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1. INTRODUCTION

Earth's Climate change is an inescapable theme of our times and is scientifically proven. The average global temperature is rising up and carries consequences such as the melting of glaciers, the increasing intensity of storms and the faster rise in sea level. While some previous climate change were originated by natural causes such as variations in Earth orbit, which causes cycles of cooling-heating, the current situation is quite different, being human activity the main cause, especially with the coming of Industrial era about 150 years ago [1].

The energy used by humans comes mostly from fossil fuels like coal, natural gas or oil. The fact that the reserves of fossil fuels on our planet are finite and the environmental problems associated with their use need a change in mindset and the use of alternative forms of energy production.

These alternatives are called renewable energies and are examples the nuclear energy, hydroelectricity, biomass, wind power, solar energy, geothermal power and tidal power.

Biomass

Biomass is the fourth largest source of energy in the world, after coal, oil and natural gas, that are fossil fuels and non-renewable[2].

The majority of the biomass components are cellulose, hemicellulose and lignin. In smaller amounts can be found terpenes, triglycerides, some organic proteins and some inorganic elements[3].

Many products can be produced from biomass after being processed in a biorefinery. The biorefinery operates in a mode similar to a oil-based refinery, applying hybrid technologies of various areas such as bioengineering and chemistry of polymers[4].

Current products of biorefineries include biofuels such as bioethanol, biodiesel or biogas. The most interesting material for the production of biofuels should have high

energy content (as the case of terpenes and vegetable oils) and should preferably be liquid. However biomass constituents like terpenes, occur in a limited amount. Lignocellulosic biomass arises then as a very attractive material because of its high abundance and it has gained much importance in large-scale production of biofuels.

Biodiesel

Biodiesel is generally defined as a mixture of long chain fatty esters (preferably methyl and ethyl esters), derived from renewable sources [5]. Nowaday, this alternative fuel has gained increasing popularity because it is renewable, non-toxic and biodegradable.

Figure 1 shows the variation of the exhaust emission replacing diesel with biodiesel. In the environmental point of view, the use of biodiesel is advantageous, since its combustion does not emit heavy metals, sulphur oxides or aromatics compounds. However, as biodiesel contains about 10% oxygen more in its composition, the combustion occurs at temperatures higher when compared with fossil fuels, contributing to superior emission levels of nitrogen oxides (NOx) [6-8].

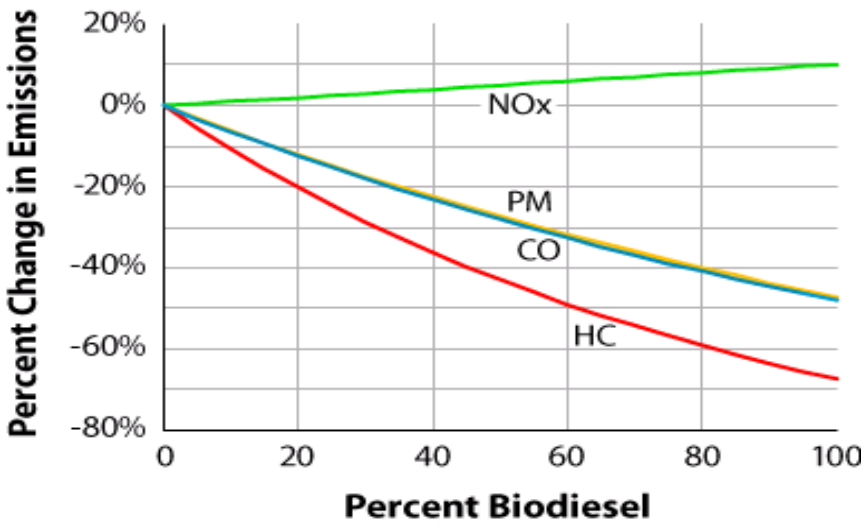


Figure 1: Percent change in emission by the replacing of diesel for biodiesel (Nitrogen oxides, Particulate matter, Carbon monoxide, Hidrocarbonets)[9]

Biodiesel has a flash point higher than the diesel (for diesel is between 54 and 71 °C ant for biodiesel is about 93 °C [8]), making its storage and transport safer. Biodiesel also has an excellent lubricity and is fully miscible with diesel in any proportion [7, 8, 10].

Biodiesel can be produced from, for example, vegetable oils, animal fats, fried oils and algae oils [11] being vegetable oils the main raw material used because they are abundant and easily accessible.

Vegetable Oils

Vegetable oils are triglycerides (TAG) which are triesters of glycerol of various fatty acids with an aliphatic chain of, at least, 8 carbons [12-14] . By the hydrolysis of triglycerides present in vegetable oils, we can get mono and diglycerides, along with some free fatty acids (FFA).

The Table 1 lists the major fatty acids found in vegetable oils and animal fats that are used for biodiesel production. A special focus have been on lauric acid, oleic acid and linoleic acid that have high abundance in some of these raw materials.

Vegetable oils have a higher viscosity (about 5-10 times [7]) that the diesel and a high molecular weight. Their direct use as fuel is not recommended because problems such as a poor atomization, low volatility and the accumulation of residuals in several parts of the engine can occur[6] .

There are several methods to decrease the viscosity of vegetable oils such as dilution, micro-emulsification, pyrolysis and transesterification [10], being the last one the most widely used in industry.

Table 1:Composition of Biodiesel obtained from different sources[12]

Oil Or Fat	Fatty acid composition (%)							
	12:0 Lauric Acid	**14:0** Myristic acid	**16:0** Palmitic acid	**18:0** Stearic acid	**18:1** Oleic acid	**18:2** Linoleic acid	**18:3** linolenic acid	**22:1** Erucic acid
Babassu	44-45	15-17	5.8-9	2.5-5.5	12-16	1.4-3		
Canola			4-5	1-2	55-63	20-31	9-10	1-2
Coconut	44-51	13-18.5	7.5-10.5	1-3	5-8.2	1.0-2.6		
Corn			7-13	2.5-3	30.5-43	39-52	1	
Cottonseed		0.8-1.5	22-24	2,6-5	19	50-52.5		
Linseed			6	3.2-4	13-37	5-23	26-60	
Olive		1.3	7-18.3	1.4-3.3	55.5-84,3	4-19		
Palm		0.6-2.4	32-46.3	4-6.3	37-53	6-12		
Peanut		0.5	6-12.5	2.5-6	37-61	13-41		1
Rapeseed		1.5	1-4.7	1-3.5	13-38	9.5-22	1-10	40-64
Safflower			6.4-7.0	2.4-2.9	9.7-13.8	75.3-80.5		
Safflower (high-oleic)			4-8	2.3-8	73.6-79	11-19		
Sesame			7.2-9.2	5.8-7.7	35-46	35-48		
Soybean			2.3-11	2.4-6	22-30.8	49-53	2-10.5	
Sunflower			3.5-6.5	1.3-5.6	14-43	44-68.7		
Tallow(beef)		3-6	25-37	14-29	26-50	1-2.5		

Transesterification

In transesterification reaction an ester group reacts with an alcohol which has a different structure of the original alcohol moiety of the ester. The transesterification of triglycerides is the most common reaction to obtain biodiesel and is represented on Figure 2. The three ester groups of the triglyceride molecule, each with a moiety of fatty acid, react with 3 molecules of alcohol on consecutives steps. The products are the molecules of alkyl esters (biodiesel), that contain the single fatty acid and the alcohol moieties, and glycerol[15].

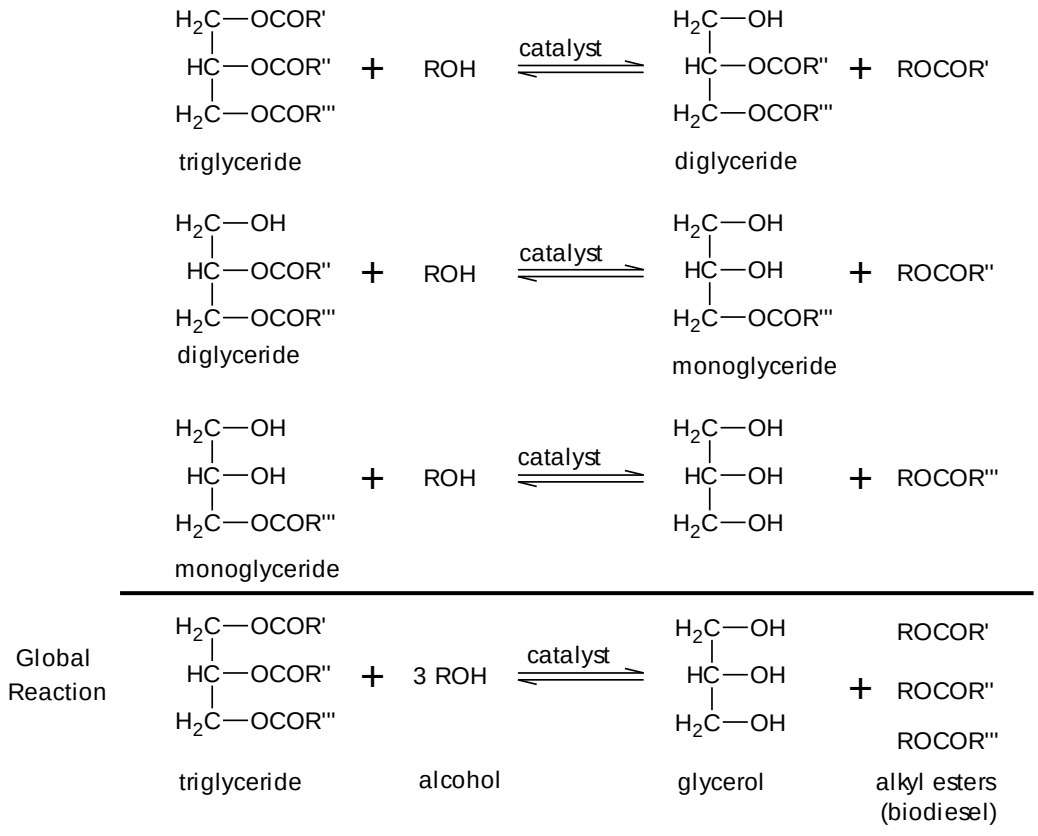


Figure 2: Transesterification reaction for biodiesel production

Several factors affect the transesterification as the type of catalyst, the alcohol/oil molar ratio , temperature, the reagents purity, the amount of free fatty acids, the alcohol used and the mixing as this is a two phase reaction [16, 17] .

