OPTIMIZATION OF ENZYMATIC TRANSESTERIFICATION OF RAPESEED OIL USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT: Enzymatic synthesis of methyl esters from crude rapeseed oil has been studied. The commercial immobilized lipases Novozym® 435, Lipozyme® TL IM and Lipozyme® RM IM have been tested as biocatalyst. At the same experimental conditions, Lipozyme® TL IM showed the best performance allowing a final product with an ester content within the biodiesel specifications (> 96.5%). This enzymatic process was then optimized through a central composite design of four variables - temperature, methanol-to-oil molar ratio, catalyst amount and water content. Based on the response surface methodology, the optimal values of the variables were as follow: reaction temperature of 37.5ºC, protein amount of 0.22 wt%, methanol-to-oil molar ratio of 6.2:5 and water content of 22.3 wt%, with 15 h reaction time. Different oil sources (rapeseed, soybean and used frying oils) were also tested in the enzymatic process using Lipozyme® TL IM. The experimental data obtained, at the same reaction conditions, showed that raw material properties have influence on the biocatalytic process.
Keywords: biodiesel, enzymatic process, rapeseed oil, transesterification.

1 INTRODUCTION

Biodiesel, a product obtained by conversion of glycerides in methyl esters, is an alternative fuel for diesel engines that can be used as a substitute or in mixture with diesel, due to the similarity of their physical and chemical properties. This biofuel has received considerable attention in the recent past as a biodegradable and non-polluting fuel.

Although biodiesel is usually produced by chemical processes using basic or acid catalysts, it can also be obtained using enzymes as catalysts. The enzymatic processes may be advantageous because they do not promote secondary reactions, thereby reducing the number of purification steps, and the presence of the enzyme in the glycerol phase can even increase its value to produce foodstuff. Furthermore, the enzyme can be immobilized into solid supports and thus be used in continuous mode, for example in a packed bed bioreactor, or reused in batch processes after simple filtration, which is economically advantageous for industrial purposes. However, the high cost of enzymes and some problems concerning methanol and/or glycerol inhibition still cause enzymatic processes to be unattractive for the large-scale production of biodiesel.

Lipases can be used to catalyse the reaction in mild conditions and, in the last years, an increasing number of researches on this subject have been reported [1-3]. The yield of biodiesel products through lipase catalysis is modulated by the substrate ratio (alcohol/oil), alcohol type, temperature of the reaction, water content, purity of the triacylglycerol, and enzymes immobilisations. The quality of the source oil is also important for efficient conversion to biodiesel.

Lipases are fatty-acid-chain-length-specific, substrate-specific, and region- and enantioselective, and therefore biodiesel production will require different types of lipases and substrates from the various available renewable resources. Those lipases which are found suitable for biodiesel production can be produced in large quantities, while those that are not so selective and active can be engineered to improve their biocatalytic activity for biodiesel production [4].

The aim of the present study is to investigate lipase-catalysed alcoholysis of crude rapeseed oil in the presence of the commercially available immobilized lipases Novozym® 435, Lipozyme® TL IM and Lipozyme® RM IM in an organic/aqueous biphasic system. The tested enzymes have different enzyme-substrate specificity (1,3-specific and nonspecific lipases). Optimum reaction parameters such as reaction temperature, enzyme amount, methanol-to-oil molar ratio and water content, were determined by response surface methodology. This statistical methodology employs multiple regression and correlation analyses as tools to assess the effects of the four independent factors on the dependent variable (fatty acid methyl ester content). Its principal advantage is the reduced number of experimental runs required to generate sufficient information for a statistically acceptable result.

2 MATERIALS AND METHODS

2.1 Materials

Crude rapeseed oil obtained from a Portuguese biodiesel industry (PRIO-Biocombustíveis), commercial soybean oil (Valouro) and used frying oil obtained at LNEG canteen, were used as raw materials for the enzymatic transesterification.

