Optimization, kinetic, and scaling-up of solvent-free lipase-catalyzed synthesis of ethylene glycol oleate emollient ester

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Short title: Solvent-free synthesis of emollient ester

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ABSTRACT

The use of enzymatic catalysts is an alternative to chemical catalysts since they can help to obtain products with less environmental impact, considered sustainable within the concept of green chemistry. The optimization, kinetic, lipase reuse, and scale-up of enzymatic production of ethylene glycol oleate in the batch mode were carried out using the NS 88011 lipase in a solvent-free system. For the optimization step, a 2^3 Central Composite Design was used and the optimized condition for the ethylene glycol oleate production, with conversions above 99%, was at 70 °C, 600 rpm, substrates molar ratio of 1:2, 1 wt% of NS 88011 in 32 hours of reaction. Kinetic tests were also carried out with different amounts of enzyme, and it showed that by decreasing the amount of the enzyme the conversion also decreases. The lipase reuse showed good conversions until the second cycle of use, after which it had a progressive reduction reaching 83% in the 4^{th} cycle of use. The scale-up (9-fold increase) showed promising results, with conversion above 99%, achieving conversions similar to small-scale reactions. Therefore, this work proposed an environmentally safe route to produce an emollient ester using a low-cost biocatalyst in a solvent-free system.

Keywords: Ethylene glycol oleate, NS 88011, Emollient ester, Lipase, Solvent-free.

HIGHLIGHTS

- Ethylene glycol oleate was successfully synthesized through enzymatic route.
- Maximum conversion of 99% was achieved under the selected conditions.
- Scale-up showed promising results, with conversion above 99%, for the use of this process on an industrial scale.

1. INTRODUCTION

The Personal Care, Perfume and Cosmetics (PCPC) market moves billions of dollars worldwide [1]. Global beauty and personal care recorded dynamic 6% value growth in 2018, which was the strongest for over a decade and will continue unabated in 2019 [2].

Brazil is currently the 4th largest PCPC consumer market in the world (6.9% - US$ 30 billion), according to the ABIHPEC [3], surpassed only by United States (18.3% - US$ 89.5 billion), China (12.7% - US$ 62 billion) and Japan (7.7% - US$ 37.5 billion). This information shows that the use of cosmetics, perfumes and personal hygiene is increasingly present in people's daily lives and has been indispensable for basic health care, well-being and quality of life.

The brazilian industry of cosmetics presented an average growth near to 11.4% in the last 20 years, increasing the liquid turnover of sales tax from R$ 4.9 billion in 1996 to R$ 42.6 billion in 2015 [3]. This can be explained by the fact that PCPC is one of the sectors of the industry that invests the most in inquiries to identify the transformations of the society and to develop products that meet the new demands of the society.

The cosmetics and pharmaceutical industries have increased their interest in esters; therefore they have been looking to invest in the use of esters produced through green technologies that attend the appeal of the society for a product considered ecologically correct [4-5]. However, to obtain the esters, the method conventionally used in industrial

processes is the esterification of Fischer, which uses chemical catalysts, such as the sulfuric acid (H_2SO_4). This method uses severe conditions, such as high temperature and pressure, in addition to requiring a final stage of purification of the product for the removal of residual acid, as well as the treatment of the generated residues [6]. The obtained final product can present toxic residues to human beings and environment, and, in this way, is not considered a natural ester [7-8].

Given the need to develop cleaner technologies, biotechnology began to invest in the use of techniques that use living organisms or part of them to produce or even modify products [9]. Enzymes are a set of proteins that have catalytic activity produced by all living organisms, are solid substances that act as non-toxic biological biocatalysts, and are applied to accelerate a chemical reaction and increase the rate of conversion of different substrates into specific products [10].

The use of enzymes as biocatalysts instead of strong acids and bases, for esterification processes, for example, has significant environmental advantages such as minimizing the generation of toxic waste [11]. In this way, the enzymes used to produce esters are an attractive option when compared with the use of chemical catalysts. Because of using mild reaction conditions, low temperature and pressure, as well as eliminating the use of harmful substances and thus producing less toxic waste, it can be considered environmentally friendly. Due to the high specificity of enzymes, the final product obtained is purer and free from unwanted by-products [12].

The ethylene glycol oleate (2-hydroxyethyl(*Z*)-octadec-9-enoato – $C_{20}H_{38}O_3$), molar mass of 326.52 $g.mol^{-1}$, is an ester of monoethylene glycol and oleic acid. It acts as a pearlizing agent, skin conditioner and emulsion stabilizer, and it is a moisturizer widely used in cosmetics, shampoos, bath products, personal cleansers, and other skin and hair products.

The production of esters with emollient and emulsifying properties that are widely used in cosmetic and personal care compositions using alternative synthesis processes, such as enzymatic ones, makes the processes much more attractive not only to industry and to consumers but also to the environment, for being considered sustainable within the concept of green chemistry. In this context, the present work was conceived to obtain ethylene glycol oleate through enzymatic esterification reaction in a solvent-free system, operating in batch mode, and to determine the best processing conditions, as well as evaluating kinetic, lipase reuse, and the scale-up of the process.

