Accepted Manuscript

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Please cite this article as: Sánchez, Daniel Alberto, Tonetto, Gabriela
Marta, Ferreira, María Luján, An insight on acyl migration in solvent-free
ethanolysis of model triglycerides using Novozym 435.Journal of Biotechnology
http://dx.doi.org/10.1016/j.jbiotec.2016.01.001

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An insight on acyl migration in solvent-free ethanolysis of model triglycerides using Novozym 435

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An insight on acyl migration in solvent-free ethanolysis of model triglycerides using Novozym 435

Highlights:

- Ethanolysis of S, M and LCFA triglycerides (TG) catalyzed by Novozym 435
- Focusing on the secondary reaction of acyl migration
- 2-monoglyceride was favored by long reaction times and large biocatalyst loading with saturated S and MCFA TG
- Acyl migration increased the yield of ethyl esters and minimized the content of 2-monoglyceride.
- Conversion of acylglycerides with unsaturated LCFA was low due to limitations in their access to the active site of the lipase

ABSTRACT

In this work, the ethanolysis of triglycerides catalyzed by immobilized lipase was studied, focusing on the secondary reaction of acyl migration. The catalytic tests were performed in a solvent-free reaction medium using Novozym 435 as biocatalyst.

The selected experimental variables were biocatalyst loading (5-20 mg), reaction time (30-90 min), and chain length of the fatty acids in triglycerides with and without unsaturation (short (triacetin), medium (tricaprylin) and long (tripalmitin/triolein)).

The formation of 2-monoglyceride by ethanolysis of triglycerides was favored by long reaction times and large biocatalyst loading with saturated short- to medium-chain triglycerides. In the case of long-chain triglycerides, the formation of this monoglyceride was widely limited by acyl migration.

In turn, acyl migration increased the yield of ethyl esters and minimized the content of monoglycerides and diglycerides. Thus, the enzymatic synthesis of biodiesel was favored by long-chain triglycerides (which favor the acyl migration), long reaction times and large biocatalyst loading.

The conversion of acylglycerides made from long-chain fatty acids with unsaturation was relatively low due to limitations in their access to the active site of the lipase.

Abbreviations

1,2-DAG	1,2-diacylglyceride (s)
1,3-DAG	1,3-diacylglyceride (s)
1-MAG	1-monoacylglyceride (s)
2,3-DAG	2,3-diacylglyceride (s)
2-MAG	2-monoacylgliceride (s)
3-MAG	3-monoacylglyceride (s)
AM	Acyl Migration
CALB	*Candida antarctica* lipase B

ED1   Experimental Design 1

ED2   Experimental Design 2

FAEE   Fatty acid ethyl ester (s)

FID   Flame ionization detector

GGG   Glycerol

GPG   2-Monopalmitoylglycerol

IS   Internal calibration standards

MM2   Molecular Mechanics v.2

MSTFA   N-methyl-N-(trimethylsilyl) trifluoroacetamide

PAEE   Palmitic acid ethyl ester

PGG   1-Monopalmitoylglycerol

PGP   1,3-Dipalmitoylglycerol

PPG   2,3-Dipalmitoylglycerol

PPP   Tripalmitoylglicerol (Tripalmitin)

TAG   Triacylglyceride (s)

X_{TAG} Triacylglyceride conversion

Key words: ethanolysis, triglycerides, acyl migration, Novozym 435

1. INTRODUCTION

The ethanolysis reactions of triglycerides (TAG) catalyzed by enzymes have been widely studied in the last few years for the production of biodiesel using a short-chain alcohol that can be obtained without resorting to fossil fuels [1-6], for the synthesis of acyl glycerides (specific mono- and diglycerides) [7-9], or for the synthesis of ethyl esters used in different industrial applications [10].

However, acyl migration (AM), a secondary reaction that occurs during the ethanolysis of TAG, has not been given due consideration. This reaction is responsible for the generation of undesired isomers during the synthesis of acyl glycerides [11-12]. But in turn, it is a desired reaction during the production of biodiesel, because most of the lipases used in this synthesis act at a specific position on the glycerol backbone [13-15]. If not for acyl migration, biodiesel yields would not exceed 66% and they would be high in monoglycerides.

In this work, important factors affecting the acyl migration reaction during the ethanolysis of TAG were studied. Reaction time, chain length of the saturated fatty acid in TAG and the biocatalyst loading were analyzed. The effect of a fatty acid chain with unsaturation was also studied. Understanding how these factors influence acyl migration would help to minimize or maximize that migration according to the product of interest.

2. EXPERIMENTAL

2.1. Materials

Novozym 435, which is a commercial form of *Candida antarctica* lipase B (CALB) immobilized on acrylic resin, was kindly provided by Novo Nordisk A/S (Brazil). Ethyl decanoate and ethyl palmitate were purchased from SAFC. Tripalmitin and silylation reagents were obtained from Fluka. Monoolein, monocaprin, dipalmitin, glyceryl tridecanoate, glyceryl trioleate and glyceryl trioctanoate were supplied by Sigma-Aldrich. Absolute ethanol was provided by Dorwil, and pyridine was purchased from Anedra S.A. All products used were of analytical grade.

2.2. Ethanolysis reaction catalyzed by lipase

The ethanolysis reaction was performed in 10 mL flasks, which were kept in a thermostatic bath with temperature control and magnetic stirring. The reaction was carried out as follows: 100 mg of triglyceride were mixed with 2 mL of absolute ethanol. When the reactant mixture reached the selected temperature, the reaction was started by adding the amount of enzyme to be studied in each test.

The values of reaction time, chain length of the fatty acid of TAG with and without unsaturation, and loading of immobilized lipase were established according to the experimental designs explained below.