The enzymatic reactions were catalyzed by immobilized preparations of lipases: macroporous resin immobilized lipase Novozym® 435 (*C. antarctica* fraction B lipase), acrylic resin immobilized Lipozyme® TL IM (*T. lanuginosus* lipase) and anion-exchange resin immobilized Lipozyme® RM IM (*Rhizomucor miehei* lipase), kindly supplied by Novozymes A/S (Bagsvaerd, Denmark).

The chemicals used, of analytical grade, were supplied by Merck (Darmstadt, Germany), and the GC standard (heptadecanoic acid methyl ester) for FAME quantification was obtained from Sigma-Aldrich (Diesenhofen, Germany).

2.2 Experimental design

The enzymatic conversion of crude rapeseed oil into fatty acid methyl esters was optimised using the central composite design (CCD). A five-level-four-factors CCD was adopted in this study, requiring 27 experiments, which included sixteen factorial points, eight axial points and three central points. The effect of the following parameters on the transesterification reaction was evaluated: reaction temperature (T), protein amount (P), methanol-to-oil molar ratio (MR) and water content (W). Table I shows the parameters and their levels.

Table I: Factors and their levels for CCD

Variable	Levels				
	-2	-1	0	+1	+2
Temperature (ºC)	25	31.2	37.5	43.7	50
Protein (wt%)	0.06	0.1	0.14	0.18	0.22
Molar ratio (-)	3:1	4:1	5:1	6:1	7:1
Water (wt%)	0	6	12	18	24

The response variable used to build the model corresponded to the fatty acid methyl ester content (FAME, %), at a reaction time of 15h, that was obtained in each experiment after phase separation (Table II).

Table II: Experimental design results

Exp.	T (ºC)	P (wt%)	MR (-)	W (wt%)	FAME content
1	-1	-1	-1	-1	71.8
2	+1	-1	-1	-1	53.4
3	-1	+1	-1	-1	75.2
4	+1	+1	-1	-1	5.0
5	-1	-1	+1	-1	40.0
6	+1	-1	+1	-1	29.0
7	-1	+1	+1	-1	43.0
8	+1	+1	+1	-1	21.2
9	-1	-1	-1	+1	80.1
10	+1	-1	-1	+1	88.2
11	-1	+1	-1	+1	86.7
12	+1	+1	-1	+1	87.8
13	-1	-1	+1	+1	92.6
14	+1	-1	+1	+1	96.0
15	-1	+1	+1	+1	96.1
16	+1	+1	+1	+1	97.0
17	-2	0	0	0	95.0
18	+2	0	0	0	20.8
19	0	-2	0	0	89.2
20	0	+2	0	0	95.0
21	0	0	-2	0	74.0
22	0	0	+2	0	63.5
23	0	0	0	-2	0.28
24	0	0	0	+2	91.5
25	0	0	0	0	94.3
26	0	0	0	0	91.3
27	0	0	0	0	93.0

The results were analyzed with Yates' algorithm to determine the effects of each factor and the main interactions. The experimental data were analysed via response surface methodology, in order to calculate the coefficients of the second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} x_i x_j \qquad (1)$$

where Y is the response factor (fatty acid methyl ester content), β_0 the constant coefficient, β_i, β_{ii} and β_{ij} the coefficient for linear, quadratic and interaction effect, respectively, and x_i and x_j the factors (independent variables).

2.3 Experimental procedure

The enzymatic reactions were performed in 250 mL screw-capped Erlenmeyer flasks, incubated at different temperatures with an orbital shaking at 150 rpm. The biphasic system consisted of 50 g of organic phase - the vegetable oil - and an aqueous phase composed of Millipore water, methanol and the immobilized lipase. Samples were collected after 15 h of reaction time and centrifuged to phase separation. The organic phase was then prepared to be analyzed by gas chromatography.

2.4 Analytical methods

(i) Raw material characterization

The vegetable oils were characterized in terms of the acid, iodine and saponification values according to NP EN ISO 660 [5], NP EN ISO 3961 [6] and ISO 3657 [7], respectively. The water content was analyzed using the EN ISO 12937 Karl-Fisher Coulometer method [8]. To determine the fatty acid composition of each raw material, oil samples (~150 mg) were chemically derivatized using the borum trifluoride method described in the EN ISO 5509 [9]. The organic phase obtained was analysed by gaseous chromatography. Standards of FAME were used to identify the FAME in the sample. Fatty acid composition was calculated as percentage of the total fatty acids present in the sample determined from the peak areas.