2. MATERIALS AND METHODS

2.1 Materials

The substrates used for the esterification reaction were vegetable acid oleic (ALMAD, oils fats, and derivates - Brazil) and the monoethylene glycol (99.5% of purity, Vetec). n-Hexane (99% of purity, Vetec) was used to recover the catalyst. For product quantification, the reagents were: ethanol (99.8% of purity, Vetec), potassium hydroxide (99% of purity, Vetec) and phenolphthalein alcoholic solution (10%). NS 88011 commercial lipase, produced by *Candida antarctica* and immobilized in a hydrophobic polymer resin, used as catalyst, was kindly donated by Novozymes (Brazil/Araucária – PR).

2.2 Optimization of Ethylene Glycol Oleate Synthesis

For the ethylene glycol oleate synthesis, the experimental apparatus proposed by Pereira et al. [13] was used, composed by a 150 mL jacketed reactor with mechanical stirring (IKA RW 20) and with a propeller-shaped stirring rod with four blades (Ø 3 cm). The temperature was controlled by a thermostatic bath (TECNAL TE-184).

The optimization of the ester production was made by a 2^3 Central Composite Design (CCD), evaluating the effects of temperature, agitation and the acid to alcohol molar ratio (Table 1). The variables and process conditions were selected based on previous group studies (Table 2). First, oleic acid and monoethylene glycol were added and, after complete solubilization by mechanical stirring and temperature (2 min), an aliquot of about 1 g of the sample was taken to determine the acidity index (without the presence of lipase), and then lipase was added. The experiments were carried out in duplicate runs, totalizing 22 assays. The time of reaction (24 hours) and concentration of lipase (1% (w/w) of lipase regarding the total mass of the substrate) were established based on the work of Pereira et al. [13]. Furthermore, reactions were carried out without the presence of the lipase. The ester production was accompanied by the reduction of the acidity of the reaction medium (acidity index - AOCS Cd 3d-63) [14].

The statistical analysis was carried out by using the online software Protimiza Experimental Design (http://experimental-design.protimiza.com.br/), considering a significance level of 95% ($p < 0.05$).

2.3 Kinetic Evaluation

The kinetic evaluation was carried out varying the concentration of lipase in the best condition found in the CCD (molar ratio of 1:2 (acid to alcohol), temperature of 70 °C and agitation of 600 rpm), and NS 88011 lipase concentrations of 0.6, 0.8, 0.9 and 1% (w/w total substrate mass) were studied. Samples were taken at predetermined time during 48 hours to evaluate the conversion. All tests were performed in triplicate.

2.4 NS 88011 Lipase Reuse

The NS 88011 lipase reuse was evaluated in the optimized experimental condition previously determined. The reuse of the biocatalyst was performed based on the method described by Lerin et al. [15]. The lipase was separated from the reactional medium through filtration with filter paper under vacuum, followed by 3 washes with n-hexane (20 mL) to remove traces of the previous reaction; then the lipase was oven-dried at 40 °C for 4 h. After the enzyme was kept in a desiccator, using the gel of silica blue as a drying agent, for at least 12 h. After this procedure, the biocatalyst was successively employed in other reactions until a significant conversion decay was observed. In this step, all tests were also performed in triplicate.

2.5 Scale-Up

For scale-up, in the optimized condition of the CCD, the amount of substrate was increased by about 9-fold (35 to 300 g). This increase is extremely important to propose the industrial process. Therefore, the reactor volume went from 150 to 500 mL and a larger propeller-shaped stirring rod (Ø 5 cm) was used to improve the agitation effectiveness. All tests were also performed in triplicate.

2.6 Measurement of Acidity Index

The quantification of the ethylene glycol oleate was performed following the reduction of the acidity index in the reactional medium, indicating acid consumption and, consequently, the formation of the product. The acidity index (or acid number) is defined as the amount of potassium hydroxide needed to neutralize 1 g of sample. This index was determined following the AOCS methodology Cd 3d-63 [14]. First, approximately 1 g of the

sample was diluted in ethanol (20 mL), and 3-4 drops of phenolphthalein were added. This mixture was then titrated with 0.1 M potassium hydroxide (KOH) under vigorous stirring until the colour changed to pink. The acidity index was determined according to Equation 1:

$$AI = \frac{(56.1 * C_{KOH} * V_{KOH})}{m} \qquad (1)$$

Where: *AI* is the acidity index (wt %), *56.1* is the molecular mass of potassium hydroxide (g.mol^{-1}), C_{KOH} is the concentration of KOH solution (mol.L^{-1}), V_{KOH} is KOH solution volume (mL) employed in the titration, and *m* is the sample mass (g).