Usually short-chain alcohols are used in the reaction [7], being the methanol the mostly used due to is low price and because his absolute form is easily obtained. Moreover, the process is easy to control and biodiesel purification easier with methanol than with heavier alcohols. However, methanol is toxic and obtained from fossil fuels. On the other hand, ethanol has been widely studied as a potential replacement for methanol because it can be obtained from renewable sources and is a more effective solvent for the oil than methanol [18]. However is it more difficult to obtain pure and its production from renewable resources is today limited to a number of countries. Moreover ethanol can be used as a biofuel itself, so its transformation may not be economically favorable. Although the stoichiometry of the reaction is 1:3 (mole triglyceride: mole alcohol), an excess of alcohol is beneficial to increase the conversion to alkyl esters and facilitate the separation of glycerol [19].

The catalysts mostly used industrially are the homogeneous basic catalysts such as NaOH or KOH but the use of heterogeneous acid or biological catalysts as enzymes has gaining increased interest in particular for oils of poor quality with a high acidity or fatty acids for which the basic catalysis is not adequate.

Homogeneous process

Transesterification catalyzed by bases

Currently, the alkaline catalyst is the most widely used in industrial production of biodiesel because it can be used at relatively low temperatures (between 40-65 ° C [20]), atmospheric pressure and the reaction is fast allowing high conversions (> 98%) in about 3 hours of reaction time [7].

The catalysts used in catalysis are basic alkali metal hydroxides (NaOH and KOH) and metal alkoxides (CH_3ONa). The absence of water in the raw material is required as this can lead to hydrolysis of triglycerides and successive saponification of free fatty acids with the salt, reducing the catalytic efficiency and consuming the catalyst. The presence of water further increases the viscosity, leading to the formation of gels and difficult the separation of glycerol [17]. Another requirement for the base catalyzed transesterification is a low content of free fatty acids, because the saponification, mentioned earlier, can occur.

In the base catalyzed transesterification the catalyst is removed at the end along with the glycerol and cannot be reused. The glycerol needs to be neutralized increasing the difficulty of purification of this compound and preventing the reuse of the catalyst [5].

Transesterification Catalyzed by acids

In a transesterification catalyzed by acids are used Brönsted acids such as HCl and H_3PO_4. With acid catalysts can be obtained a high conversion to alkyl esters, but the reaction is slower than the alkaline catalysis [21] and usually are required temperatures above 100°C and more than three hours of reaction [22] .

When vegetable oils or animal fats contain a high level of free fatty acids, the acid catalysis is more effective than alkaline because both reactions of esterification (free fatty acids) and transesterification (triglycerides) occur and the reaction of saponification doesn't happen.

The molar ratio alcohol/oil is one of the major factors influencing the transesterification. On one hand an excess of alcohol increases the yield of the reaction but then makes the recovery of glycerol more difficult [23].

Heterogeneous process

Transesterification catalyzed by enzymes

Enzymes are globular proteins that act as biological catalysts [14]. For the production of biodiesel are generally used lipases that catalyze the transesterification of triglycerides, the esterification of free fatty acids and the hydrolysis of triglycerides in fatty acids, diglycerides, monoglycerides and glycerol. Lipases are usually classified as hydrolases since they catalyze mainly the hydrolysis reaction.

An excess of water can lead to the occurrence of the hydrolysis reaction instead of transesterification [24] but the saponification doesn´t occur. On other hand the excess of water might produce enzyme deactivation [25].

The excess of alcohol used to ensure higher conversions can have an inhibitory effect on the catalytic activity of the enzyme during the reaction resulting in a reduction of the conversion because the lipase is easily deactivated by hydrophilic and polar compounds such as lower linear alcohols [5, 15, 26].

The transesterification catalyzed by enzymes may be able to compete in terms of reaction time and yields with transesterification catalyzed by acids and bases [27] but its biggest problem at present is the high cost of enzymes [28].

Enzymes can be used in its pure form but recent studies show that they are more effective when they are immobilized, making them more attractive for industrial use. Immobilization techniques include physical adsorption on solid supports, covalent bonding to solid supports and physical entrapment in polymer matrix support [24].

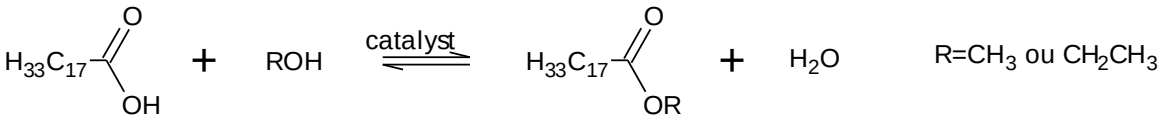
The catalysis can be carried out using intracellular lipases (whole cells) as *Rhizopus oryzae* and extracellular as *Candida antartica* or *Mucor miehei*. In the case of extracellular enzymes, after cultivation and separation, they must be submitted to complex purification steps prior to immobilization. In Intracellular lipases, the cultivation and immobilization steps occur together, which may reduce the cost of the process [29].

Immobilized enzymes can be recycled and reused several times. Immobilization prevents the agglomeration of enzymes and increases their thermal stability. When

immobilized enzymes are used there's no toxic catalyst on the effluents and the separation step is more versatile. Among the disadvantages are an additional cost and some loss of enzyme activity during the immobilization [30].

Esterification

An alternative way to transesterification reaction for the production of biodiesel is the esterification, in which a fatty acid, instead of a triglyceride, reacts with an alcohol. The by-product of this reaction is water, instead of glycerol. On Figure 3 is represented the esterification of oleic acid. The reaction is reversible and to shift the equilibrium in favour of the products two methods can be used: removal of one of the products, preferably water, or using an excess of alcohol.



oleic acid | alcohol | alkyl ester (Biodiesel) | water

Figure 3: Esterification reaction of oleic acid with an alcohol

Free fatty acids are a by-product of the refining of edible oils, which are removed in a neutralization step in the chemical refining (for oils with low acidity) or physical refining by deodorization (in oils with high acid content) in order to be marketed. These acids recovered on the deodorization process have the potential to be converted into biodiesel also by processes of acid or enzymatic catalysis.

2. EXPERIMENTAL PROCEDURE

2.1 Materials

The fatty acid used in this work was oleic acid, *cis*-9-Octadecenoic acid, represented on Figure 4. Oleic acid (90.0%w/w) was acquired from Aldrich. This acid is present in high amounts in oils such as olive, palm, peanut or canola oil. The oleic acid and the alcohol (methanol or ethanol) are miscible, which represents an advantage over the transesterification because the triglycerides and alcohol are immiscible.

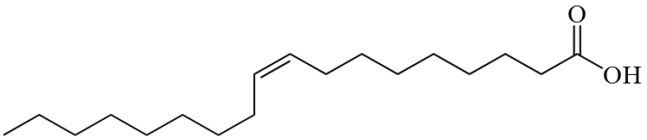


Figure 4: Oleic acid molecule

The catalyst used was the Novozym® 435, a lipase B from *Candida antarctica*, adsorbed on a macroporous acrylic resin. The Novozym 435® was obtained from Novozymes (≥10000 U/g). This immobilized enzyme has a high robustness and thermostability [31].

Methanol (99.9%w/w) was acquired from Lab-Scan and the ethanol (99.8%w/w) from Riedel-de-Haën. To remove the water from the samples we used anhydrous sodium sulphate.

For the silylation the chemicals used were BSTFA (*N,O*-Bis(trimethylsilyl)trifluoroacetamide), (CTMS) chlorotrimethylsilane and Pyridine. The BSTFA and the CTMS were acquired from Fluka and the Pyridine (99.0%w/w) from Aldrich.

For the gas-chromatography analysis the samples were diluted in dichloromethane with analytical grade (99.9%w/w) acquired from Fisher Scientific.

2.2 Experimental Apparatus and Procedure

Esterification Reactions

Esterification reactions were carried out in a ground erlenmeyer. The reagents where weighed on analytical balance (±0.0001g) by the following order: enzyme, oleic acid and alcohol (methanol or ethanol). The mass of enzyme corresponding to a desired molar ratio was calculated with reference to the 90% oleic acid present in the reaction.

For the kinetic study 100mL ground erlenmeyers were used and the total volume of reagents was 30mL, for the other reactions 50mL ground erlenmeyers were used and the total volume of reactants was 10mL. The erlenmeyers were introduced on an orbital stirrer at 150rpm and 40 ± 0.05 ºC.

After the defined time of reaction two samples were collected from the bottom of the final product in each erlenmeyer. To remove the water, the samples were passed through anhydrous sodium sulfate.

Kinetic study

An initial study of the conversion vs. time was made in order to find a reaction time that could be used on the other experiments. The alcohol/oleic acid molar ratio was 6 and the enzyme concentration was 2%. The agitation used was 150 rpm with 40ºC of temperature.

Several samples of 200μL each were collected at the times of 5, 10, 20, 30, 40 minutes and 1, 1.5, 2, 4, 5, 6, 7, 8 and 24 hours.

Sample derivatization

In order to increase the volatility of the samples for GC analysis we proceed to a derivatization of the samples collected. In a glass tube 30 μL of each sample was introduced and 100 μL of Pyridine, the solvent, 100 μL of BSTFA, the silylant agent, and

50 μL of CTMS, the catalyst were added. The tubes were hermetically closed and kept at temperature between 70 and 80ºC, into an oil bath, for 30 minutes.

Chromatography analysis

The fatty acid and ester were analyzed by capillary gas chromatography. Each sylilated sample of 40 μL was diluted in 200 μL of dichloromethane and 0.5 μL were injected into a gas chromatographer with a flame ionization detector (Varian 3800 GC-FID) in a split injection system with the ratio of 1:20.

On the analysis a DB1-ht column (length: 15m, internal diameter:0.32mm and film thickness:0.1μm) coated with 0.1μm film of dimethylpolysiloxane was used.

The column temperature was set at 100ºC and then programmed to increase up to 200ºC, at 8ºC/min with a final step of 5 minutes at that temperature. Detector and injector temperatures were set at 220ºC and 250ºC, respectively. The carrier gas was Helium with a flow rate of 2mL/min.