2.3. Box-Behnken experimental designs

2.3.1. Experimental Design 1 (ED1)

In this study, a three-level design including a subgroup of runs of a full 3-level factorial design was developed, with a total of 15 experiments. The variables analyzed were reaction time, immobilized lipase loading and chain length of the saturated fatty acid of the triglyceride. The levels studied for these variables are presented in Table 1. As shown in Table 2, the studied responses were triglyceride conversion (X_{TAG}) and mole fraction of 1,2-diglyceride (1,2-DAG), 1,3-diglyceride (1,3-DAG), 2-monoglyceride (2-MAG), 1-monoglyceride (1-MAG), fatty acid ethyl ester (FAEE) and glycerol (GGG). The order of the experiments was fully randomized to provide protection against the effects of lurking variables.

Insert Table 1

Insert Table 2

2.3.2. Experimental Design 2 (ED2)

This design is similar to ED1, but in this case the long-chain TAG is changed (for triolein) to study the effect of the unsaturation on the reaction of interest. The variables and their levels are presented in Table 3. The 15 experiments carried out for ED2 are shown in Table 4.

Insert Table 3

Insert Table 4

2.4. Statistical analysis

The complete statistical analysis was performed using the software STATGRAPHICS Centurion version XV.2. The responses were fitted by multiple regression, and the models generated were used to evaluate the effect of the selected experimental factors. The goodness of fit was assessed using the coefficient of determination (R^2). The statistically significant effect of the variables was tested using ANOVA. Non-significant coefficients were removed from the models (p-value > 0.05).

2.5. Gas chromatography analysis

Samples were diluted with pyridine and siliylated with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). The analysis of the samples was performed in a PerkinElmer AutoSystem XL gas chromatograph equipped with on-column injection, a flame ionization detector (FID) and a high temperature capillary column ZB-5HT Inferno (15 m x 0.32 mm, with an ID of 0.10 μm), using H_2 as carrier gas. The temperature detector was maintained at 380 ºC. The initial column temperature was maintained at 50 ºC for 1 min, increased to180 ºC at a rate of 15 ºC/min, then increased to 230 ºC at 7 ºC/min, further increased to 370 ºC at 10 ºC/min, and finally maintained there for 5 min. Results are the mean of two injections with an average relative error lower than 2%. The determination of elution times of reactants and products was performed with high purity standards.

3. THEORETICAL METHOD AND APPROACH

Molecular Mechanics v.2 (MM2) as implemented in Chem3D Ultra 5.0 (by Cambridge Soft) was used to obtain the conformational steric minima for each initial configuration explored. For

better visualization, ChemBioOffice 2008 was used. The configuration included the CALB structure, with the alcohol and fatty acid pockets following Gudiño et al [16]. Besides, the full structures of tripalmitin, tricaprylin, triacetin and triolein were modeled. Three different conformations for each triglyceride were obtained: at the CALB active site entrance (at the surface of the enzyme), at the "middle" in the road to catalytic serine (near the walls of the catalytic triad), and near the serine group of CALB. After the minimization step, a PM3 (parameterized model 3) calculation was performed to obtain the formation enthalpy (ΔH°_f) for each conformational minimum for the triglyceride *only*. This approach was selected to analyze the structural changes of the triglycerides due to the interaction with the lipase at different points of the road to the catalytic triad.

The reported ΔH°_f for the triglycerides were obtained as follows:

$$\Delta H^{\circ}{}_{f} = \Delta H^{\circ}{}_{\text{conformation of TAG}}$$

4. RESULTS AND DISCUSSION

4.1. Identification of the products

The theoretical products of the enzymatic ethanolysis of TAG catalyzed by Novozym 435 were 1,2-diglycerides (1,2-DAG), 2,3-diglycerides (2,3-DAG), 2-monoglycerides (2-MAG) and fatty acid ethyl esters (FAEE). Acyl migration enables the production of another diglyceride isomer (1,3-diglycerides (1,3-DAG)) and two isomers of monoglycerides: 1-monoglycerides (1-MAG) and 3-monoglycerides (3-MAG).

The capillary column used in this study allows differentiation between positional isomers, and their identification was carried out based on the work reported by Bruschweiler and Dieffenbacher [17], who proposed a method to determine mono- and diglycerides by gas chromatography.

A typical chromatogram of the ethanolysis of tripalmitin, showing the reaction products and unconsumed reactants, is presented in Supplementary information.

4.2. Acyl migration reaction

Candida antarctica lipase B has been reported as a sn-1,3 specific lipase in alcoholysis reactions [18-23], so that for the ethanolysis of TAG it could be expected to obtain 1,2- and 2,3-diglycerides and 2-monoglycerides. The positional specificity of lipases to attack the sn-1 and sn-3 positions is given by steric restrictions that block the access of the fatty acid esterified at the sn-2 position to the active site of CALB [24]. However, 1,3-DAG, 1-MAG and even glycerol were obtained. As mentioned above, the formation of these glycerides and glycerol can be explained in terms of the acyl migration reaction. The migration observed in this work occurred mainly from sn-2 to sn-3 position, and to a lesser extent from sn-2 to sn-1. This reaction has been considered thermodynamically favored [25].

A mechanism for the ethanolysis of TAG catalyzed by Novozym 435 is shown in Figure 1. The gray arrow indicates the reaction path without acyl migration.

Insert Figure 1

4.3. Importance of the analysis of the samples

Since the migration of acyls from sn-2 to sn-1 or sn-3 position is a spontaneous reaction, the samples must be analyzed in the shortest time possible. Acyl migration has been observed in samples stored at -20 °C.

Two overlapping chromatograms obtained for the reaction samples of the ethanolysis of tricaprylin catalyzed with 20 mg of Novozym 435 for 90 min are presented in Supplementary Information. Plotted in black are the results of the chromatographic analysis of the sample after the reaction ended, and plotted in red are the results of the analysis of the same sample after storage for 48 h at -20 °C. As it can be observed, acyl migration can occur after the reaction ended even at considerably low temperatures. That is why the samples must be analyzed in the shortest time possible to avoid that acyl migration occurs during storage of the samples.