2.7 Determination of Reaction Conversion

The fatty acid to ester conversion was calculated through the relation between the indexes of final and initial acidity [13], according to Equation 2:

$$C(\%) = \frac{(AI_f - AI_i)}{AI_i} * 100 \qquad (2)$$

Where: *C(%)* is fatty acid to ester conversion in percentage, AI_f is the final acidity index (mg KOH.g^{-1}), and AI_i is the initial acidity index (mg KOH.g^{-1}). The experimental errors were calculated based on the replicates or triplicates of the experiments.

3. RESULTS AND DISCUSSION

3.1 Optimization of Ethylene Glycol Oleate Synthesis

For the synthesis of the ethylene glycol oleate, the optimization of process variables (temperature, agitation, and molar ratio) was performed using a 2^3 CCD. The CCD matrix with coded and real values and responses (conversion of oleic acid to ethylene glycol oleate) for the conditions tested are shown in Table 1. The good reproducibility of the experimental data can be seen in Table 4 by the low pure error ((107/4405.75) x 100 = 2.42%) and that

tends toward zero, which is a consequence of the high reproducibility of the repetitions performed in the central point (run 17 to 22 - Table 1).

With the results of the CCD, the estimated effect for each variable was determined and reported in Table 3 for the synthesis of the ethylene glycol oleate. In the CCD for the three factors (molar ratio, temperature, and agitation) and the interaction between molar ratio and temperature the effects were statistically significant in ethylene glycol oleate production in the range analyzed. Analysis of variance (ANOVA) was performed to verify whether the empirical model can reproduce the experimental data (Table 4). From this analysis, an *F* test was performed, which indicates whether there are significant differences between the means. The results found ($p < 0.05$) in this test were $F_{tabled} = 2.79$ and $F_{calculated} = 56.1$, and the $F_{calculated}$ is 20.1 times higher than F_{tabled}, which means that there are statistically significant differences between the averages. The probability of significance (p-value) was 0.008. It was also possible to verify the percentage of the total variance model, evaluated through the R^2, which showed a value of 95.74%. Through the results obtained, it can be concluded that there is a relationship between the variables and whose analysis of variance showed a good agreement of the model with the experimental data. Furthermore, the R^2 value shows that the adjusted Equation (3) was able to explain the variability of the experimental values:

$$X_{AO} = 67 - 2.75 * RM + 15 * T + 5.13 * AG + 4 * RM * T - 0.37 * RM * AG - 0.62 * T * AG \quad (3)$$

Where: X_{AO} is the conversion of the acid oleic in ethylene glycol oleate, RM is the molar ratio (acid to alcohol), T is the temperature and AG is the agitation.

The obtained statistical model was used to describe the conversion predicted of ethylene glycol oleate (column 6 - Table 1) and allowed the construction of the response surface, which is shown in Fig. 2, 3 and 4.

It is observed in Table 1 that the temperature had a significant positive effect (Fig. 1) on the ester conversion, where the highest temperatures led to high conversions. This can be seen in the runs at 70 °C that led to the highest conversions (run 13 and 16), while the runs with the lowest temperatures (40 °C) led to the lowest conversions (run 4 and 12). Higher reaction temperatures improve the solubility of the substrates and reduce the viscosity, promoting increased collision of substrate molecules with the enzyme, and in this way, it is avoided that there takes place a limitation of transfer of mass [13, 16-18].

As mentioned by Badgujar and Bhanage [19], the substrates molar ratio is an essential factor to determine the influence of the reagents in the enzyme activity and, consequently, in the conversion of the reaction. Therefore, for any catalysis system that has the objective to reach high conversions, without loss of the enzyme activity and waste of reagents, there must be done the study of the molar ratio (acid to alcohol) to favour high conversions of the acid studied [17]. Thus, the significant negative effect of the molar ratio can be observed in Fig. 1, when the highest temperature and agitation were used (run 13 and 16 - Table 1), where good conversions (85 and 92%, respectively) were obtained in all the molar ratios (Fig. 2 and 3). Meantime, the runs with the lowest temperature (40 ºC) and highest agitation (600 rpm) showed that the lower molar ratio positively influenced to obtain good conversions of the ester. On the other side, in run 10, using the lowest molar ratio (1:2) the conversion of 69% was observed, and, for run 11, with a molar ratio of 1:6 a lower conversion (52%) was obtained (Fig. 4). The increase of the molar ratio influenced positively the synthesis because an excess of alcohol favours the reaction towards the synthesis of products, minimizing the

diffusion limitations. However, excessive alcohol levels provoke the inhibition of the enzyme activity [20-22].

The agitation may be a relevant parameter in esterification reactions since it helps the miscibility between the substrates, promotes a homogeneity mixture and can also prevent the enzyme decantation in the reactor [23-24]. A significant positive effect was observed in this study (Fig. 1) where the best conversions were achieved with the agitation of 600 rpm and temperature of 70 °C, regardless of the molar ratio (run 13 to 16 - Table 1). On the other hand, a conversion of 75% was observed in run 7 using 200 rpm of agitation, while in run 16 using 600 rpm the ester conversion was 92%.