Conversion Quantification

The percentage of conversion (alkyl esters formed) on the reaction was quantificated by comparison between the areas from the peaks of alkyl esters and oleic acid on the chromatogram according to equation 1.

$$\boldsymbol{Conversion} = \frac{\boldsymbol{A_{ae}}}{\boldsymbol{A_{ae}+A_{oa}}} \times \mathbf{100} \qquad \textbf{(1)}$$

where A_{ae} is the area of the peak of alkyl ester and A_{oa} is the area of the peak of oleic acid.

Design of experiments

An optimization of a process involves identifying the most important parameters. One way to accomplish this identification would be testing each variable at a time which would be an exhaustive task that would take too long and would involve too many costs. This kind of approach would also ignore the interactions between variables. A procedure to overcome these problems is the definition of an experimental design where the factors are analyzed simultaneously and can also focus on more than one answer at the same time [5, 32].

To optimize the conditions for enzymatic esterification of oleic acid with methanol or ethanol, an experimental design was defined with the purpose of identifying the most important parameters and their interactions. For the enzymatic esterification of oleic acid with methanol three parameters were tested: methanol/oleic acid molar ratio(R), enzyme concentration (E) and temperature (T). A central composite design, 2^3, with six replications of the central point was used. The conditions were defined for zero level (central point) and one level (+1 and -1, the factorial points). The design was extended up to the axial points which are at a distance of α coded units from the central point,

$$\alpha = \sqrt{k}$$

being k the number of variables.

The data obtained were fitted to the following second order polynomial equation that contains (k+1)(k+2)/2 parameters

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_i \beta_{ii} X_i^2 + \sum_{i<j} \sum_j \beta_{ij} X_i X_j \qquad (2)$$

where Y is the dependent variable (conversion in %) and β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for the intercept, linear, quadratic and interaction terms, respectively. X_i and X_j are the independent variables [33].

The point with the conditions of maximum conversion for the mathematical model was tested for the design of experiments of two variables.

The conditions that define each level of the experimental design adopted for the enzymatic esterification of oleic acid with methanol are represented on Table 2.

Table 2: Level of variables for central composite design 2^3

Variables	Levels				
	-1.682	-1	0	+1	+1.682
R	5	7.03	10	12.97	15
E	1	1.81	3	4.19	5
T	35	37.03	40	42.97	45

The statistical analysis of the experimental data for the central composite design 2^3 showed that the temperature had no statistical significance for the range of values studied. A new experimental design was defined excluding the temperature, this time with only two variables: methanol/oleic acid molar ratio(R) and enzyme concentration (E). The temperature was kept at 40ºC.

Two different designs of experiments were defined. First, a factorial design was used and, on the region with the conditions of higher conversion values, a central composite design 2^2 was defined with six replications of the central point. The aim of this procedure was to obtain a good response surface that would allow us to find the optimal conditions of the reaction. The range of values studied for each variable of factorial design and central composite design are indicated on Table 3 and Table 4, respectively.

The influence of temperature on reaction in a larger range will be studied later under fixed conditions in which the temperature is the only variable.

Table 3: Levels of variables for factorial design 2^2 for methanol

Variables	Levels	
	-1	1
R	0.5	10
E	1	5

Table 4: Levels of variables for central composite design 2^2 for methanol

Variables	Levels				
	-1.414	-1	0	1	1.414
R	0.78	1.5	3.25	5	5.72
E	2.59	3	4	5	5.41

For the esterification of oleic acid with ethanol the approach was similar to the one used for the methanol and again it was chosen to study only two variables: ethanol/oleic acid molar ratio (R) and the enzyme concentration (E). An initial central composite design 2^2 was defined and, in the region with the conditions of higher conversion values, a new factorial design was defined. The range of values studied for each variable for central composite design and for factorial design are indicated on Table 5 and Table 6, respectively.

Table 5: Levels of variables for central composite design 2^2 for ethanol

Variables	Levels				
	-1.414	-1	0	1	1.414
R	0.26	1.5	4.5	7.75	8.74
E	0.17	1	3	5	5.83

Table 6: Levels of variables for factorial design for ethanol

Variables	Levels	
	-1	1
R	3	6
E	5	7

Study of temperature influence on the reaction

For the study of temperature influence on the reaction the conditions were: alcohol/oleic acid molar ratio, R=6 and enzyme concentration, E=2%. The values of

temperature studied were between 20ºC and 60ºC for methanol or 70ºC for ethanol .The highest temperature was limited by the boiling point of the alcohol which is 64.7ºC for methanol and 78.4ºC for ethanol.

Study of immobilized enzymes reuse

To study the reuse of the immobilized enzymes was carried out a set of ten consecutive tests of three hours for each alcohol. The conditions set were T = 40 º C, R = 6 and E=2%.

After collecting the samples for further analysis, the remaining solution in the erlenmeyer was removed with a Pasteur pipette having the attention of not removing any granule of immobilized enzyme. The immobilized enzymes were washed with distilled hexane, and dried in the erlenmeyer. The same erlenmeyer was used in the following test, in which the other reagents were added to the existing enzymes.

2.3 Software

All statistical analysis were made using Statistica 8.0 from Statsoft© (ANOVA, pareto chart and predicted values vs observed values graph), Matlab R2009b from The MathWorks™ (response surface and contour plot) and Microsoft Office Excel 2007 from Microsoft©. The confidence level used was 90%.

3. EXPERIMENTAL DATA AND STATISTICAL ANALYSIS

3.1 Experimental Data

Kinetic study

The times of sampling for the study of the conversion versus time and their conversions are indicated in Table 7.

Table 7: Experimental data for the kinetic study

Time(h)	Methanol(%)	Ethanol(%)	Time(h)	Methanol(%)	Ethanol(%)
0.08	5.54	4.87	2	65.40	59.94
0.17	11.50	10.74	4	84.53	85.53
0.33	21.69	13.58	5	89.29	94.66
0.50	37.10	18.55	6	92.62	93.53
0.67	35.64	26.45	7	93.80	92.19
1	60.07	34.75	8	99.77	92.33
1.5	58.73	49.42	24	99.05	98.92

The reaction time of 3 hours (between 60 and 70% conversion) was chosen as the reference time for our other tests, since it seemed to be appropriated to define our experimental designs.

Design of experiments, central composite design 2^3, for the enzymatic esterification of oleic acid with methanol

On Table 8 the experimental conditions according to the central composite design 2^3 and their respective conversion is represented.

Table 8: Experimental data for the central composite design 2^3 of reaction with methanol

X_1	X_2	X_3	E(%)	T	R	Conversion(%)
-1	-1	-1	1.82	37.03	7.03	46.04
1	-1	-1	4.17	37.03	7.04	80.41
-1	1	-1	1.90	42.97	7.19	66.43
1	1	-1	4.20	42.97	7.42	81.40
-1	-1	1	1.86	37.03	13.21	12.94
1	-1	1	4.19	37.03	12.97	50.53
-1	1	1	1.81	42.97	12.91	13.62
1	1	1	4.27	42.97	12.96	19.03
-1.682	0	0	0.98	40.00	9.97	7.75
1.682	0	0	4.88	40.00	9.87	76.40
0	-1.682	0	3.10	35.00	10.70	58.58
0	1.682	0	2.94	45.00	9.80	47.55
0	0	-1.682	3.00	40.00	5.04	88.51
0	0	1.682	3.04	40.00	14.99	7.17
0	0	0	3.14	40.00	10.00	54.54
0	0	0	3.02	40.00	10.00	57.39
0	0	0	3.04	40.00	10.00	50.47
0	0	0	3.02	40.00	10.01	52.29
0	0	0	3.02	40.00	10.01	57.02
0	0	0	3.00	40.00	10.00	56.46

Design of experiments, central composite design 2^2, for the enzymatic esterification of oleic acid with methanol

On Table 9 the experimental data from the central composite design 2^2 of the reaction with methanol is represented.

Table 9: Experimental data for the central composite design 2^2 of reaction with methanol

X_1	X_2	R	E(%)	Conversion(%)
Factorial design				
-1	-1	0.50	1.00	44.11
-1	1	0.50	5.00	42.48
1	-1	9.97	0.98	7.75
1	1	9.87	4.88	76.40
Central composite design				
-1	-1	1.51	3.00	94.74
-1	1	1.50	5.02	96.23
1	-1	5.04	3.00	88.51
1	1	5.00	5.01	97.12
-1.414	0	0.50	3.99	46.61
1.414	0	5.99	3.99	92.87
0	-1.414	3.26	1.98	89.92
0	1.414	3.26	6.00	98.08
0	0	3.25	4.028	95.54
0	0	3.25	4.004	95.90
0	0	3.25	4.013	95.69
0	0	3.25	4.004	96.30
0	0	3.26	3.999	95.46
0	0	3.26	3.999	95.63

Design of experiments, central composite design 2^2, for the enzymatic esterification of oleic acid with ethanol

On Table10 the experimental data for the central composite design 2^2 of the reaction with methanol is represented.

Table 10: Experimental data for the central composite design 2^2 of reaction with ethanol

X_1	X_2	R	E(%)	Conversion(%)
Central composite design				
-1	-1	1.498	1.045	63.12
-1	1	1.497	5.001	91.88
1	-1	7.491	1.000	37.52
1	1	7.479	4.972	83.83
-1.414	0	0.2636	2.994	22.46
1.414	0	8.753	2.987	67.53
0	-1.414	4.491	0.176	10.19
0	1.414	4.485	5.81	90.02
0	0	4.499	3.040	77.38
0	0	4.487	3.005	78.21
0	0	4.476	2.997	77.64
0	0	4.489	3.016	83.99
0	0	4.488	2.996	79.20
0	0	4.507	3.016	88.08
Factorial design				
-1	-1	2.998	5.019	89.88
-1	1	2.994	7.070	91.47
1	-1	6.016	5.037	87.83
1	1	6.001	7.003	91.15

Study of the temperature influence on the reaction

The experimental data for the study of temperature on the reaction are presented on Table 11.