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4.4. Model fitting

The model results obtained for each response variable are analyzed in detail in the following subsections. The factor settings and measured responses are given in Table 1 to 4.A detailed analysis using second order models is used to adjust the responses. Equation 1 was used to adjust all the responses:

$$Response = A_0 + A_1 L + A_2 T + A_3 C + A_4 LT + A_5 LC + A_6 TC + A_7 L^2 + A_8 T^2 + A_9 C^2 \quad (1)$$

where L is the mass of the lipase, T is the reaction time, and C is the type of triglyceride with different chain length.

This equation was refined by a decreasing selection of variables, starting with a model that involved all the variables specified and then removing one variable at a time based on its statistical significance in the model. In each stage, the algorithm removed from the model the variable that was statistically less important. The removal of the variables was based on an evaluation of the F-value. If the less significant variable had an F value that was lower than that specified in the analysis, it was removed from the model. When all the remaining variables had a large F value, the procedure stopped.

Table 5 includes all the equations obtained after multiple regression using the Statgraphics Centurion XV.2 (Equations 2 to 15), the description of the response, the equation numbers and the values of the percentage of the variation in the parameter explained using the equation.

Insert Table 5

4.4.1. Triglyceride conversion

Triacetin shows the highest conversion at 60 minutes with 20 mg Novozym 435 (43.28%, Table 2), whereas with tripalmitin the conversion achieves 29.7%. Only 15.18% triolein is converted using the same reaction conditions.

Eq. 2 describes the behavior of TAG conversion when the ethanolysis was carried out considering only saturated TAG (ED1).Both the increase in biocatalyst loading and reaction

time favored the increase in TAG conversion, but the increase in chain length of the fatty acids had a negative effect. Eq. 3 was used to adjust TAG conversion for ED2. The effect of biocatalyst content and chain length on TAG conversion for ED1 (a) and ED2 (b) is presented in the Supplementary Material

4.4.2. Mole fractions of diacylglycerides

CALB has been reported as being sn-1,3-specific [18-22]; however ,when the ethanolysis of TAG was performed at short reaction times, a marked selectivity toward sn-3 position could be observed. The alcoholysis at the sn-1 position was considerably slower and this situation became more significant when the chain length of the acids constituting the TAG increased or with unsaturation in the fatty acid.

The results are in agreement with reported data on the preference of CALB for short-chain fatty acids [26]. Main results of the ethanolysis of tripalmitin are shown in Table 2: absence of 2,3-dipalmitin (GPP) and sn-3 as the preferentially attacked position (The absence of 2,3-dipalmitin shown in a comparative chromatogram presented in Figure 11 of the Supplementary Material).

4.4.2.1. Mole fraction of 1,2-diglyceride

When analyzing ED1, the mole fraction of 1,2-diglycerides was increased by the increase in reaction time and Novozym 435 loading. The increase in chain length of saturated TAG had a significant negative effect on the fraction of this diacylglyceride.

Eq. 4 was used to adjust the fraction of 1,2-DAG with ED1. The molar fraction in products of 1,2 diglyceride was from near 27% to 5.5% with TAG conversion higher than 5 %.

The presence of unsaturation increased the negative effect of the increase in chain length on the 1,2-diacylglycerol fraction.

Eq. 5 was used to describe the relationship between the 1,2-diglyceride fraction and the presence of unsaturation (ED2).

Two response surface plots showing the variation of the 1,2-diacylglyceride mole fraction are presented in Supplementary Material, for a)ED1 and in case b)for ED2.

4.4.2.2. Mole fraction of 1,3-diglyceride

Long-chain fatty acids present in diglycerides favored acyl migration, because the formation of 1,3 DAG is only explained in terms of this reaction from 1,2 DAG. Apparently the ethanolysis at the sn-1 position (when esterified with LCFA) is complex and this situation favored acyl migration from sn-2 to the sn-3 position.

The increase in biocatalyst loading had a positive effect on this response [27]. Increasing the reaction time had a negative effect on the 1,3-diglyceride fraction. The rate of the ethanolysis at sn-3 position was faster than the migration, and thus the 1,3-diacylglyceride was converted to 1-monoglyceride.

Eq. 6 and 7 were used to adjust the 1,3-diglyceride fraction for ED1 and ED2, respectively. The molar fraction in products was below 1% for both cases.

Even thought the behavior was similar in ED1 and ED2 a considerably poorer adjustment can be observed for the latter.

4.4.3. Mole fractions of monoacylglycerides

This monoglyceride is an important precursor for the synthesis of structured TAG. It is assumed that the 2MAG is generated by the alcoholysis of TAG with 1,3-specific lipases [28].

The mole fraction of 2-MAG varied from near 0 to 8.5% whereas mole fraction of 1-MAG was lower than 0.7 % for ED1 and lower than 0.3% for ED2.

4.4.3.1. Mole fraction of 2-monoglyceride

The main factors affecting the 2-monoglyceride fraction were biocatalyst loading and reaction time. Both variables had a positive effect on this response. The increase in chain length of TAG had a slightly negative impact on this fraction (as can be seen in Eq. 8). Acyl migration takes place, either in the diglyceride from 1,2-DAG to 1,3-DAG and then generating 1-monoglyceride by ethanolysis, or directly in monoglycerides by converting 2-MAG to 1-MAG.

The presence of unsaturation in the long-chain TAG (ED2) increased the negative effect of the increase in chain length.

Eq. 8 and 9 describe the relationship between the mole fraction of 2-MAG and the variables studied for ED1 and ED2, respectively. The variation of this fraction with respect to biocatalyst loading and chain length of TAG for both experimental designs is presented in Figure 2

Insert Figure 2

4.4.3.2. Mole fraction of 1-monoglyceride

The formation of 1-MAG can be explained in terms of the acyl migration reaction. 1-MAG can be obtained by acyl migration from 1,2-DAG to 1,3-DAG and subsequent ethanolysis at the sn-3 position, or by acyl migration from sn-2 to the sn-1 position in 2-MAG. Increase in biocatalyst loading and in chain length fatty acid had a positive effect on the formation of 1-MAG. The presence of unsaturation limited the access of the substrate to the active site of the lipase. Eq. 10 describes the relationship between the mole fraction of 1-MAG and the variables studied for ED1.