CCD results (Table 1) showed that runs 15 and 16 had the highest conversion of fatty acid to ester (~90%) using the highest temperature (70 °C), the molar ratio (1:6) and agitation (600 rpm). However, runs 13 and 14 showed excellent conversion (~85%) using the lowest molar ratio (1:2) and the highest temperature (70 °C) and agitation (600 rpm). As it is known, in industrial processes, product purification processes are a costly and challenging step. So, to facilitate them, one could use stoichiometric concentrations or a small excess of alcohol. Given these disadvantages, for the next steps, the condition of runs 13 and 14 was used. This strategy was also performed by Sá et al. [25], which used the lowest molar ratio in order to overcome the disadvantages related to excess of alcohol in the reaction medium and to maintain the minimum reagent waste that occurs when higher molar ratios are used. Besides, excess of alcohol can lead to enzyme inactivation and, consequently, decreased conversion, making enzyme processes even more expensive. Therefore, esterification studies for different molar ratio are essential to minimize excessive alcohol use and to achieve high conversion values.

3.2 Influence of Enzyme Concentration

It is known that industrial enzymatic processes, but not only, require processes with low production costs. For this, the enzymatic processes must present high yields and the use of minimal amounts of substrates and biocatalysts to achieve the desired results. Thus, the amount of lipase used also affects the economic and industrial viability of the processes [26]. On the other side, an excess of lipase can generate its agglomeration and thus hinder the access of substrates to the enzyme [7, 26-27], negatively interfering with the rate of the substrate. Therefore, the amount of lipase should be controlled to ensure high conversions and low cost [4, 28]. In order to further reduce the amount of lipase used, the reaction kinetics for the effect of the amount of enzyme was evaluated using the condition optimized in the previous step, that is, run 13 (Table 1 - molar ratio of 1:2 (acid to alcohol), 70 °C, 600 rpm) and reaction time of up to 48 hours. The results are shown in Fig. 5.

The reaction catalyzed by NS 88011 showed a decrease in the conversion of oleic acid to ethylene glycol oleate with a reduction in the amount of enzyme used (Fig. 5). This behavior was also observed in other studies evaluating the effect of biocatalyst concentration [29-30]. The esterification reaction catalyzed by the lowest concentration of lipase (0.6% w/w) showed the lowest conversion (70%) of the substrates to ester. Moreover, with the gradual increase in the enzyme concentration of 0.8, 0.9 and 1% (w/w) there was also an increase in the conversion to ester, reaching conversions of 80, 83 and 99% in 48 hours of reaction, respectively. It is also observed that when used 1% of conversion enzyme, close to 99% is achieved in 32 hours of reaction. Similar results were obtained by Holtz et al. [31] to produce cetoestearyl stearate using 1% (w/w) of NS8011, showing 99% conversion in 32 hours of reaction. Nonetheless, for Pereira et al. [13], the amount of lipase (Novozym 435)

above 0.5% (w/w) led to high conversions (~ 99%) of ethylene glycol monostearate in 24 hours of reaction.

The initial rate of the enzymatic esterification reaction kinetics, corresponding to the concentrations of 1, 0.9, 0.8 and 0.6% (w/w) are 0.031, 0.028, 0.010 and 0.004 $mmol.g.min^{-1}$, respectively. These results of the esterification kinetics show that, at the beginning of the reaction, the conversion of oleic acid to ester increases rapidly, with the higher initial rate for the highest enzyme concentrations, as expected, so that the speed of an enzymatic reaction depends positively on the concentration of the catalyst.

These results indicate that it is feasible to use a relatively small amount (1%) of NS 88011 to obtain practically total conversions, thus contributing to cost reduction by facilitating the ester purification step.

3.3 Reuse of the biocatalyst

This step is crucial because it shows how efficient the biocatalyst can be in this reaction system. The higher the reuse of the biocatalyst, the lower the costs and, consequently, the industry has a greater interest in changing from chemical catalysts (strong acids and strong bases) to ecological biocatalysts [13, 31-32].

For evaluation of the reuse, the optimized condition in CCD (run 13 - Table 1) was tested, and the conversion results of the ethylene glycol oleate, along with successive uses of the biocatalyst, are represented in Fig. 6, where it is observed that good conversions were achieved until the second cycle of use. The conversions for the second, third and fourth cycles were 89, 52 and 17%, respectively, what means a loss of 83% during the reuse.

As seen in the second cycle of use, lipase did not lose its catalytic efficiency after the first reaction and subsequent washing with n-hexane. This indicates that after 48 hour reaction, washing with n-hexane and drying in an oven, the enzyme remains bound to the

support and with their functional active sites. However, from the third cycle of use, the conversion and, consequently, the catalytic efficiency dropped abruptly. This fact, which may related to time of exposure of the enzyme to high temperature, the reaction substrates and products or the washing solvent of the enzyme support are inactivating the enzyme, or the enzymes are being removed from the support between cycles, as already described by other authors [13, 29, 32].