Table 11: Experimental data for the study of temperature influence on the reaction

TºC	Methanol(%)	Ethanol(%)
30	66.96	62.04
40	69.54	71.32
50	72.07	73.15
55	66.44	-
60	61.12	80.87
70	-	84.23

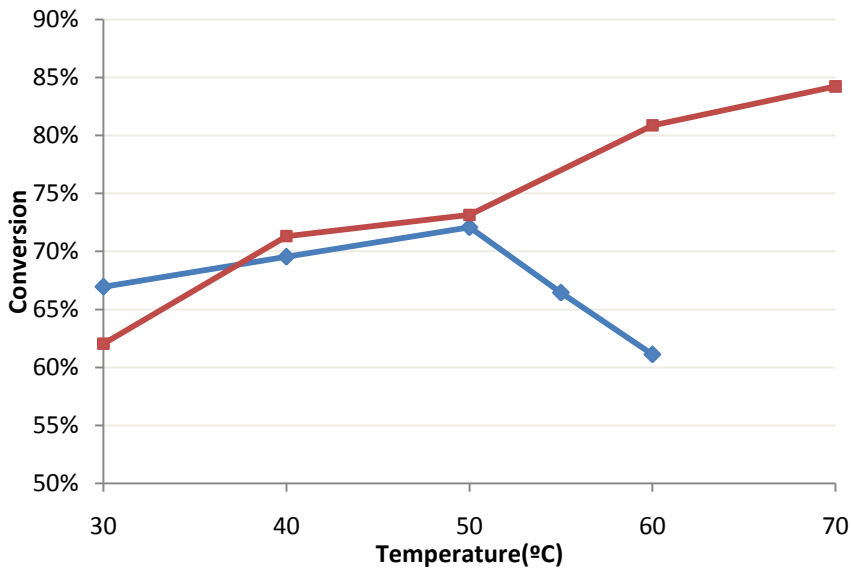


Figure 5: Influence of the temperature on the reaction using (——) methanol or (——) ethanol

In Figure 5, the influence of the temperature on the reaction is presented. As can be seen, the conversion increases monotonously with increasing temperature when ethanol is used for the range of values studied. For methanol the conversion increases up to approximately 50ºC and decreases with increasing temperature for higher temperatures. This behaviour can be explained by the fact that methanol is more deleterious to the enzyme than ethanol. So, for higher temperatures, the enzyme is deactivated by methanol leading to lower conversions.

Study of immobilized enzymes reuse

Table 12: Experimental data for the study of immobilized enzymes reuse

Cycle Number	Methanol(%)	Ethanol(%)	Cycle number	Methanol(%)	Ethanol(%)
1	68.19	61.65	**6**	35.95	57.58
2	61.67	58.16	**7**	28.21	57.71
3	59.79	58.23	**8**	22.34	55.80
4	47.92	56.68	**9**	19.36	55.60
5	36.85	56.26	**10**	16.91	56.17

The results obtained for the study of the reuse of the enzyme are shown in Table 12 and Figure 6. Can be verified that, in the case of enzymatic esterification of oleic acid with ethanol, doesn't occur a significant loss of enzyme activity after ten cycles. In the case of methanol there is a significant decrease in conversion caused by a decrease in enzyme activity as a consequence of the deleterious effect of methanol.

It was not possible to quantify the loss in mass of immobilized enzymes resin once they still contained oleic acid in the final residue, after dried, and the final mass of immobilized enzymes was higher than the initial, however it was the method used to remove the solution from the flask was effective, without causing significant loss of immobilized enzymes.

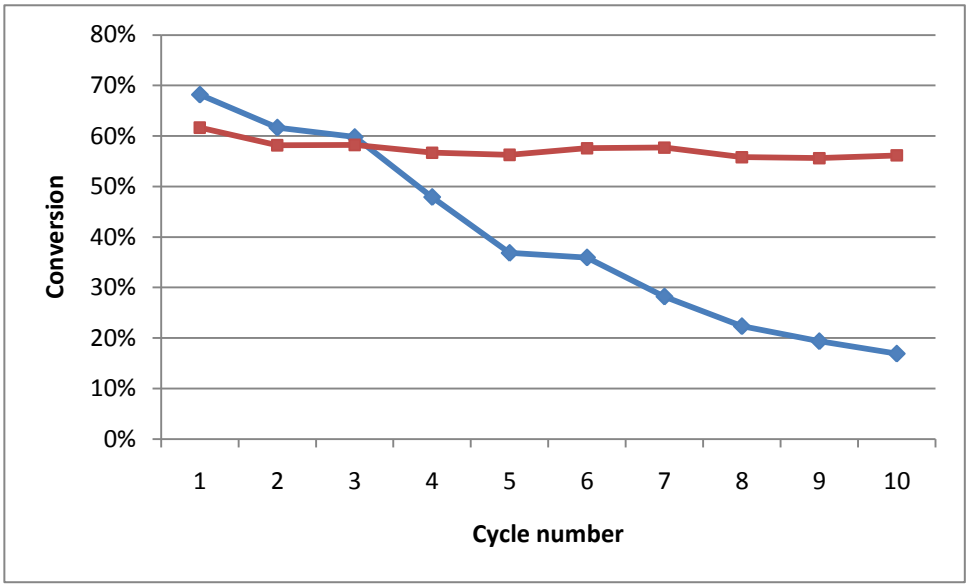


Figure 6: Study of immobilized enzyme reuse using (——) methanol or (——) ethanol

3.2 Statistical Analysis

With the experimental results of each experimental design a second order mathematical model was generated, where not only the linear effects for each variable, but also the quadratic effects and the interaction between each variable were considered. It were considered as significant parameters those with p under 10% ($p<0.1$), due to the large variability inherent to bioprocesses.

Statistical analysis for enzymatic esterification of oleic acid with methanol

Design of experiments for three variables

For the central composite design 2^3 the second order mathematical model derived can be described by the following equation:

$$Conversion = -598 + 107.8 \times E + 19.17 \times T + 28.38 \times R - 1.824 \times E \times T - 0.2246 \times E \times R - 0.7383 \times T \times R - 3.275 \times E^2 - 0.08754 \times T^2 - 0.2965 \times R^2 \quad (3)$$

On the Pareto chart shown on Figure 7 are represented the effects considered for the reaction. The height of the bars shows the absolute value of t calculated and the bars are arranged in descending order of significance. The vertical line gives the t tabulated ($t_{10,0.1/2}$ =1.81) from which the effects are considered significant for a confidence level of 90%. The parameters with no statistical significance are incorporated to residuals on ANOVA analysis (Table 14).

In agreement with Figure 7 and Table 13, Enzyme concentration is the most significant variable. The effects that show statistical significance ($p<0.10$) are the enzyme concentration (E), methanol/oleic acid molar ratio (R), enzyme concentration-temperature interaction (ExT), temperature-methanol/oleic acid molar ratio interaction (TxR) and quadratic enzyme concentration (E^2).

The proposed method has a very good coefficient of determination (0.971) and F calculated is highly significant ($p=0.000002$), as can be seen on Table 14, which demonstrate a good adjustment of the model to the experimental values.

The statistical analysis of this procedure is not detailed as the following since the experimental design was redefined excluding the temperature

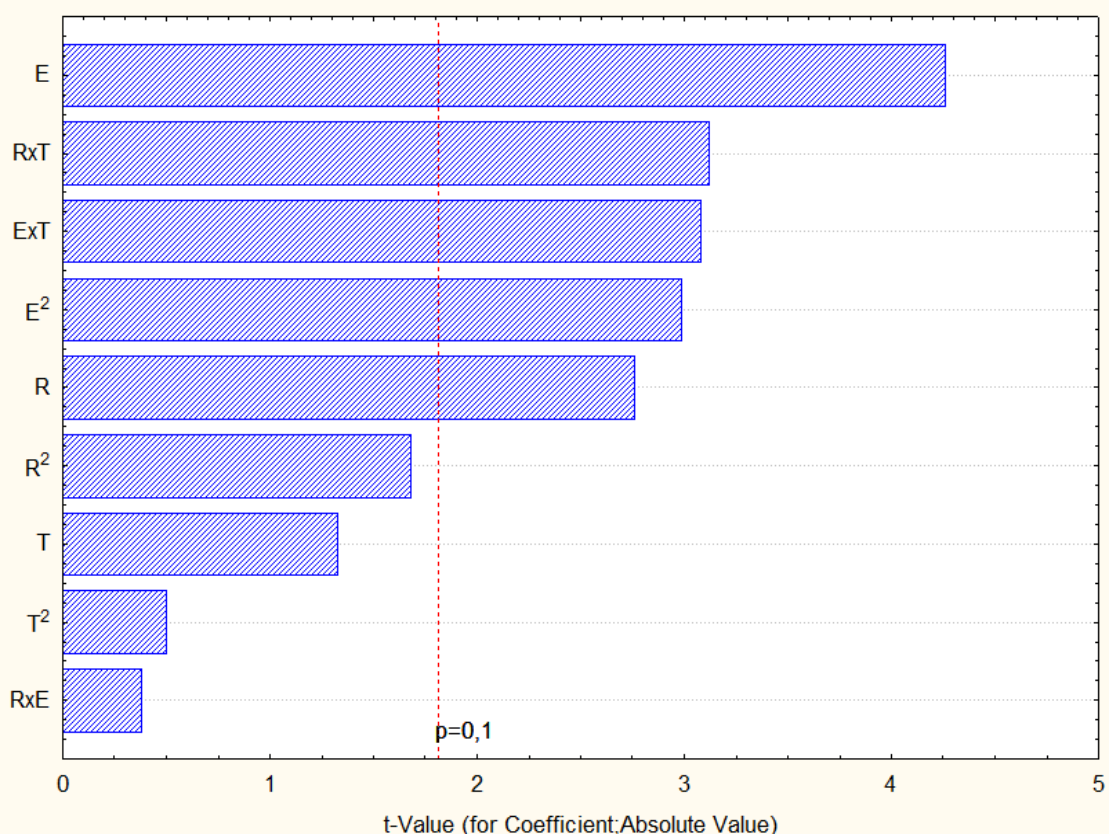


Figure 7: Pareto chart for the effects on conversion rate for esterification with methanol for experimental design with 3 variables.

Table 13: Regression coefficients of the response conversion rate for esterification with methanol for experimental design with 3 variables.

	Coefficients	Standard deviation	t	p value
Interception	-598.89	309.28	-1.94	0.08
R	28.38	10.27	2.76	0.02
E	107.79	25.32	4.26	0.00
T	19.17	14.42	1.33	0.21
RxE	-0.22	0.59	-0.38	0.71
RxT	-0.74	0.24	-3.12	0.01
ExT	-1.82	0.59	-3.08	0.01
R^2	-0.30	0.18	-1.68	0.12
E^2	-3.30	1.10	-2.99	0.01
T^2	-0.09	0.18	-0.50	0.63

Table 14: ANOVA table for central composite design 2^3 for esterification with methanol

Source	SS	DF	MS	F_{calc}	P
Regression	11542.22	9	1282.47	36.63	0.000002
Residuals	350.09	10	35.01		
Total	11892.31	19			

Design of experiments of two variables

For the statistical analysis the experimental values obtained from both experimental designs of two variables, factorial and central composite design were considered.