Eq. 11 describes the relationship between the mole fraction of 1-MAG and the variables studied for ED2.

4.4.4. Mole fraction of glycerol

The formation of glycerol during the ethanolysis of TAG catalyzed by Novozym 435 was a result of the acyl migration reaction. To detect this polyalcohol, it is necessary to sample the reaction with care and perform an adequate analysis of those samples.

The increase in reaction time, biocatalyst loading and chain length of TAG favored the formation of glycerol. The three variables favored acyl migration when only saturated TAG were considered. Eq. 12 was used to model the mole fraction of glycerol for ED1 with molar fractions for glycerol from 0 to 1.4 %.

The effect of reaction time and biocatalyst loading favored the formation of GGG in ED2. However, the presence of unsaturation had a negative effect on this response. Probably the

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access of acylglycerides formed by unsaturated fatty acids to the active site of the lipase was restricted, limiting the progress of the reactions.

Eq. 13 represents the relationship between the mole fraction of glycerol and the variables studied for ED2 with molar fraction in products for glycerol from 0 to 1.1 %.

4.4.5. Mole fraction of ethyl ester

The generation of fatty acid ethyl esters (FAEE) was favored by the increase in reaction time and biocatalyst loading, considering ED1 or ED2.

The increase in the chain length of TAG had a negative effect on the fraction of ethyl esters en more when the long-chain fatty acids of the TAG presented an unsaturation. Apparently the access of these acids to the active site of the lipase is limited. Eq. 14 and 15 describe the relationship between the fraction of FAEE and the variables studied for ED1 and ED2, respectively.

Two response surface plots for the FAEE fraction are presented in Supplementary Material.

4.5. Multi-response analysis using desirability functions

In the previous sections, the effects of experimental variables on different responses when performing the enzymatic ethanolysis of triglycerides were evaluated. Second order regressions were developed for each response, and optimal conditions were obtained for the responses. In this section, responses are analyzed with the objective of obtaining a global optimum. However, when considering all the responses at the same time, it is unlikely to achieve optimality in the same place, especially when some of the responses should be maximized and others minimized. For this analysis, the approach based on a utility function called "desirability function" was used. The desirability function was first introduced by Harrington [29], who suggested the calculation of desirability values associated with each outcome of an experiment.

In this work, we tried to develop a model that maximizes TAG conversion, the FAEE fraction and the mole fraction of glycerol (focused on the enzymatic synthesis of biodiesel), and

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also a model that maximizes TAG conversion and the 2-MAG fraction and that minimizes the mole fraction of glycerol and 1-MAG (focused on the synthesis of 2-MAG minimizing the acyl migration).

The plots of the estimated surface of the desirability function for the maximization of the synthesis of ethyl esters are presented in the Supplementary Material. As it can be observed, the highest values of the desirability function were obtained when the synthesis was carried out with long-chain TAG and high biocatalyst loading.

The conversion of TAG composed of long-chain fatty acids with unsaturation was low due to restrictions to access the active site of the lipase. The desirability function exhibited the highest values when reaction time and biocatalyst loading were at the maximum levels analyzed, but when TAG had a short or medium chain length.

The plots of the estimated response surface for the maximization of the 2-MAG fraction and minimization of acyl migration are included in Supplementary MAterial. In these graphs only saturated TAG were considered (ED1), because the presence of unsaturation widely limits TAG conversion and the 2-MAG fraction.

The desirability function, when maximizing the 2-MAG fraction, reached the highest values when the biocatalyst loading and reaction time were at the maximum levels tested in this work and with short- and medium-chain TAG (See Supplementary Material).

4.6. Results of Theoretical model

Table 6 shows the results of the three different conformations for each triglyceride: at the CALB active site entrance (position A), at the "middle" in the road to catalytic serine (position B), and near the serine group of the catalytic triad of CALB (position C).

Insert Table 6

The minimization steps produced structures that clearly are not really different, except than they are "compacted", through a process like the one that takes place in a bellows for the

saturated TAG. There are 10 kcal/mol of the difference for the triolein outside CALB and inside CALB and near 3 Å to the serine (but the structure is located near the active site of serine and the hydrocarbon chains are placed inside the CALB structure). The interaction of the double bonds of the hydrocarbon chains of triolein with the walls of the CALB is strong when the triolein is introducing in the active site of CALB. The main point here is how the triolein reaches serine from CALB, because the movement is severely hindered.

This simple theoretical study gave us clues about the potential explanation for two main facts that emerged from the data:

a) the lower activity of the CALB in Novozym 435 for triolein (even with similar selectivity than to other TAG).

It is clear from the experimental design results that selectivity is similar but activity is much lower for triolein than for the other studied triglycerides. The supposed causes of the difference were the steric and electronic characteristics of the triolein versus the saturated TAG. Figure 3 shows the different conformations found for the tripalmitin at the lipase surface in the road to the catalytic site (position A) and near serine (position C), and triolein at the lipase surface (position A) and located at the walls of the pockets of the active site of CALB (position B). The shown structures were obtained after steric minimization of the CALB active site and neighborhood plus the coordinated TAG near the entrance to the active site (structures A and B) or near the serine (structure C).

Insert Figure 3

b) the sn-3 stereoselectivity of CALB.

It is clear that CALB has sn-3 stereoselectivity for all the triglycerides. Trying to find an explanation, the structure of triacetin was carefully analyzed

Insert Figure 4

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Figure 4 shows a potential explanation for the sn-3 selectivity in CALB. The steps a to c show how the triacetin can be twisted without a substantial steric hindrance around. To visualize, think in a fork changing in location through the change in the location of the fork handle. Now the main changes take place in the glycerol structure and the bonds are rotated without being broken or changed. Now, to coordinate to serine the group attached to C1 of glycerol, the steric hindrance would be impossible to accommodate with the walls of CALB. Instead, the coordination of the group attached to the C3 of glycerol allow the right location of the remaining of the TAG with the adequate space to avoid strong interactions with the alcohol and fatty acid pockets of CALB.