All of these parameters (with exception for the water content) were determined after sample preparation according to the techniques specified in the standard ISO 661:2003 [10].

(ii) Protein determination

To evaluate the protein amount present in each enzymatic preparation, a modified Lowry method was used [11]. The protein content (P) on an immobilization support was calculated as:

$$\mathrm{P\ (mg/g)} = \frac{\text{protein (mg)}}{\text{immobilization support (g)}} \qquad (2)$$

(iii) Gas-chromatography analysis of FAME

The FAME contents were analyzed on a Varian 3300 gas chromatograph with a flame-ionization detector (Walnut Creek, CA, USA). A Supelcowax 10 capillary column (30 m x 0.32 mm i.d.; film thickness 0.25 μm; Bellefonte, PA, USA) with helium as the carrier gas was used. The column oven temperature was maintained at 200 ºC with injector and detector temperatures at 200 and 250 ºC, respectively.

The sample for analysis was prepared according to the procedure described in EN 14103 [12]. For this, 150 mg of the organic phase were weighted and 3 mL of internal standard solution (methyl heptadecanoate, 10 mg/mL) were added. The fatty acid methyl esters content was calculated by the following equation:

$$C = \frac{\sum A - A_{IS}}{A_{IS}} \times \frac{C_{IS} \times V_{IS}}{m} \times 100\% \qquad (3)$$

where C is the fatty acid methyl ester content (wt%), $\sum A$ is the total peak area, A_{IS} is the peak area of methyl

heptadecanoate, C_{IS} is the concentration of methyl heptadecanoate solution (mg/mL), V_{IS} is the volume of methyl heptadecanoate solution (mL) and m is the weight of the sample (mg).

3 RESULTS AND DISCUSSION

3.1 Characterization of raw materials

Crude rapeseed and soybean oils as well as used frying oil were characterized according to some parameters that are relevant when the final purpose is to obtain a good quality biodiesel (Table III). Exception for the saponification values, all the parameters showed values significantly different depending on the raw material. The saponification value showed that the studied raw materials should have a similar molar esterification capacity. This parameter also allowed the determination of the molecular weight of the oils – 913.04, 878.20 and 892.67 g mol^{-1} for rapeseed, soybean and used frying oils, respectively.

Table III: Crude rapeseed oil (RO), soybean oil (SO) and used frying oil (UFO) characterization

Parameter	Unit	RO	SO	UFO
Acid value	mg KOH/g	2.27	0.75	1.01
Iodine value	$gI_2/100g$	115	132	119
Water content	mg/kg	1048	278	1267
Saponification value	mg KOH/g	184	191	188
Fatty acid composition				
C16:0		5.13	15.0	10.2
C18:0	wt%	1.71	5.34	3.96
C18:1		59.0	22.7	29.3
C18:2		22.5	48.9	53.2
C18:3		9.31	6.56	1.38
Others		2.35	1.50	1.96

The iodine value for rapeseed oil and used frying oil was lower than the one of soybean oil (>120) and, consequently, more favourable to obtain a biodiesel according to the European standard in what concerns this parameter. In terms of fatty acid composition, all the raw materials were mainly composed of unsaturated fatty acids (78 to 91 wt%), namely oleic and linoleic acid (Table III). The linolenic acid content, a parameter considered in the EN 14214 [13], was lower than the standard specifications (< 12 wt%).

In order to perform the enzymatic transesterification with similar initial water content values in the raw material (< 1000 mg/kg), rapeseed oil and used frying oil were dried in the presence of anhydrous sodium sulfate and a final water content of 572 and 784 mg/kg were achieved, respectively.

