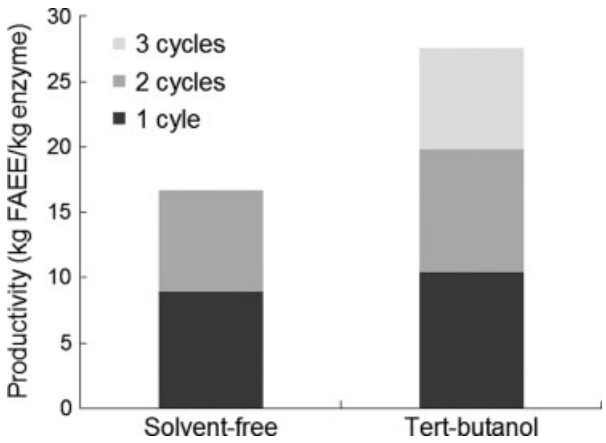


**Figure 6.** Productivity of FAEE in solvent-free and tert-butanol system.



  

85.4%. Tert-butanol used as co-solvent increased ethyl esters yield, maintaining the enzyme activity for ethyl ester yield >70% after three cycles of use. The rich miscella has great potential as a low cost feedstock for biodiesel production when enzymatic catalysts like Novozym®435 are used, simultaneously introducing an environmentally friendly step by using the renewable solvent ethanol in the extraction and the reaction process.

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