5. CONCLUSIONS

Based on the results presented above, it is evident that:

1- The presence of glycerol in the reaction products is a clear indication of acyl migration. It is very important to perform an adequate sampling to detect this polyalcohol.

2- The analysis of the reaction samples must be carried out in the shortest time possible. Acyl migration from sn-2 to sn-1 or sn-3 position occurs spontaneously during storage of the samples, event at a temperature of -20 °C.

3- The formation of 2-MAG by ethanolysis of triglycerides is favored by longer reaction times and larger biocatalyst loadings with saturated triglycerides with short- or medium-chain. In the case of long-chain triglycerides, the formation of this monoglyceride is widely limited by acyl migration.

4- Acyl migration increases the yield of ethyl esters and minimizes the content of monoglycerides and diglycerides in the biodiesel. The enzymatic synthesis of biodiesel is recommended if oils composed mainly of long-chain triglycerides are used because they favor acyl migration. In order to maximize yield, considerably long reaction times and large biocatalyst loadings are necessary.

5- The conversion of acylglycerides formed by long-chain fatty acids with unsaturation is relatively low due to restrictions to access to the active site of the lipase.

6. ACKNOWLEDGMENTS

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Captions for figures

Figure 1. Scheme of the ethanolysis of triglycerides catalyzed by Novozym 435.
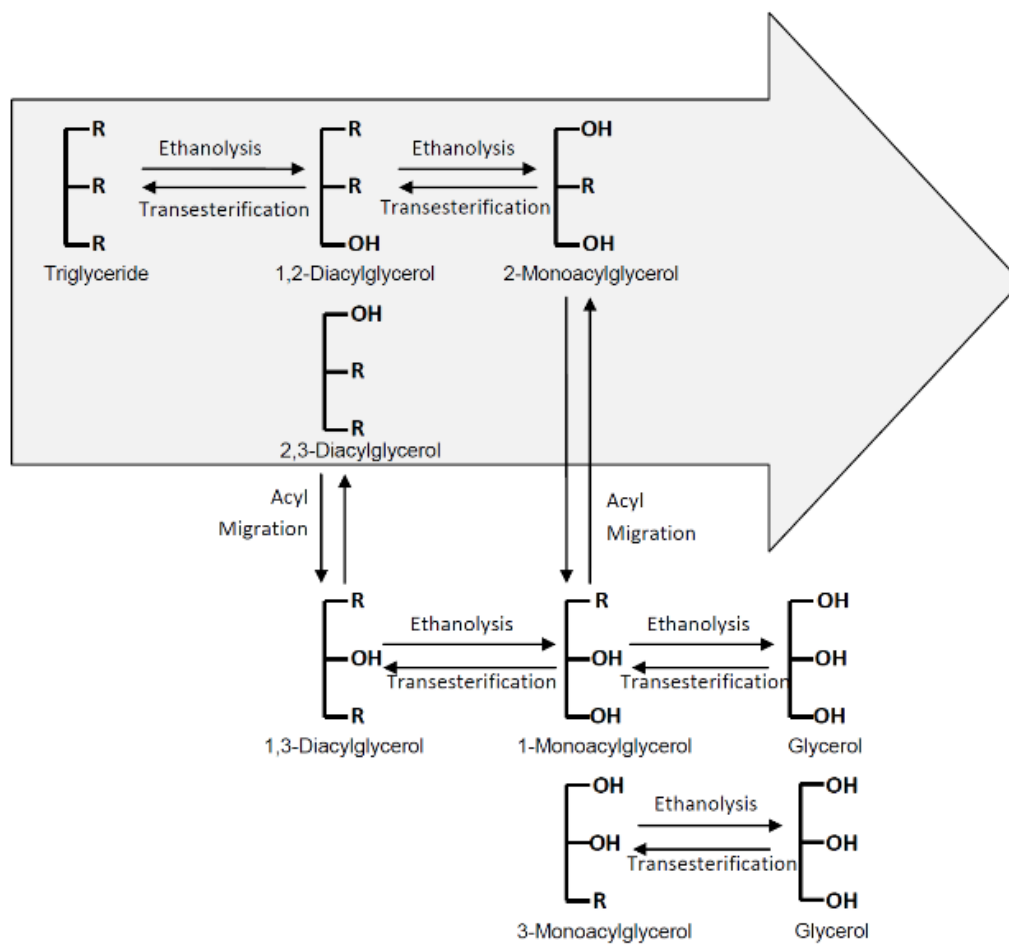
Figure 2. Variation in the mole fraction of 2-MAG in the ethanolysis of triglycerides catalyzed by Novozym 435 after 90 min of reaction. Variables: biocatalyst loading and chain length of the triglyceride. References: a) ED1, b) ED2.