3.2 Screening of lipases for transesterification

In order to select the most suitable immobilized lipase for the transesterification reaction, similar experiments were carried out using Novozym® 435 (57.8 mg protein/g support), Lipozyme® TL IM (98.2 mg protein/g support) and Lipozyme® RM IM (47.5 mg protein/g support). The transesterification of 0.274 mol of methanol with 0.054 mol of rapeseed oil (5:1 methanol-to-oil molar ratio), at a water content of 12 wt% (based on weight of oil), a reaction temperature of 37.5ºC and a protein amount of 0.14 wt% (based on weight of oil) was studied. The referred values correspond to the ones defined in the central composite design for the central point (Table I).

From the data obtained (Fig. 1) it can be observed that Lipozyme® TL IM allow to obtain a maximum ester content of 98 wt% while with the other lipases the reaction product was almost constant and near to zero. Thus, the 1,3-specific lipase - Lipozyme® TL IM - was selected for the rapeseed biodiesel production process. Also based on the obtained profile of Fig. 1, a reaction time of 15 h was selected to the subsequent studies to ensure equilibrium reaction conditions.

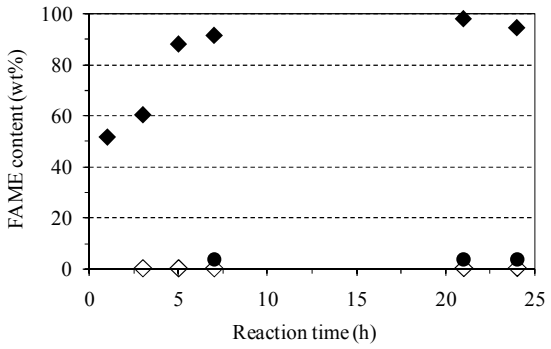

Figure 1: Fatty acid methyl ester content using Lipozyme® TL IM (♦), Novozym® 435 (●) and Lipozyme ® RM IM (◊) on transesterification reactions (T = 37.5 ºC; protein = 0.14 wt%; added water (12 wt%) molar ratio 5:1)

3.3 Optimization of enzymatic transesterification

In order to optimize the reaction conditions of rapeseed biodiesel synthesis by Lipozyme® TL IM , a central composite design with five-level-four-factors (reaction temperature, protein amount, methanol-to-oil molar ratio and water content) was applied (Table I). Since the idea of use a CCD was to simplify the optimization study by performing each experiment only once, the repeatability of the method was derived from triplicate experiments carried out at the centre point of the selected variables (Table II, Exp. 25, 26 and27). The standard deviation of these replicates was 1.23. The relevance of each factor and the interactions among them are shown in Table IV where it can be seen that the more relevant factor is the water content followed by reaction temperature. The two other factors (protein amount and methanol-to-oil molar ratio) showed an almost equal effect on the methyl ester conversion. The binary interactions T*W and MR*W showed the higher value and this demonstrated which variables are closely related (Table IV).

The highest methyl ester conversion value of 97.0 wt% was obtained at 44ºC with a protein amount of 0.18 wt%, a methanol-to-oil molar ratio of 6:1 and 18 wt% of water content (experiment 16 in Table II). By using multiple regression analysis, the experimental data (methyl ester content, wt%) were correlated with the four independent factors using the second order polynomial equation as in Eq. (1). The coefficients of the full regression model equation and their statistical significance were determined and the final model is as follow:

$$Y = -149.06 + 12.37T + 185.58P + 16.47MR - 4.65W - 0.22T^2 - 108.24P^2 - 6.01(MR)^2 - 0.32W^2 - 17.98TP + 0.51T(MR) + 0.22TW + 60.16P(MR) + 15.75PW + 1.16(MR)W \quad (4)$$

where Y, T, P, MR and W are the methyl ester content (wt%), the reaction temperature (ºC), the protein amount (wt%), and the water content (wt%), respectively. Positive sign in front of the terms indicates synergistic effect, while negative sign indicates antagonistic effects.