3.4 Scale-up

On a laboratory scale, it is possible to provide conditions of homogeneity and adequate heat and mass transfer relatively quickly and efficiently. However, as the scale of production increases, maintaining these conditions also becomes more difficult. During the scheduling of processes, it is expected to obtain yields, reproducibility and product quality equal or better compared to smaller scales. However, what is usually observed is a series of difficulties that accompany the transfer the process to industrial scale. In general, the increase in reactor size is accompanied by increased mixing time, heat and mass transfer caused mainly by the increase in the liquid column. Therefore, scaling-up is significant to direct the way the reaction will be conducted with higher quantities of the substrate, so the challenge is to produce large quantities with high productivity and product quality [33].

The results for scaling-up the glass jacketed reactor and mechanical agitation are shown in Fig. 7. This was performed from a 150 to a 500 mL reactor, with a 9-fold increase in the substrates mass. For this evaluation, there was used the optimized condition in CCD (run 13 - Table 1). It was observed that, even with the scale increased in 9-fold, the behavior of the reaction system remained unchanged and with conversions close to 100% in 32 hours of reaction. Thus, it is highlighted that the use of this solvent-free reaction system is promising to be scaled for industrial processes.

4. CONCLUSIONS

It was possible to produce ethylene glycol oleate from oleic acid and ethylene glycol in a solvent-free system with conversions of 99% using a commercial immobilized lipase from *Candida antarctica* (NS 88011) as biocatalyst. Temperature and agitation proved to be the fundamental variables for achieving high conversions. The best conversion was found in the condition of 1% (w/w) NS 88011 lipase, agitation of 600 rpm, the molar ratio of 1:2 (acid to alcohol) and temperature of 70 °C in 32 hours of reaction. The results obtained for the scale-up are considered promising and indicate that the difficulties related to the homogeneity of heat and mass transfer have been overcome. Also, this scale-up methodology can serve as a basis for other scale-up studies for the enzymatic production of bioproducts. The solvent-free esterification system has the advantage of producing emollient esters suitable for cosmetic applications. Considering the global consumption of Personal Care, Perfume and Cosmetics (PCPC), this work proposed an environmentally safe route for the production of an emollient ester using a biocatalyst in a solvent-free system with high industrial applicability.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1 - Matrix of the 2^3 Central Composite Design (CCD) experiment (coded and real values) used for the optimization of ethylene glycol oleate production in 24 hours of reaction using 1% (w/w) NS 88011 lipase.

Run	RM[1] (X_1)	Temperature (X_2) (°C)	Agitation (X_3) (rpm)	Experimental conversion (%)	Predicted conversion (%)[2]	Relative error (%)[3]
1	-1 (1:2)	-1 (40)	-1 (200)	48	52	-9.67
2	-1 (1:2)	-1 (40)	-1 (200)	55	52	4.29
3	1 (1:6)	-1 (40)	-1 (200)	44	39	9.36
4	1 (1:6)	-1 (40)	-1 (200)	41	39	2.73
5	-1 (1:2)	1 (70)	-1 (200)	78	75	2.72
6	-1 (1:2)	1 (70)	-1 (200)	79	75	3.95
7	1 (1:6)	1 (70)	-1 (200)	75	79	-5.49
8	1 (1:6)	1 (70)	-1 (200)	81	79	2.32
9	-1 (1:2)	-1 (40)	1 (600)	66	64	1.73
10	-1 (1:2)	-1 (40)	1 (600)	69	64	6.00
11	1 (1:6)	-1 (40)	1 (600)	52	50	2.65
12	1 (1:6)	-1 (40)	1 (600)	47	50	-7.70
13	-1 (1:2)	1 (70)	1 (600)	85	85	-0.73
14	-1 (1:2)	1 (70)	1 (600)	84	85	-1.93
15	1 (1:6)	1 (70)	1 (600)	88	87	0.70
16	1 (1:6)	1 (70)	1 (600)	92	87	5.02
17	0 (1:4)	0 (55)	0 (400)	68	67	1.47
18	0 (1:4)	0 (55)	0 (400)	66	67	-1.52
19	0 (1:4)	0 (55)	0 (400)	65	67	-3.08
20	0 (1:4)	0 (55)	0 (400)	63	67	-6.35
21	0 (1:4)	0 (55)	0 (400)	67	67	0.00
22	0 (1:4)	0 (55)	0 (400)	61	67	-9.84

[1] RM = acid to alcohol molar ratio.

[2] Calculated according to Equation 3.

[3] Relative error - $RE = \left(\frac{Exp.Conv.-Predict.Conv.}{Exp.Conv.}\right) x100$.

Table 2 – Summary of variables and process conditions from previous studies.

Biocatalyst	Lipase concentration (%)	Temperature (°C)	Agitation (rpm)	MR*	Ester	Ref.
Novozym 435	0.1 - 2	65 - 70	600 - 1000	1:1	Ethylene glycol monostearate	[13]
	1	61.8 - 78.4	250.8 - 889.2	1:0.8 - 1:1.6	Butyl and Ethyl stearate	[29]
NS 88011	0.5 - 2	60	150 - 250	1:1 - 1:7	Cetostearyl stearate	[28]
	0.6 - 1	40 - 70	200 - 600	1:2 - 1:6	Ethylene glycol oleate	This work

* RM = acid to alcohol molar ratio.