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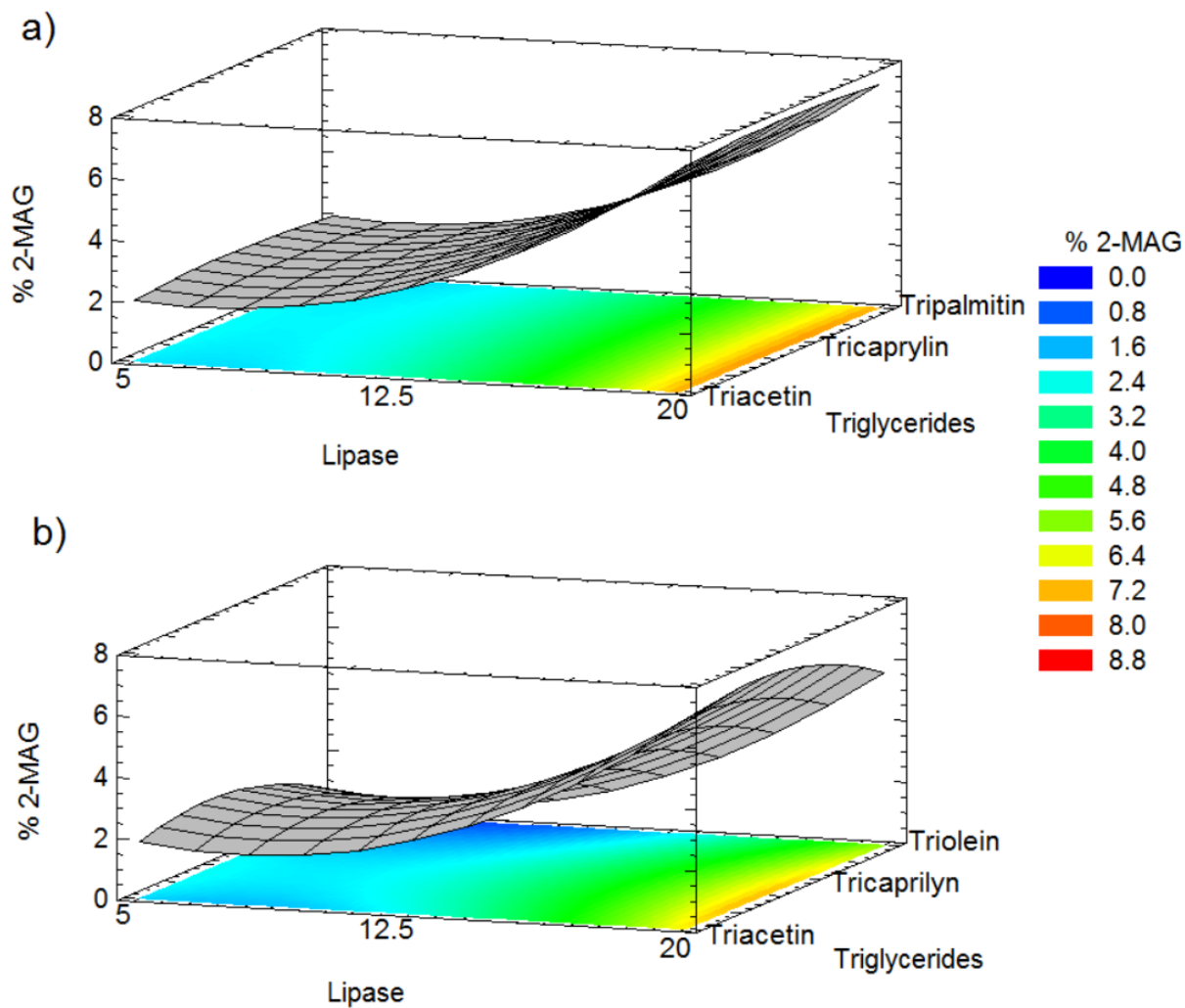
Figure 3. Schematic representación of tripalmitin (a) and triolein (b) at the CALB surface (position A), near the serine (position C) and located at the entrance of the tunnel to the catalytic triad of CALB (B) Tripalmitin (Fig.10. a) only change the opening of the 3 long hydrocarbon chains through the road to the serine. The case of triolein is more difficult (Fig.10.b). The structure is bent and therefore the double bonds introduce steric restraints for the central portion of the TAG to reach the serine.

a)

CALB   SerOH

C   A

b)

CALB   SerOH

B   A

Figure 4. Schematic representation of triacetin (without hydrogens) in: (a) position A, (b and c) position B, (d) position C.