Table IV: Primary and secondary effects observed as a result of the 2^4 experimental design with three replicates at the centre point (experiments 1 to 16 and 25 to 27 in table I)

Parameter	Value
Yield average at the centre point	92.87
Standard deviation	1.23
Main effects	
(1) T	-13.49
(2) P	-4.89
(3) MR	-4.16
(4) W	48.24
Secondary effects: binary interactions	
(12) T*P	-9.01
(13) T*MR	6.36
(14) T*W	16.86
(23) P*MR	4.81
(24) P*W	7.56
(34) MR*W	13.89

Calculated according to Yates algorithm [14].
W, water content; T, reaction temperature; P, protein amount; MR, methanol-to-oil molar ratio.

The results of the second-order response surface model fitting in the form of ANOVA are given in Table V. The Fischer *F*-test (F_{model}=15.12) with a very low probability value [(P_{model}>*F*)<0.0001] demonstrates a very high significance for the regression model. To test the model fitness, the determination coefficient (R^2) was evaluated. In this case, the value of the determination coefficient (R^2=0.9379) indicates that the sample variation of 93.79% for methyl ester conversion is attributed to the independent variables and only 6.21% of the total variations are not explained by the model.

Table V: Analysis of variance (ANOVA) for the quadratic model

Sources of variations	Sum of squares	Degrees of freedom	Mean square	*F*-value	Prob>*F*
Model	23512.59	14	1679.47	15.12	<0.0001
Residual	1555.36	12	129.61		
Total	25067.95	26			

R^2=0.9379

The second-order model was represented as response surfaces and allowed to select the most suitable conditions for the enzymatic transesterification process. The response surface for the predicted values of fatty acid methyl ester content as a function of reaction temperature and protein amount at a constant methanol-to-oil molar ratio of 5:1 and 12 wt% of water content, at a reaction time of 15 h, was plotted (Fig. 2). It is observed that, the methyl ester content initially increases when there is an increase in temperature and protein amount. The maximum methyl ester content, near 100 wt%, was achieved at a protein amount of 0.22 wt% with reaction temperature of 30ºC. Further increase in reaction temperature promotes a significant decrease in methyl ester content (wt%) at any point along the line. This behaviour was related to lipase deactivation by temperature.

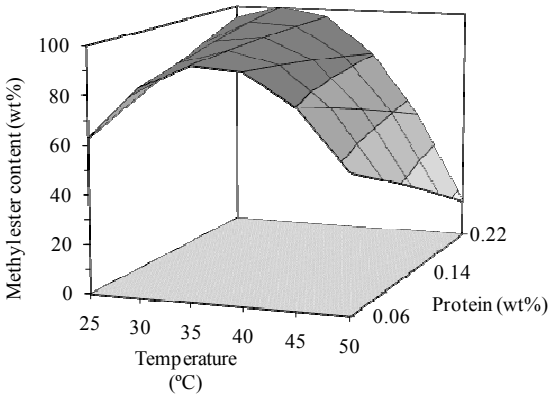

Figure 2: Response surface plots representing the effect of reaction temperature, protein amount and their reciprocal interaction on rapeseed biodiesel synthesis. Other factors are constant at zero levels.

Fig. 3 shows the effects of reaction temperature, methanol-to-oil molar ratio, and their reciprocal interaction on rapeseed biodiesel synthesis at a protein amount of 0.14 wt% and 12 wt% of water content, at a reaction time of 15h. At reaction temperatures lower than 35ºC, an increase in molar ratio led to a decrease of FAME content due to lipase inhibition effects by methanol. The observed decrease was more pronounced for methanol-to-oil molar ratio higher than 5:1.

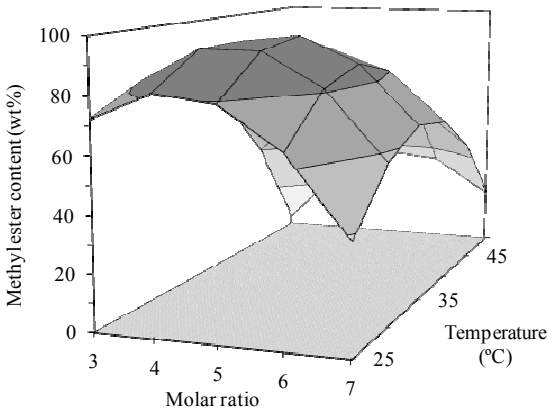

Figure 3: Response surface plots representing the effect of reaction temperature, methanol-to-oil molar ratio and their reciprocal interaction on rapeseed biodiesel synthesis. Other factors are constant at zero levels.