Based on these results it was possible to create a second order mathematical model for the studied parameters and their interactions, represented on equation 6. The enzyme-methanol/oleic acid molar ratio was also considered.

$$Conversion = 35.29 + 14.09 \times R + 9.32 \times E - 1.923 \times R^2 + 1.532 \times R \times E - 1.425 \times E^2 \quad (4)$$

By the analysis of the Pareto chart and the table of regression coefficients (Figure 8 and Table 15), it can be seen that the most significant effects($p<0.1$) are methanol/oleic acid molar ratio (R), quadratic methanol/oleic acid molar ratio (R^2) and methanol/oleic acid molar ratio-enzyme concentration interaction (RxE). The most significant variable is the methanol/oleic acid molar ratio (R).

Since it is a mathematical model, the values were extrapolated to higher conversion rates at 100% and negative conversion, which are not relevant to our study.

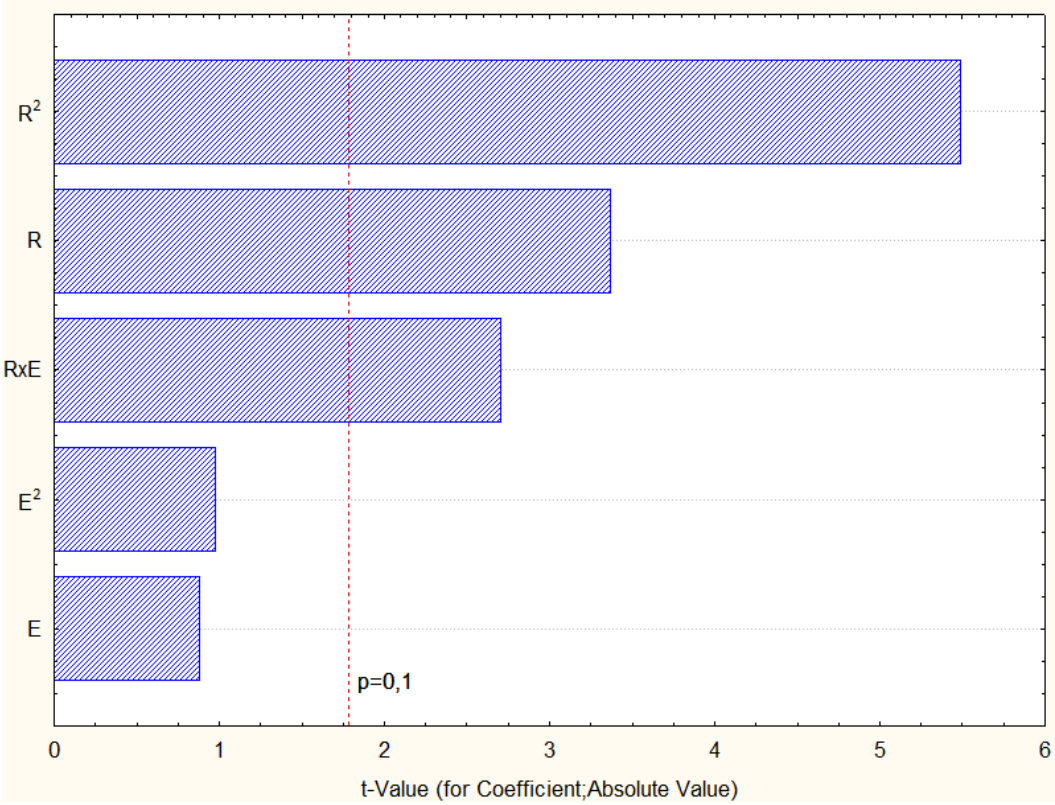


Figure 8: Pareto chart for the effects on conversion rate for experimental designs of 2 variables of esterification with methanol.

Table 15: Regression coefficients for the response conversion for experimental designs of 2 variables of esterification with methanol

	Coefficients	Standard deviation	t	p value
Interception	35.30	18.50	1.91	0.08
R	14.09	4.18	3.37	0.01
E	9.33	10.60	0.88	0.40
R^2	-1.92	0.35	-5.48	0.00
E^2	-1.43	1.46	-0.98	0.35
RxE	1.53	0.57	2.71	0.02

The proposed method has a good coefficient of determination (0.841) for biological catalysts, and by the analysis of variance on ANOVA table it can be observed that F calculated is much larger than F tabulated ($F_{5;7;0.1}$=2.88). Those results indicates

a good agreement between the experimental values and the predicted by the model as can be seen on Table 16 and confirmed on the plot of predicted values vs observed values on Figure 9.

On Figure 9, the diagonal line represents the perfect correlation between the values predicted by the model and the ones observed. The points below the line indicate that the predicted value is lower than the observed and the points above the line indicate that the predicted value is higher than the observed.

In the graph we can see that all points are randomly distributed around the diagonal and the nearby it, confirming the good fit of the model.

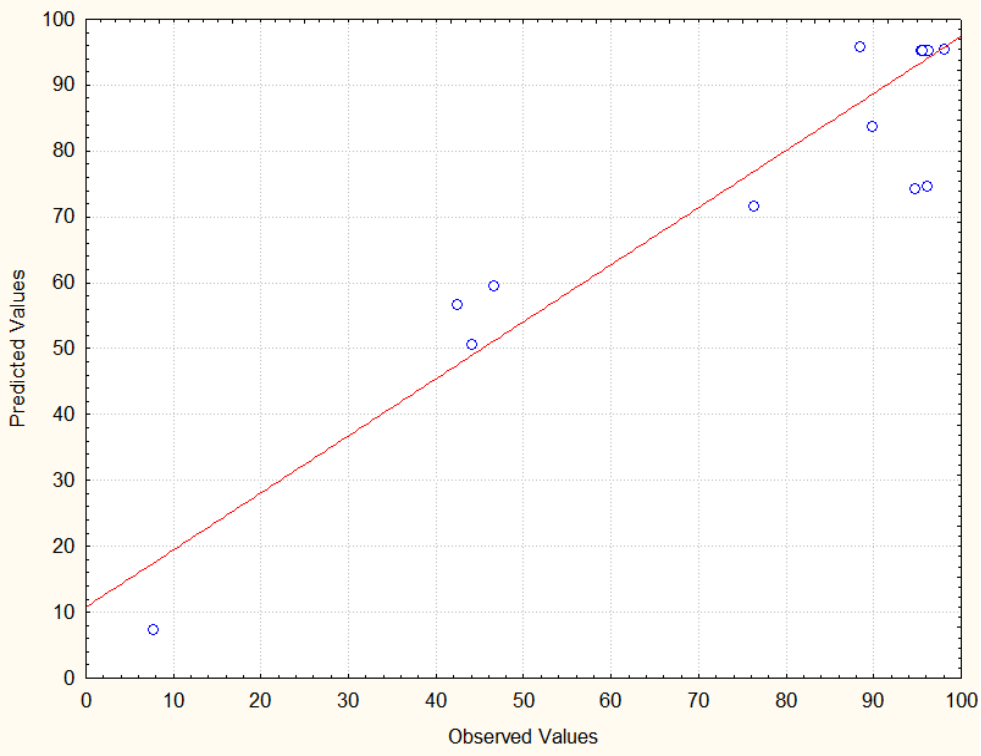


Figure 9: Predicted values vs observed values for experimental designs of 2 variables of esterification with methanol.

Table 16: ANOVA table for experimental designs of 2 variables of esterification with methanol.

Source	SS	DF	MS	F	P
Regression	10365.18	5	2073.04	15.533	0.00007
Residuals	1601.44	12	133.45		
Total	11966.62	17			

Based on the response surface and contour plot represented on Figure 10 and Figure 11, we can identify the conditions that result on a higher conversion of methyl esters. It is possible to observe that the area with higher conversion (>80%) lies between a molar ratio of 4 and 9 and an enzyme concentration between 4 and 10%.

Since this is a mathematical model, the values extrapolate to conversion rates higher than 100% and negative conversions, which are not relevant to this study.

The response surface and contour plot indicate that the reaction is favoured by a methanol/oleic acid molar ratio of about 6, and is strongly disfavoured for higher values, for low concentrations of enzyme.

For values of enzyme concentration above 6, an increase of the molar ratio up to about 5 benefits the reaction, with a decrease in the conversion for higher molar ratio values.

The oblique arrangement of curves in contour plot indicates that the interaction is significant in the tested model.

The maximum value of the Equation 4 was 110.8%, due to the mathematical extrapolation, which corresponded to the conditions of a molar ratio of 6.32 and an enzyme concentration of 6.64%. This point was calculated using the Solver from Microsoft Office Excel and it was tested experimentally in which a conversion of 100% was obtained.