c

b

a

Triacetin near the active site of CALB

Hydrogens not shown

Ser-OH

d

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Captions for tables

Table 1. Values of the variables used in ED1.
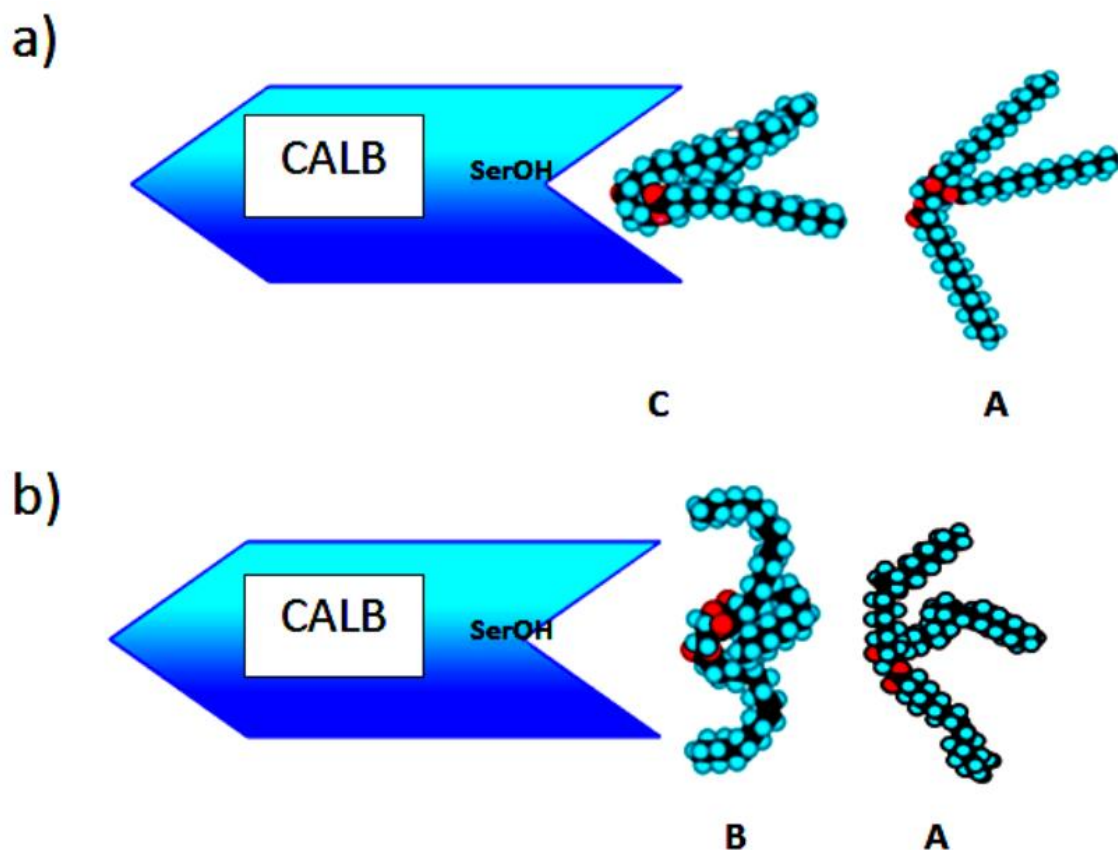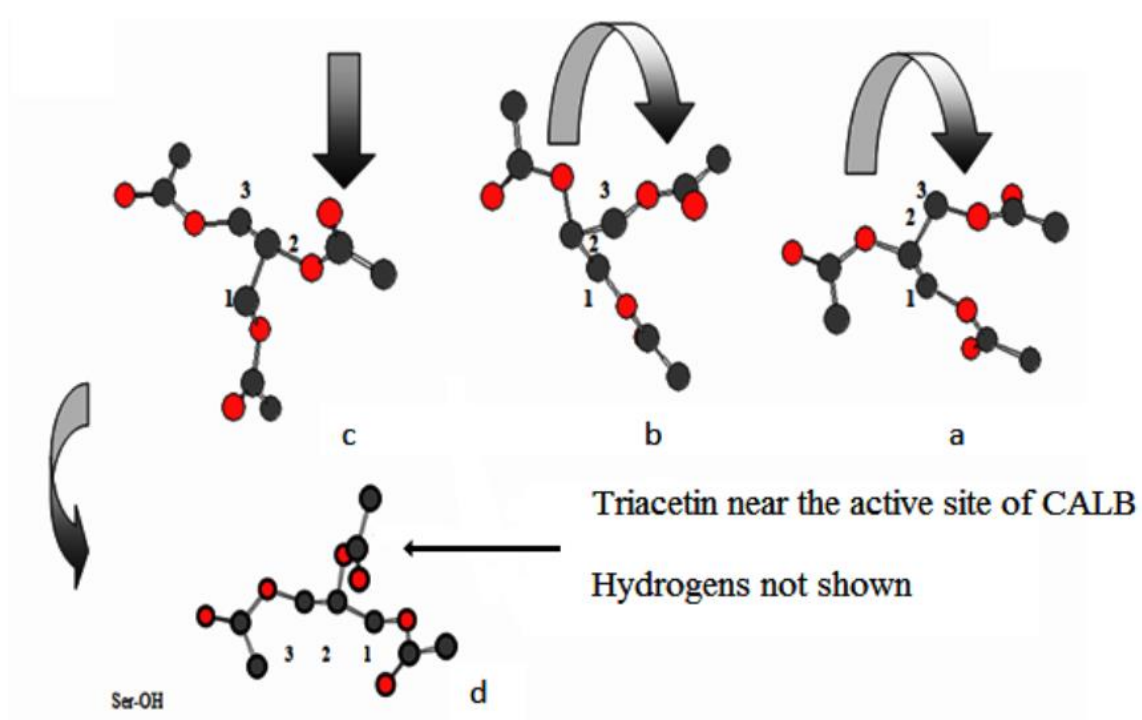
Independent variables	Symbols	Coded-variable levels		
		-1	0	+1
Amount of immobilized lipase (mg)	L	5	12.5	20
Reaction time (min)	T	30	60	90
Chain length of the saturated of triacylglycerol*	C	S	M	L

* S= Short (Triacetin), M= Medium (Tricaprylin), L= Long (Tripalmitin)

Table 2.Factors and responses evaluated in the ethanolysis of triglycerides catalyzed by Novozym 435 according to ED1.

L	T	C	X_{TAG} (%)	1,2-DAG[a]	1,3-DAG[a]	2-MAG[a]	1-MAG[a]	FAEE[a]	GGG[a]
1.0	1.0	0.0	65	26.68	0	8.46	0.07	45.03	0.52
1.0	-1.0	0.0	42.18	22.64	0.43	3.69	0.17	33.54	1.1
0.0	1.0	1.0	26.88	15.2	0.39	2.59	0.65	25.68	1.15
-1.0	1.0	0.0	29.09	19.95	0	1.97	0.1	23.74	0.16
0.0	0.0	0.0	29.93	22.97	0	0.09	0	22.96	0
-1.0	-1.0	0.0	8.28	7.35	0	0.28	0.02	7.61	0
0.0	-1.0	1.0	14.34	9.02	0.7	1.11	0.54	15.94	0.69
0.0	0.0	0.0	27.87	21.48	0	0.1	0	22.59	0
1.0	0.0	1.0	29.7	15.81	0.82	2.79	0.69	27.71	1.36
0.0	0.0	0.0	29.44	22.74	0	0.13	0	22.31	0
-1.0	0.0	-1.0	8.27	6.83	0	0.22	0.07	8.72	0.44
1.0	0.0	-1.0	43.28	25.4	0	3.07	0.29	32.93	0.27
0.0	-1.0	-1.0	13.37	10.87	0	0.47	0.1	12.72	0.24
-1.0	0.0	1.0	9.11	5.83	0.89	0.65	0.52	9.28	0.37
0.0	1.0	-1.0	37.47	23.47	0	2.33	0.26	29.61	0.32

[a] Mole percentage in relation to the total amount of compounds in the reaction sample

Table 3. Values of the variables used in ED 2.