The effect of reaction temperature and water content on methyl ester content at a molar ratio of 5:1 and a protein amount of 0.14 wt%, is provided in Fig. 4. An increase of introduced quantities of water increased the

fatty acid methyl esters contents at reaction temperatures lower than 35ºC within a water amount of 16 wt%. Further increase in water content led to FAME contents decrease showing that the hydrolysis reaction will be present and, consequently, will affect the reaction equilibrium towards product formation (methyl esters). It was also observed that experiments without water addition led to low methyl ester contents (< 20 wt%) or even to the non occurrence of reaction, meaning that it will be necessary to increment the water content in order to have a better lipase performance. From the obtained results it was clear that the water content present in the reaction medium and the reaction temperature are important parameters in the final methyl ester content as indicated by the values of the primary and secondary effects showed in table IV.

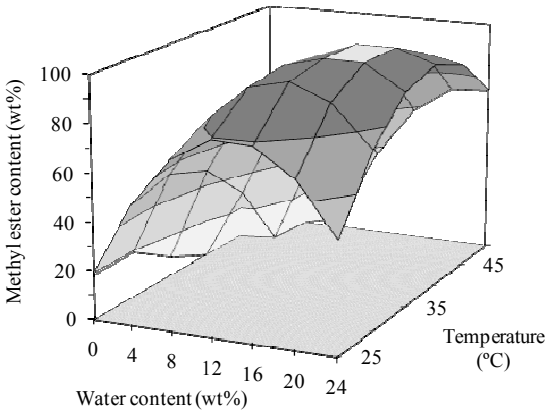

Figure 4: Response surface plots representing the effect of reaction temperature, water content and their reciprocal interaction on rapeseed biodiesel synthesis. Other factors are constant at zero levels.

Fig. 5 shows the changes of methyl ester content with varying methanol-to-oil molar ratio and protein amount, at a reaction temperature of 37.5ºC and 12 wt% of water content. The results showed that the influence of protein amount on methyl ester content is related to the amount of methanol present in the reaction medium. A decrease of methyl ester content with the increase of protein amount, for methanol-to-oil molar ratios lower than 5:1, was observed. This may be due to a mass transfer limitation as a result of immobilized lipase agglomeration in the presence of a reduced aqueous phase. Consequently, the active sites of the enzymes molecules would not be exposed to the substrate and do not contribute significantly to the reaction. An opposite behaviour was observed by using methanol-to-oil molar ratios higher than 5:1. In these cases, it was observed a methyl ester content increase with the increase of protein amount which can be explained by the easier substrate-enzyme interaction in the systems with high volumes of aqueous phase (water + methanol). At a molar ratio of 5:1, the enzymatic reaction was not significantly dependent to the protein amount.

The response surface for the predicted values of fatty acid methyl ester content as a function of methanol-to-oil molar ratio and water content at a constant reaction temperature of 37.5ºC and 0.14 wt% of protein amount, at a reaction time of 15 h, was plotted (Fig. 6). For all methanol-to-oil molar ratios, the water content increase to about 18 wt% led to an increase of methyl ester content. However, a decrease of methyl ester content for methanol-to-oil molar ratio close to 7:1 was observed. This behaviour is more pronounced for low water contents due to methanol inhibitory effects.

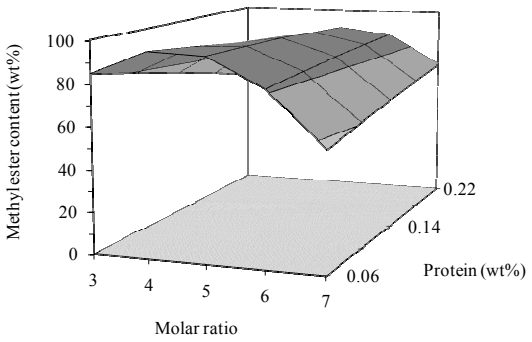

Figure 5: Response surface plots representing the effect of methanol-to-oil molar ratio, protein amount and their reciprocal interaction on rapeseed biodiesel synthesis. Other factors are constant at zero levels.