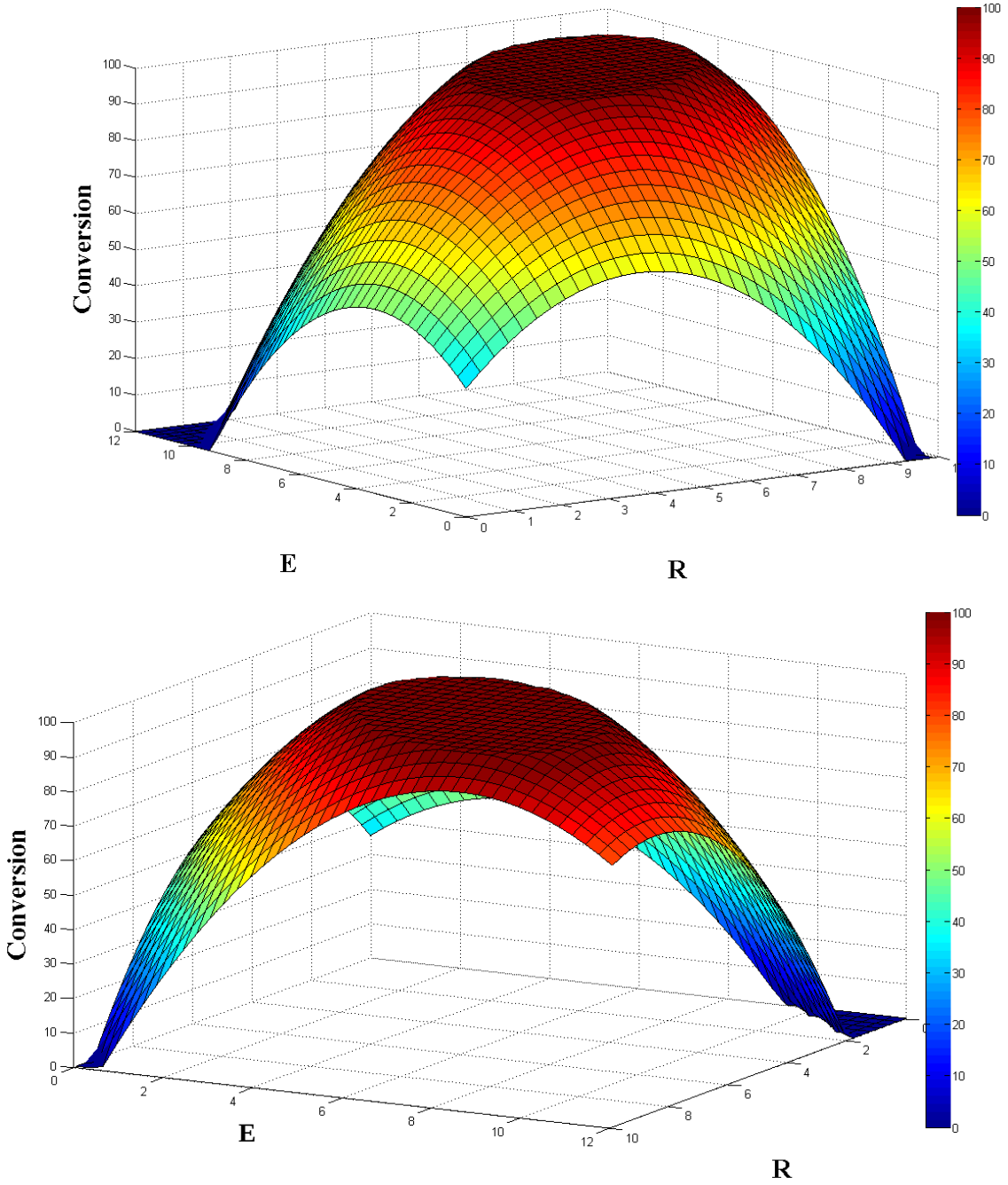


Figure 10 : Response surface for experimental designs of 2 variables of esterification with methanol.

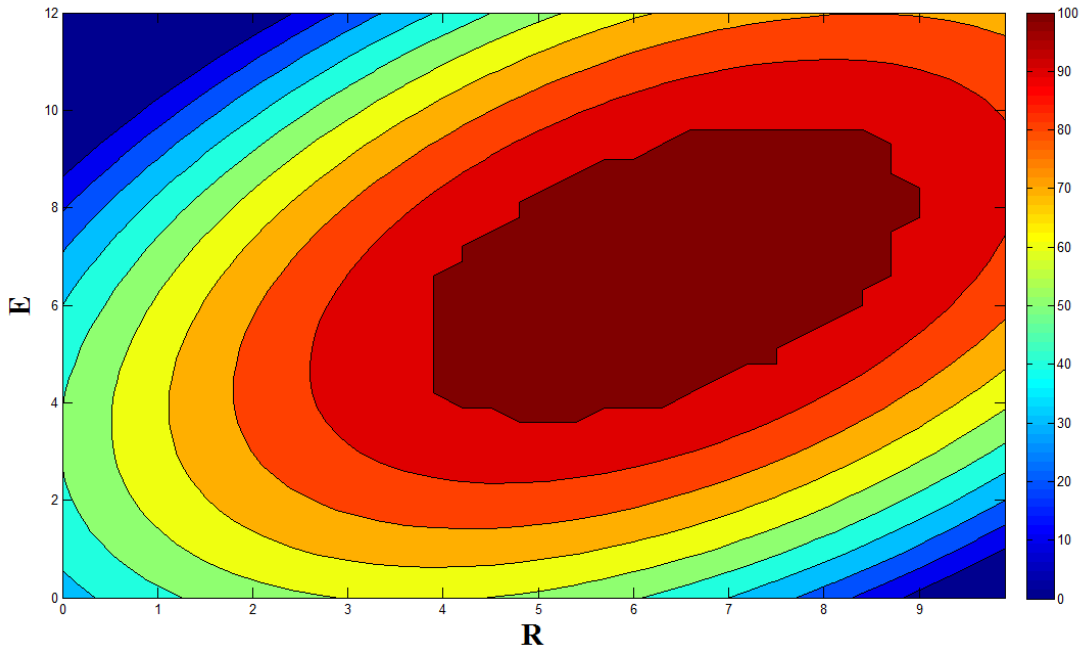


Figure 11 : Contour plot for experimental designs of 2 variables of esterification with methanol.

Statistical analysis for enzymatic esterification of oleic acid with ethanol

The equation generated by the second order mathematical model for the experimental designs of two variables for esterification with ethanol is

$$Conversion = -4.613 + 12.50 \times R + 23.87 \times E - 1.354 \times R^2 + 0.2381 \times R \times E - 2.166 \times E^2 \quad (5)$$

From the analysis of Pareto chart and the table of regression coefficients (Figure 12 and Table 18) can be observed that the interaction ethanol/oleic acid molar ratio-enzyme concentration (RxE) is the only factor that has no statistical significance with a confidence level of 90%. The enzyme concentration is the most significant variable.

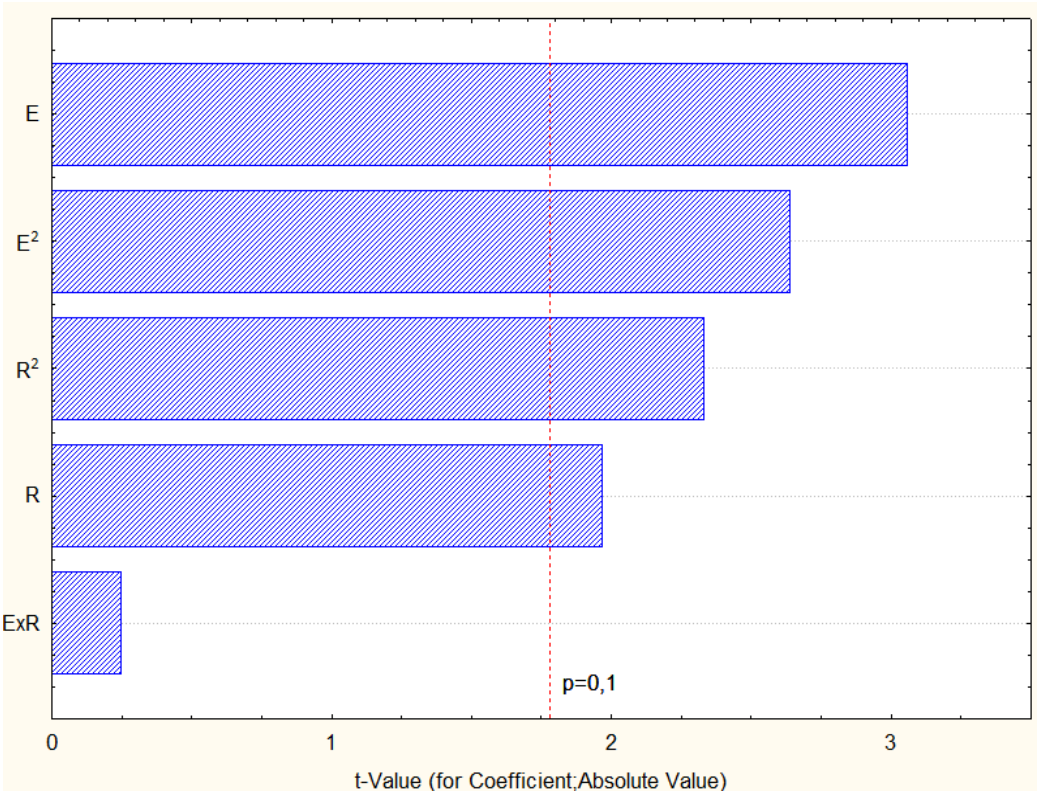


Figure 12 : Pareto chart for experimental designs of 2 variables of esterification with ethanol.

Table 17 : Regression coefficients for the response conversion for experimental designs of 2 variables of esterification with ethanol.

	Coefficients	Standard deviation	T	p value
Interception	-4.61	21.93	-0.21	0.84
R	12.50	6.36	1.97	0.07
E	23.88	7.81	3.06	0.01
R^2	-1.35	0.58	-2.33	0.04
E^2	-2.17	0.82	-2.64	0.02
RxE	0.24	0.96	0.25	0.81

The correlation of the model is 0.760, which can be considered acceptable considering that we are working with biological catalysts. The value of F calculated is good when compared to the tabled ($F_{5;12;0.1}$=2.39) as shown in the ANOVA table. The graph of predicted values vs. observed values in Figure 13 shows that the points are randomly distributed, however, some points have a greater distance from the diagonal

that leads to a decrease of the correlation. We can thus conclude that the proposed model fits well the experimental data.