Independent variables	Symbols	Coded-variable levels		
		-1	0	+1
Amount of immobilized lipase (mg)	L	5	12.5	20
Reaction Time (min)	T	30	60	90
Chain length of the triacylglycerol*	C	S	M	L

* S= Short (Triacetin), M= Medium (Tricaprylin), L= Long (Triolein)

Table 4. Factors and responses evaluated in the ethanolysis of triglycerides catalyzed by Novozym 435 according to ED2.

L	T	C	X_{TAG} (%)	1,2-DAG[a]	1,3-DAG[a]	2-MAG[a]	1-MAG[a]	FAEE[a]	GGG[a]
1.0	1.0	0.0	65	26.68	0	8.46	0.07	45.03	0.52
1.0	-1.0	0.0	42.18	22.64	0.43	3.69	0.17	33.54	1.1
0.0	1.0	1.0	9.28	7.17	0	0.12	0	10.66	0
-1.0	1.0	0.0	29.09	19.95	0	1.97	0.1	23.74	0.16
0.0	0.0	0.0	29.93	22.97	0	0.09	0	22.96	0
-1.0	-1.0	0.0	8.28	7.35	0	0.28	0.02	7.61	0
0.0	-1.0	1.0	2.52	1.61	0	0.32	0	5.59	0
0.0	0.0	0.0	27.87	21.48	0	0.1	0	22.59	0
1.0	0.0	1.0	15.18	11.21	0.12	0.98	0.23	15.34	0.32
0.0	0.0	0.0	29.44	22.74	0	0.13	0	22.31	0
-1.0	0.0	-1.0	8.27	6.83	0	0.22	0.07	8.72	0.44
1.0	0.0	-1.0	43.28	25.4	0	3.07	0.29	32.93	0.27
0.0	-1.0	-1.0	13.37	10.87	0	0.47	0.1	12.72	0.24
-1.0	0.0	1.0	0.94	0.55	0	0.31	0	1.72	0
0.0	1.0	-1.0	37.47	23.47	0	2.33	0.26	29.61	0.32

[a] Mole percentage in relation to the total amount of compounds in the reaction sample

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Table 5. Results of the data fitting with Statgraphics Centurion XV of responses following Experimental Designs 1 and 2 in Ethanolysis of Model Triglycerides. All data with a confidence level of over 95.0%. SFA: saturated fatty acids ED1; UFA: unsaturated fatty acid ED2; ED1 Experimental Design 1; ED2 Experimental Design 2

Response	Equation	Eq. n	R^2, %
Triglycerides Conversion (SFA only)	$X_{TAG} = 29.08 + 15.68L + 10.03T - 2.79C - 9.81C^2 + 3.74T^2 + 3.32L^2 - 3.61LC - 2.89TC$	2	98.96
Triglycerides Conversion (SFA + UFA)	$X_{TAG} = 33.11 + 14.88L + 9.31T - 9.31C - 16.82C^2$	3	94.70
Fraction of 1,2 DAG (SFA only)	$1,2\text{-}DAG = 21.76 + 6.32L + 4.43T - 2.59C - 2.13L^2 - 6.64C^2 - 2.14LT - 2.15LC - 1.61TC$	4	98.54
Fraction of 1,2 DAG (SFA +UFA)	$1,2\text{-}DAG = 20.54 + 6.41L + 4.35T - 5.75C - 9.66C^2$	5	93.51
Fraction of 1,3 DAG (SFA only)	$1,3\text{-}DAG = -0.015 - 0.09T + 0.35C + 0.13L^2 + 0.30C^2 - 0.11LT$	6	92.19
Fraction of 1,3 DAG (SFA+UFA)	$1,3\text{-}DAG = 0.07L - 0.05T + 0.07L^2 + 0.04T^2 - 0.04C^2 - 0.11LT$	7	76.37
Fraction of 2 MAG (SFA only)	$2\text{-}MAG = 0.11 + 1.86L + 1.23T + 1.77L^2 + 1.72T^2 - 0.20C^2 + 0.77LT$	8	93,27
Fraction of 2 MAG (SFA+UFA)	$2\text{-}MAG = 0.11 + 1.68L + 1.02T - 0.55C + 1.91L^2 + 1.58T^2 - 0.88C^2 + 0.77LT$	9	85.75
Fraction of 1-MAG (SFA only)	$1\text{-}MAG = 0.06L + 0.03T + 0.21C + 0.05L^2 + 0.04T^2 + 0.35C^2$	10	96.64

Fraction of 1-MAG (SFA+UFA)	$1\text{-}MAG = 0.01 + 0.07L + 0.02T - 0.06C + 0.07L^2 + 0.07C^2 - 0.04LT - 0.04CT$	11	83.07
Fraction of glycerol (SFA only)	$GGG = 0.29L + 0.29C + 0.23L^2 + 0.22T^2 + 0.38C^2 - 0.18LT + 0.29LC$	12	92.39
Fraction of glycerol (SFA+UFA)	$GGG = -0.01 + 0.20L - 0.04T - 0.12C + 0.28L^2 + 0.17T^2 - 0.18LT + 0.12LC$	13	80.20
Fraction of FAEE(SFA only)	$FAEE = 22.62 + 11.23L + 6.78T + 1.77L^2 + 3.09T^2 - 4.73C^2 - 1.44LC - 1.79TC$	14	99.10
Fraction of FAEE(SFA+UFA)	$FAEE = 22.62 + 10.63L + 6.20T - 6.33C + 2.45L^2 + 2.41T^2 - 10.39C^2 - 2.65LC - 2.95TC$	15	98.99

Table 6. Formation enthalpy (ΔH°_f) for the selected conformations for each studied triglyceride.

Triglyceride	Position A ΔH°_f (kcal/mol)	Position B ΔH°_f (kcal/mol)	Position C ΔH°_f (kcal/mol)
Triacetin	- 249.6	-246	-239.5
Tricaprylin	- 338.6	-337.8	-336.2
Tripalmitin	- 468.2	-462.1	-464.0
Triolein	- 419.0	-387.0	-409.0