Table 3 – Estimated effects for the 2^3 CCD for the optimization of ethylene glycol oleate production.

Factor	**Effect**	**Standard Error**	**Calculate *t***	***p-value***
Mean	67	0.771	86.881	$1.08\ e^{-21}$
Molar ratio	-2.75	0.904	-3.041	0.008
Temperature	15	0.904	16.587	$4.65\ e^{-11}$
Agitation	5.125	0.904	5.667	0.00004
Molar ratio x Temperature	4	0.904	4.423	0.0005
Molar ratio x Agitation	-0.375	0.904	-0.414	0.684
Temperature x Agitation	-0.625	0.904	-0.691	0.500

Table 4 - Analysis of variance (ANOVA) of the estimated model for the optimization of ethylene glycol oleate production.[1]

Source of variation	Sum of squares	Degrees of freedom	Mean squares	$F_{calculated}$
Regression	4405.75	6	734.29	56.12*
Residual	196.25	15	13.08	
Lack of Fit	89.25	2	44.62	5.42*
Pure Error	107	13	8.23	
Total	4602	21		

[1] Coefficient of determination: $R^2 = 0.9574$; $F_{0.95-5;15} = 2.79$; $F_{0.95-2;13} = 3.80$.

*$p < 0.05$.

FIGURES CAPTIONS

Fig. 1 – Pareto chart of the effects of the independent studied variables for the synthesis of ethylene glycol oleate ($p < 0.05$). Experimental data and conditions are shown in Table 1.

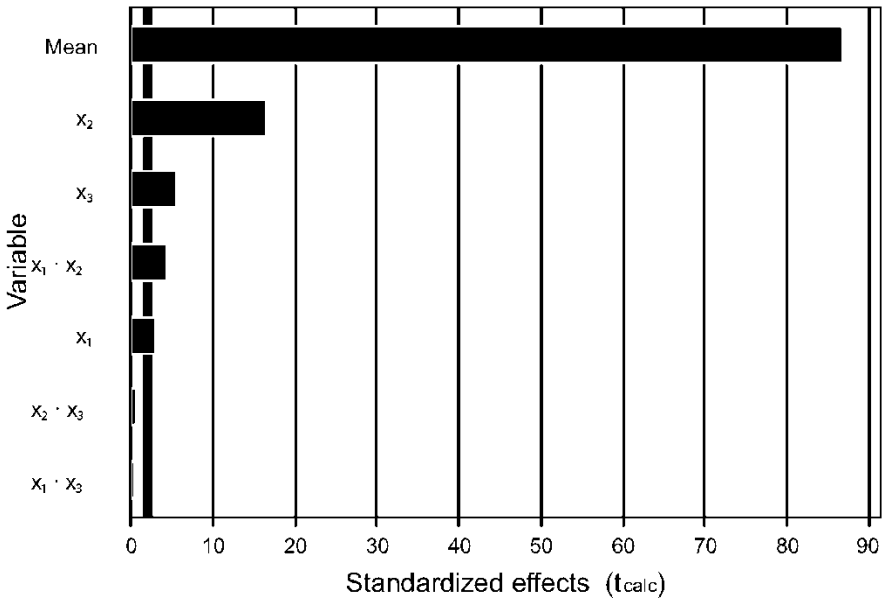

Fig. 2 - Response surface for the synthesis of ethylene glycol oleate in the function of agitation and temperature. Experimental conditions were described in Table 1. The lighter area represents the lowest conversions (<50%) in esters, while the darker area represents the highest conversions (>85%) in esters.