Table 18 : ANOVA table for experimental designs of 2 variables of esterification with ethanol

Source	SS	DF	MS	F	P
Regression	7814.24	5	1562.85	7.610	0.00197
Residuals	2464.56	12	205.38		
Total	10278.80	17			

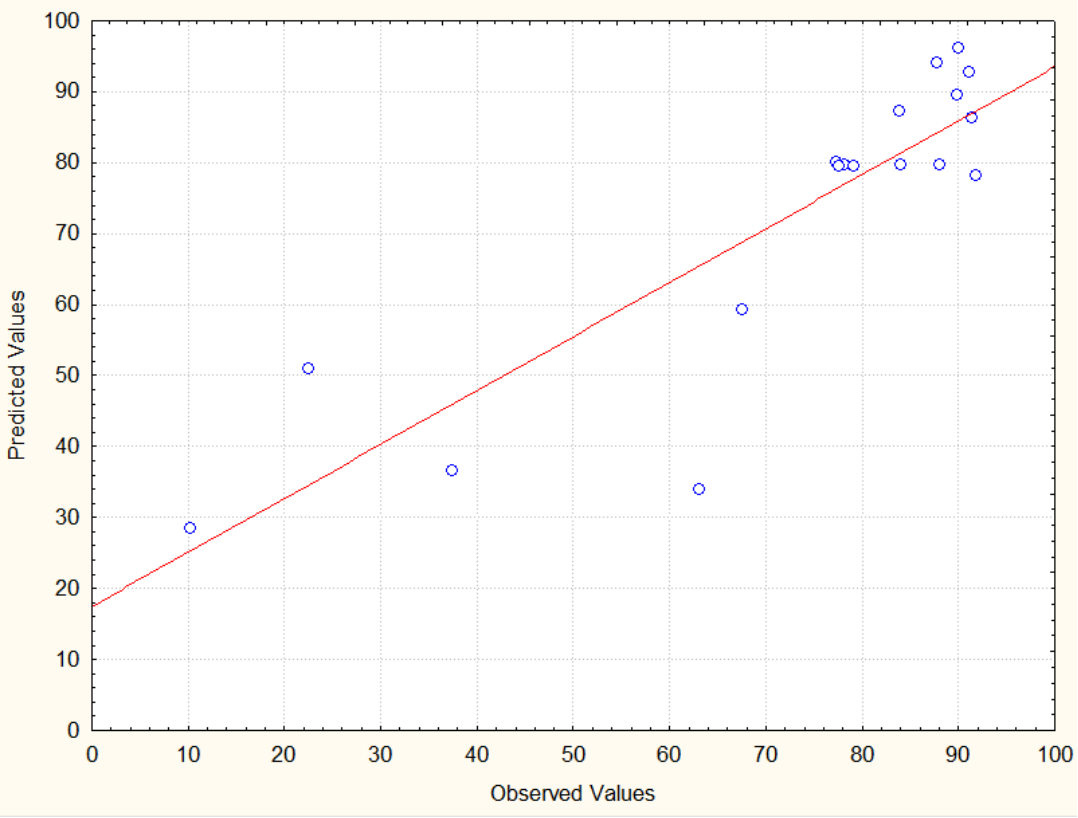


Figure 13: Predicted values vs observed values graph for experimental designs of 2 variables of esterification with ethanol.

Based on the response surface and contour plots represented on Figure 14 and Figure 15 the conditions that result on a higher conversion of ethyl esters can be identified. It is possible to verify that the area with higher conversion (>80%) lies between a molar ratio of 3 and 7 and an enzyme concentration between 4 and 7%. These graphs indicate that the conversion increases with increasing ethanol/oleic acid molar ratio up to about 5 and there is a decrease on the conversion for higher molar ratio values. The

increase in enzyme concentration has a positive effect in response up to values of about 6%, and from that value, the conversion decreases.

The maximum value of the Equation 5 was about 93.4%, which corresponded to the conditions of a molar ratio of 4.87 and an enzyme concentration of 5.65%. The result obtained experimentally for these conditions was 95.5%, a value close to expected, confirming the suitability of the model.

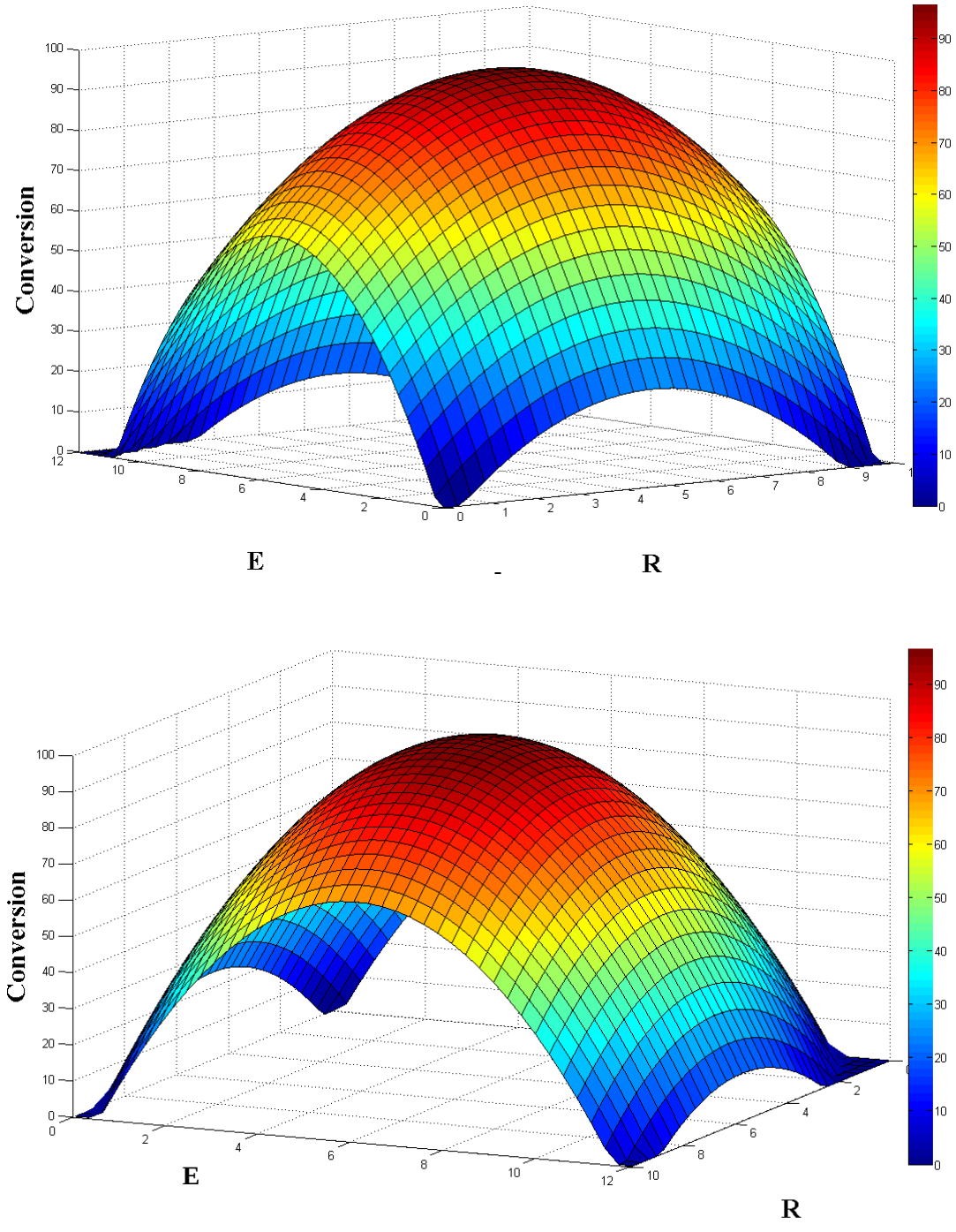


Figure 14 : Response surface for experimental designs of 2 variables of esterification with ethanol.

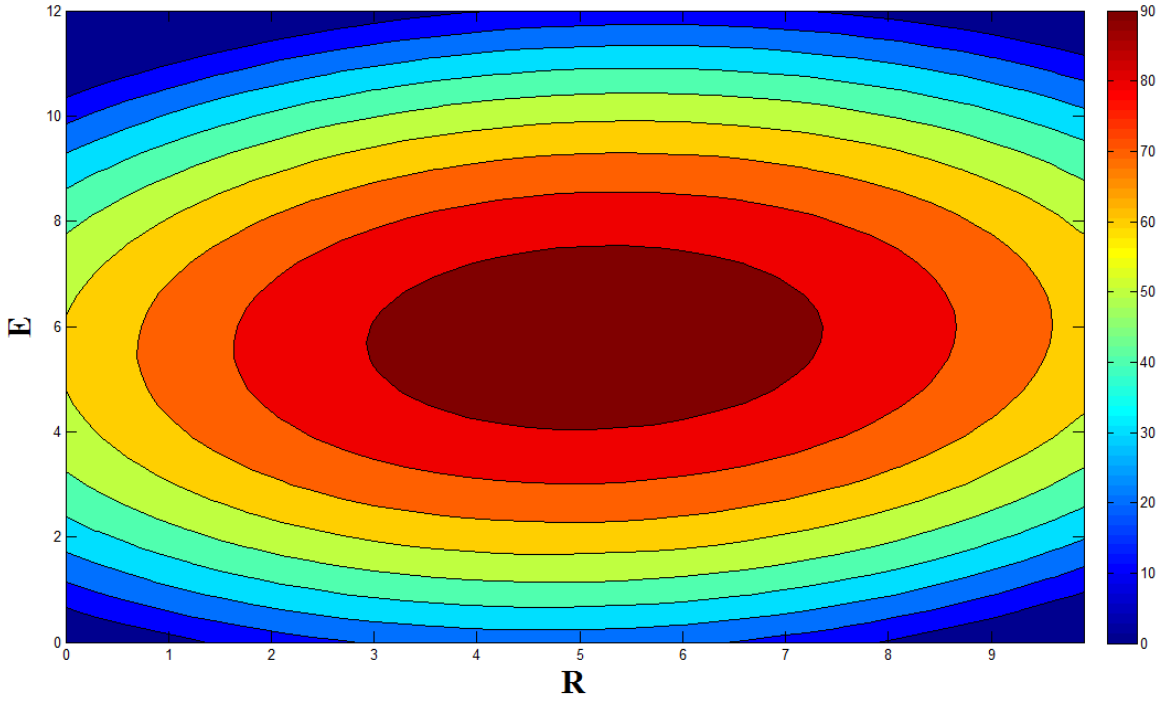


Figure 15 : Contour plot for experimental designs of 2 variables of esterification with ethanol.

4. CONCLUSIONS

It was found that design of experiments is a good method for process optimization and the experimental designs set were effective in our process of production of biodiesel by enzymatic esterification of oleic acid with methanol or ethanol, as the experimental values obtained had a good fit to ones predicted by the model. In the enzymatic esterification of oleic acid with methanol the most significant variable is the methanol/oleic acid molar ratio, but when the methanol is replaced by ethanol it is verified that the variable most significant is the enzyme concentration.

The interaction molar ratio-enzyme concentration is much more significant on the enzymatic esterification with methanol than with ethanol.

It observed that the area with higher conversion (>80%) lies between a molar ratio of 4 to 9 for methanol and 3 to 7 for ethanol, and an enzyme concentration between 4 to 10% for methanol and 4 to 7% for ethanol. It appears that the zone with higher conversions is wider for the case of methanol.