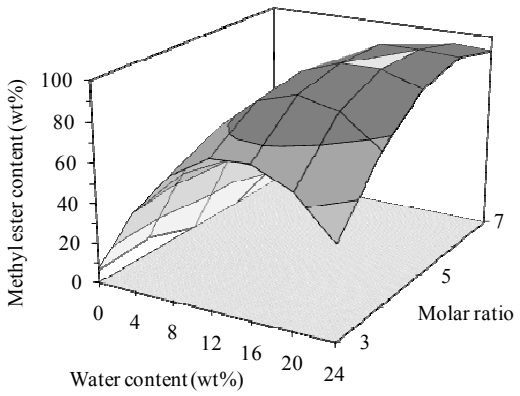

Figure 6: Response surface plots representing the effect of methanol-to-oil molar ratio, water content and their reciprocal interaction on rapeseed biodiesel synthesis. Other factors are constant at zero levels.

The optimal values of the studied variables were obtained by a MATLAB optimization procedure applied to the regression equation (Eq.(4)). The maximum conditions for rapeseed biodiesel synthesis estimated by MATLAB software were as follow: T= 37.5ºC, 0.22 wt% of protein amount, methanol-to-oil molar ratio of 6.21:1 and 22.3 wt% of water content. In this case, a methyl ester content higher than the one specified in the biodiesel European standard EN 14214 [13] will be achieved (> 96.5 wt%)..

One of the main problems associated with the use of the immobilized lipase Lipozyme® TL IM in the biphasic system studied in this work was its inability to be reused. In fact, it was observed that the physical characteristics of the support changed during the first experiment, not being possible to recover it to be used in subsequent studies (Fig. 7).

Figure 7: Aspect of the enzymatic preparation of Lipozyme® TL IM (white residue) after the first experiment.

3.4 Enzymatic biodiesel production from different feedstocks

Lipase-catalyzed reactions for production of biodiesel from various vegetable oils were studied to elucidate the effects of the oil source on the final methyl ester content. Methanolysis reactions of rapeseed, soybean and used frying oils were carried out using 50 g oil, 0.3 g immobilized Lipozyme® TL IM lipase (30 mg protein), 4.5:1 methanol-to-oil molar ratio and 15 wt% water content. Although rapeseed and soybean oils are characterized by different fatty acid compositions (Table III), no significant differences were observed in the methyl ester content reached in 15 h at 37 ºC (Table VI). However, in the experiments carried out with used frying oil as raw material, only 79.6 wt% FAME content was achieved. In this case, the different compounds that may be present in the oil due to degradation during its use may have inhibitory effects on lipase with consequent final ester content reduction.

Table VI: Conversions of rapeseed, soybean and used frying oils into FAME, using Lipozyme® TL IM

Oil source	Methyl ester content (wt%)
Rapeseed	89.1
Soybean	87.5
Used frying oil	79.6

Conditions: 15 h, 37 ºC, 50 g oil, 0.06 wt% protein, 4.5:1 molar ratio, 15 wt% water content

4 CONCLUSIONS

The comparative study of different immobilized lipase preparations for the methanolysis of rapeseed oil on an oil/aqueous biphasic system demonstrated that the 1,3-regiospecific lipase Lipozyme® TL IM is the more suitable one allowing to obtain 98 wt% of methyl ester content. The response surface methodology based on central composite design was employed for optimization and analysis of transesterification of rapeseed oil and methanol catalyzed by the selected lipase. According to the response surface methodology experiments, the optimal values of the variables were as follows: reaction temperature of 37.5 ºC, protein amount of 0.22 wt% (based on weight of oil), methanol-to-oil molar ratio of 6.2:1 and water content of 22.3 wt%, with a 15 h reaction time. It was observed that the enzyme undergoes significant inhibition when high levels of methanol and reduced levels of water were used. Also, high reaction temperature values resulted in lipase deactivation.

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