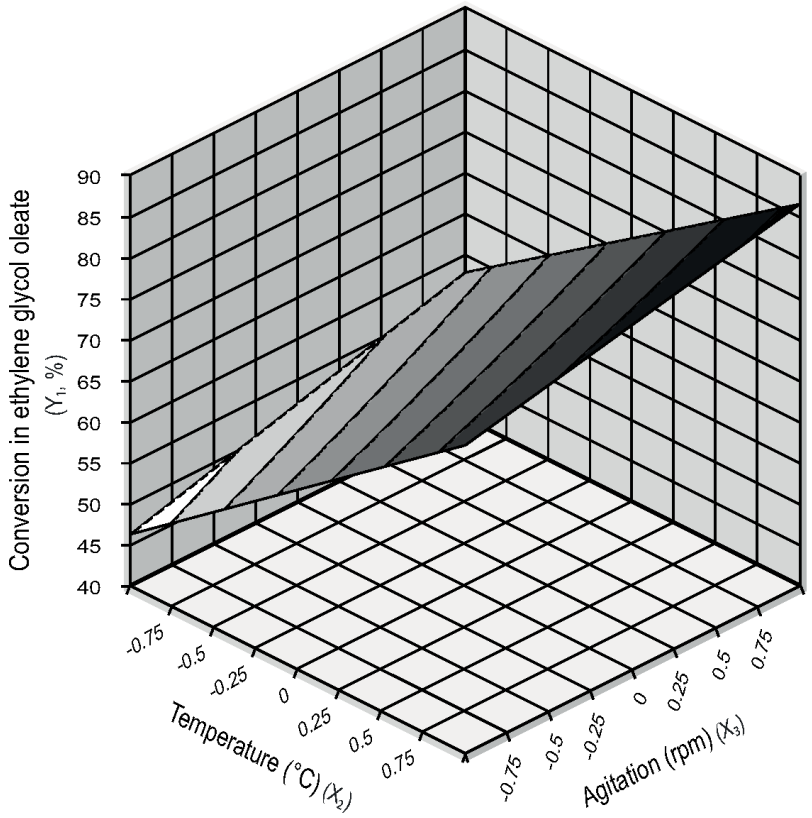

Fig. 3 - Response surface for the synthesis of ethylene glycol oleate in the function of agitation and molar ratio (acid to alcohol). Experimental conditions were described in Table 1. The lighter area represents the lowest conversions (<60%) in esters, while the darker area represents the highest conversions (>75%) in esters.

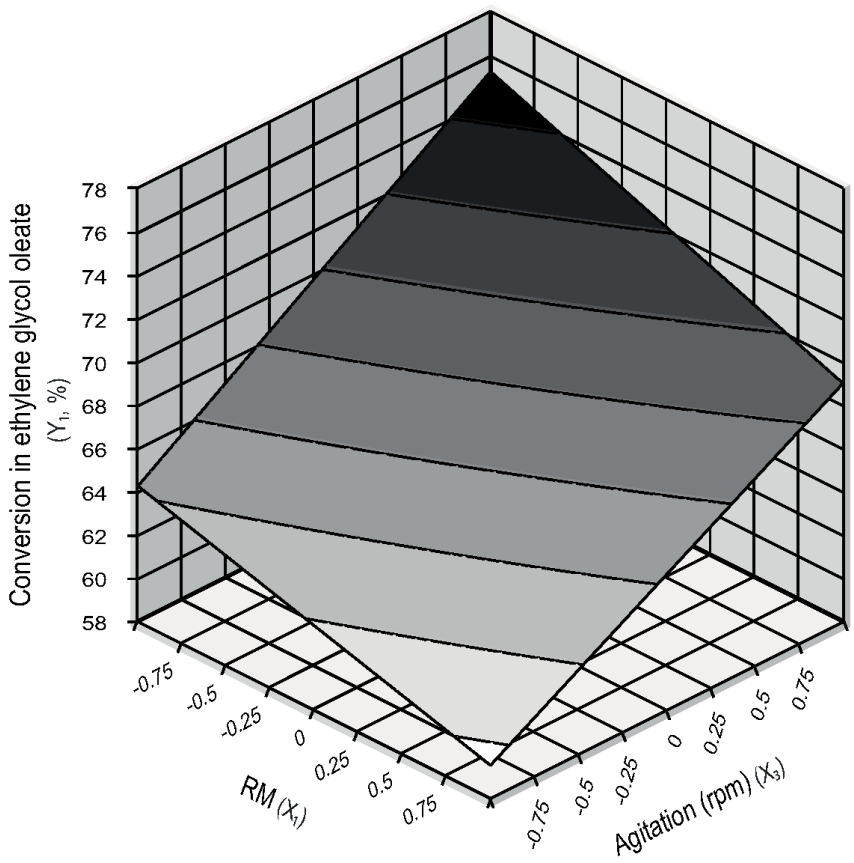

Fig. 4 - Response surface for the synthesis of ethylene glycol oleate in the function of temperature and molar ratio (acid to alcohol). Experimental conditions were described in Table 1. The lighter area represents the lowest conversions (<50%) in esters, while the darker area represents the highest conversions (>80%) in esters.