According to the second order mathematical equations the conditions with the maximum conversion were R=6.32 and E=6.64% for methanol (corresponding to 110% of conversion, due to the mathematical extrapolation of the values), and R=4.87 and E=5.65% for ethanol (93,4% of conversion). The experimental values were 95.5% and 100% for methanol and ethanol, respectively.

The conversion increases with increasing temperature when ethanol is used for the range of values studied. For methanol the conversion increases at approximately 50ºC and decreases with increasing temperature for upper values.

The same enzyme can be used 10 times in the enzymatic esterification of oleic acid with ethanol without significant loss of enzymatic activity by washing the immobilized enzymes resin with distilled hexane at the end of each test.

It was verified. by the study of temperature influence, by the fact that the molar ratio between the alcohol and oleic acid is the variable with higher significance on esterification with methanol, contrary to esterification with ethanol, and in the study of enzyme reuse, that methanol is more deleterious to the enzyme than ethanol.

The esterification of oleic acid using lipase Novozym 435® is a clean and an easy process in which the catalyst can be easily removed from the final product.

Future work

As future work it would be interesting do a similar study using other enzymes.

Currently underway is a study of the influence of pressure in the reaction using gases such as nitrogen, methane or carbon dioxide to introduce the desired pressure in the cell reactor.

5. REFERENCES

1. **Emily Feket, N.G., et al, *Clima em mudança*, in *National Geographic*. 2007, RBA: Lisbon.**

2. **Saxena, R.C.A., D.K. et al, *Biomass-based energy fuel through biochemical routes: a review.* Renewable sustaintable energy reviews, 2007. 13: p. 167-178.**

3. **Noodermeer, L.P.M.A., *Biomass to fuels, a chemical perspective.* Green Chemistry, 2006. 8: p. 861-867.**

4. **Ohara, H., *Biorefinery.* Appl Micribiol Biotechnol, 2003. 62: p. 474-477.**

5. **A.P. de A. Vieira, M.A.P.d.S., M.A.P. Langone, *Biodiesel Production Via Esterification Reactions catalyzes by lipase.* Latin American Applied Research, 2006. 36: p. 283-288.**

6. **Maris Silvana Aranda Moraes, L.C.K., et al, *Tallow biodiesel:Properties Evaluation and Consumption Tests in a Diesel Engine.* Energy & Fuels, 2008. 22: p. 1949-1954.**

7. **S. Sivaprakasam, C.G.S., *Optimization of the transesterification Process for Biodiesel Production and Use of Biodiesel in a Compression Ignition Engine.* Energy Fuels, 2007. 21: p. 2998-3003.**

8. **Gerhard Knothe, J.V.G., *The biodiesel Handbook*. 2005: AOCS Press.**

9. **Park, Y.M.e.a., *The heterogeneous catalyst system for the continuous conversion of free fatty acids in used vegetable oils for the production of biodiesel.* Catalyst Today, 2008. 131((1-4)): p. 238-243.**

10. **Demirbas, A., *Comparison of transesterification methods for production of biodiesel from vegetable oils and fats.* Energy Conversion and Management 2008. 49: p. 125-130.**

11. **Ranganathan, S.V.N., S.L.; et al, *An overview of enzimatic production of biodiesel.* Bioresource technology, 2007. 99: p. 3975-3981.**

12. **Gerhard Knothe, R.O.D., *Biodiesel:The Use of Vegetable Oils and Their Derivatives as Alternative Diesel Fuels.***

13. **Edgar Loreto, Y.L., Dora E. Lopez, *Synthesis of Biodiesel via Acid Catalysis.* Ind. Eng. Chem. Res., 2005. 44: p. 5353-5363.**

14. **Campos, L.S., *Entender a bioquímica*. third edition ed, ed. E. Editora. 2002, Lisboa: JMM.**

15. **Sivasamy, A.C., Kien Yoo, et al, *Catalytic Applications in the production of biodiesel from vegetable oils.* ChemSusChem, 2009. 2: p. 278-300.**

16. **Demirbas, A., *Biodiesel Production via Rapid Transesterification.* Energy Sources, 2008. 30: p. 1830-1834.**

17. **Divya Bajpai, V.K.T., *Biodiesel:Source, Production, Composition, Properties and Its Benefits.* Journal of Oleo Science, 2006. 55: p. 487-502.**

18. **Benjamas Cheirsilp, A.H.-K., Suchart Limkatanyu, *Impact of transesterification mechanisms on the kinetic modeling of biodiesel production by immobilized lipase.* Biochemical Engineering Journal, 2008. 42: p. 261-269.**

19. **Umer Rashid, F.A., *Production of Biodiesel through Base-Catalyzed Transesterification of Safflower Oil Using an Optimizated protocol.* Energy Biofuels, 2007. 22: p. 1206-1312.**

20. **Pinto, A.C.G., L.L.N. ; et al, *Biodiesel: An overview.* J.Braz. Chem. Soc, 2005. 16, Nº6B: p. 1313-1330.**

21. **Umer Rashid, F.A., *Production of Biodiesel through Base-Catalyzed Transesterification of Safflower Oil Using an Optimizated protocol.* Energy Biofuels, 2008. 22: p. 1306-1312.**

22. **Ulf Schuchardt, R.S., Rogério Matheus Vargas, *Transesterification of vegetable oils.* J. Braz. Chem. Soc, 1998. 9: p. 199.**

23. **Demirbas, A., *Comparison of transesterification methods for production of biodiesel from vegetable oils and fats.* Energy Conversion and Management 2007. 49: p. 125-130.**

24. **Noureddini, H.G., X.; Philkana, R.S., *Immobilized Pseudomonas cepacia lipase for biodiesel fuel production from soybean oil.* Bioresource Technology 2005. 96: p. 769-777.**

25. **Marchetti, J.M.E., A.F., *Comparison of different heterogeneous catalysts and different alcohols for esterification reaction of oleic acid.* Fuel 2008. 87: p. 3477-3480.**

26. **Jegannathan, K.R.A., S. et al, *Production of biodiesel using immobilized lipase- A critical review.* Critical reviews in Biotachnology, 2008. 28: p. 253-264.**

27. **Shah, S.S., S. ; et al, *Biodiesel Preparation by Lipase-Catalysed Transesterification of Jatropha Oil.* Energy & Fuels 2004, 2004. 18: p. 154-159.**

28. **Xu, X., *Biodiesel using bioprocessing technologies: The time is coming?* Eur. J. Lipid. Sci. Technol., 2008. 110: p. 863-864.**

29. **Ranganathan, S.V.N., S.L., *An overview of enzymatic production of biodiesel.* Bioresource Technology, 2008. 99: p. 3975-3981.**

30. **Coelho, M.A.S., A.M. ; et al, ed. *Tecnologia Enzimática*. 2008, EPUF: Rio Janeiro.**

31. ***www.novozymes.com*. [cited 21-10-2009].**

32. **Rodrigues, M.I.I., António Francisco, *Planejamento de Experimentos e Otimização de processos*, ed. C.d.P. Editora. 2005, Campinas. 326.**

33. **Netos, B.B.S., I.S.; Bruns, R.E., *Como fazer experimentos*, ed. E. Unicamp. 2007.**

34. **Martín, E.S.O., C., *Different enzyme requirements for the synthesis of biodiesel:Novozym 435 and Lypozyme TL IM.* Bioresource technology, 2007. 99: p. 277-286.**

35. **Watanabi, Y.P., P; et al, *Production of FAME from acid oil model using Immobilized Candida antartica lipase.* JAOCS, 2005. 82(11): p. 825-831.**

36. **Watanabi, Y.P., P; et al, *Conversion of acid oil by-produced in vegetable oil refining to biodiesel fuel by immobilized Candida antartica lipase.* Journal of Molecular Catalysis, 2006. 44: p. 99-105.**

6. APPENDIX

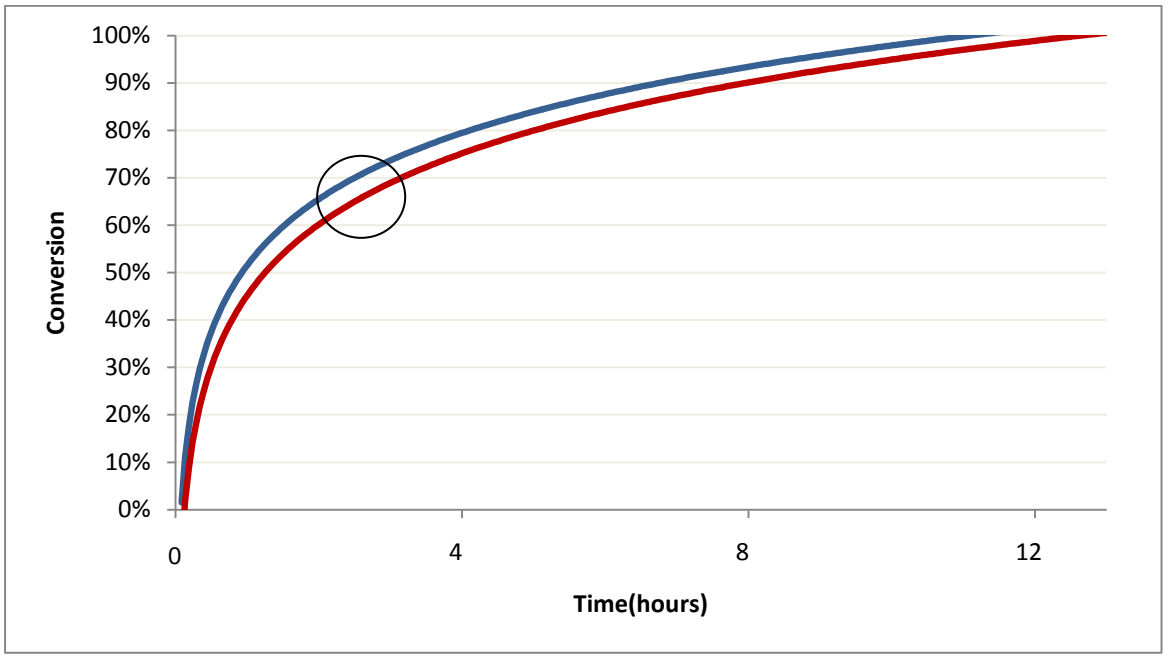


Figure 16: Experimental points for the kinetic study using (♦) methanol or (♦) ethanol

Figure 16 shows the logarithmic trend line for the experimental values of conversion versus time for the esterification with methanol or ethanol.