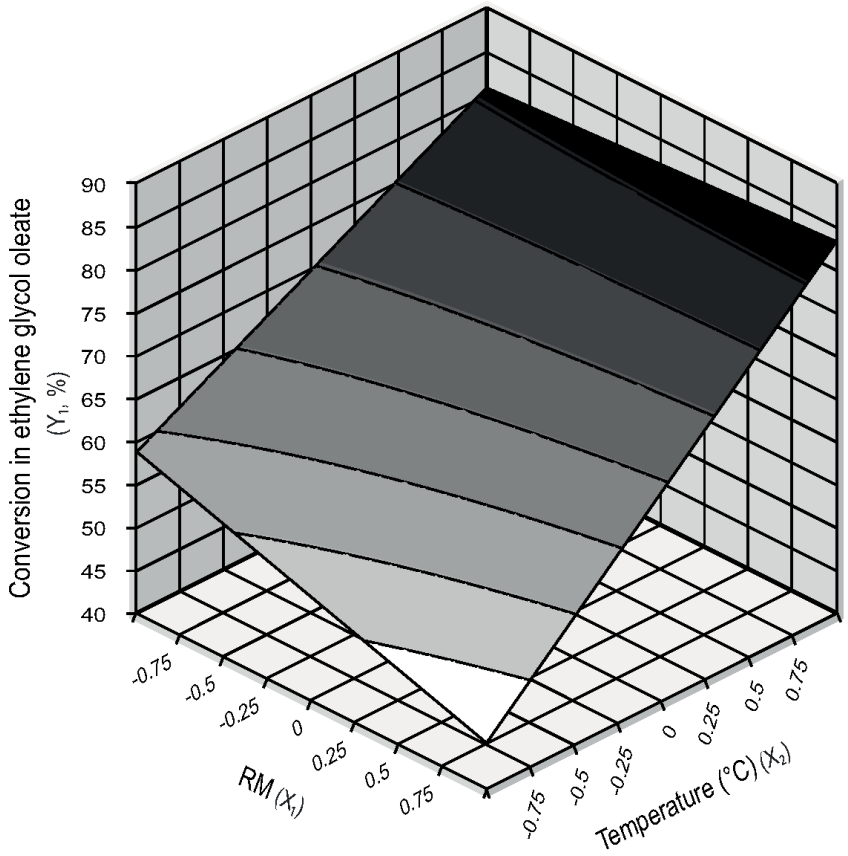

Fig. 5 - Kinetics of conversion of oleic acid to the esterification reaction at various concentrations of lipase NS 88011 (% w/w). Reaction conditions maintained fixed: 70 °C, 600 rpm, molar ration 1:2. The standard deviation was ≤0.5% for all the assays.

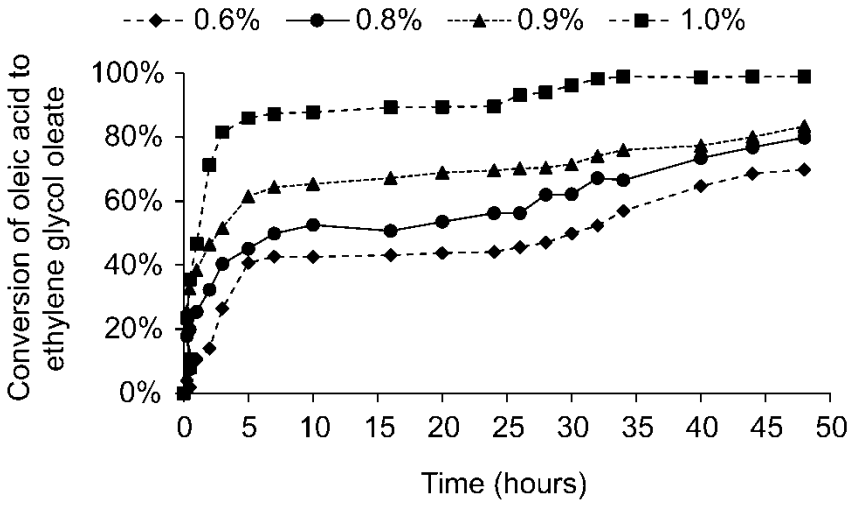

Fig. 6 - Reuse cycle of NS 88011 lipase for conversion in ethylene glycol oleate. Reaction conditions: temperature of 70 °C, agitation of 600 rpm, the molar ratio of 1:2, 1% (w/w) of NS 88011 lipase and 48 hours of reaction.

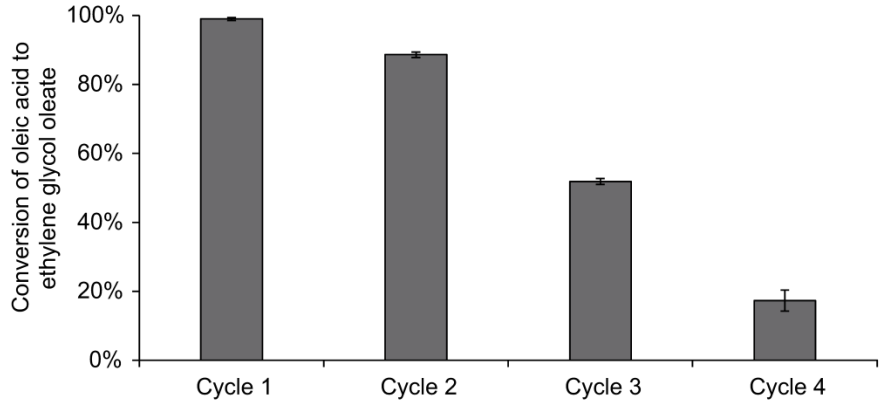

Fig. 7 - Conversion in ethylene glycol oleate in the scale-up. Reaction conditions: temperature of 70 °C, agitation of 600 rpm, the molar ratio of 1:2 and 1% (w/w) of NS88011 lipase. The standard deviation was ≤0.5% for all the assays.