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Contribution of response surface design to the development of glycerolysis systems catalyzed by commercial immobilized lipases

S. Ferreira-Dias [a,*], A.C. Correia [a], F.O. Baptista [a], M.M.R. da Fonseca [b]

[a] *Instituto Superior de Agronomia, Tapada da Ajuda, 1349-017 Lisbon, Portugal*

[b] *Instituto Superior Técnico, Centro de Engenharia Biológica e Química, Av. Rovisco Pais, 1049-001 Lisbon, Portugal*

Abstract

Two commercial immobilized lipases ("Lipozyme® IM" and "Novozym® 435") were tested as biocatalysts for the glycerolysis of olive residue oil in *n*-hexane aimed at the production of monoglycerides (MG) and diglycerides (DG). A central composite rotatable design (CCRD) was followed to model and optimize glycerolysis as a function of both the amount of biocatalyst (*L*) and of the molar ratio glycerol/triglycerides (Gly/TG). For both biocatalysts, the production of free fatty acids (FFA) was described by second order models. In terms of MG and DG production, as well as of TG conversion, the best fits were obtained with first-order models. The highest MG productions were in the range 43–45% (w/w, on the basis of total fat) for both biocatalysts tested at a (Gly/TG) ratio of one. In the case of "Novozym 435", the lowest load used (12%, w/w) gave the best results, in contrast with "Lipozyme IM" with which a concentration of about 26% (w/w) was necessary to obtain the highest production. Under these conditions, the amount of FFA produced was about 2% and 10% (w/w), respectively, for "Novozym 435" and "Lipozyme IM" catalyzed systems. Considering both FFA production and lipase loading, "Novozym 435" was shown to be a better biocatalyst for the glycerolysis of olive residue oil in *n*-hexane, aimed at the production of MG, than "Lipozyme IM". © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Diglycerides; Glycerolysis; Lipase; Monoglycerides; Response surface design

1. Introduction

In food and pharmaceutical industries, monoglycerides (MG) and diglycerides (DG) are among the most important emulsifiers. MG represent about 70% of the synthetic emulsifiers used in these industries [1].

Current processes for the production of MG and DG are based on the interesterification of triglycerides with glycerol (glycerolysis) in the presence of inorganic catalysts at high temperatures (200–250°C) [2].

The replacement of inorganic catalysts by lipases (E.C. 3.1.1.3.) is a potential attractive way to avoid side product formation and to reduce pollution ef-

* Corresponding author. Tel.: +351-21-3638161; fax: +351-21-3602036.

E-mail address: suzanafdias@mail.telepac.pt (S. Ferreira-Dias).

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fects and energy consumption because mild conditions are used.

Lipase-catalyzed glycerolysis experiments, aimed at the production of MG and/or DG, have been carried out with free [3–6] or immobilized lipases [7–10], with lipases in reversed micelle systems [11] and with liposomes in reversed micelles [12].

In this work, two commercial immobilized lipases ("Lipozyme® IM" and "Novozym® 435") were tested as biocatalysts for the glycerolysis of 30% (w/v) refined olive residue oil in *n*-hexane aimed at the production of MG and DG. Crude olive residue oil is obtained from olive cake by solvent extraction (*n*-hexane), once the physical extraction of olive oil has been completed. After refining, this oil may be used for edible purposes in blends with olive oil or other vegetable oils. The potential use of this vegetable oil as a substrate for the production of added-value compounds such as MG and DG is an attractive way to valorize the olive oil industry.

In addition, the response surface methodology (RSM) was followed as an attempt to model and optimize the glycerolysis reaction, catalyzed by both biocatalysts, as a function of both the amount of immobilized commercial lipase and the molar ratio glycerol/triglyceride (Gly/TG) used.

RSM is a set of mathematical and statistical methods developed for modelling phenomena and to find combinations of a number of experimental factors that will lead to optimum responses [13–16]. This methodology was first developed by Box and Wilson in 1951 [14]. The RSM has been recently used on modelling and optimization of several bioprocesses such as fermentations [17,18], enzymatic reactions [19–21], in product recovery [22], in enzyme immobilization techniques [23] as well as in bleaching of crude oils by adsorption [24].

2. Materials and methods

2.1. Materials

Refined olive residue oil was a gift from José Carvalho Coimbra, Avanca, Portugal; triolein, trimyristin, diolein (mixed isomers), monoolein, oleic acid and glycerol (99%) of analytical grade were purchased from Sigma. All other chemicals were of analytical grade and obtained from various sources.

The following commercial lipase preparations, kindly donated by NOVO Nordisk, Denmark, were tested as biocatalysts for glycerolysis: "Lipozyme® IM" and"Novozym® 435". "Lipozyme® IM" is the lipase from *Rhizomucor miehei* immobilized by adsorption on a macroporous anion exchange resin (phenolic type, particle size of 0.2 to 0.6 mm). This is a thermostable 1,3-specific lipase preparation that can be used at temperatures between 30°C and 70°C (Novo Nordisk, Product sheet). "Novozym® 435" is the lipase from *Candida antarctica* immobilized on a macroporous acrylic resin (bead-shaped particles with 0.3 to 0.9 mm diameter) (Novo Nordisk, Product sheet). It is also a thermostable lipase preparation with a maximum activity in the range 70–80°C. However, working temperatures in the range of 40–60°C are recommended to extend the operational stability. The positional specificity of this lipase towards the fatty acids in glycerides depends on the substrates used. Both lipase preparations may be used in solvent-free media or in the presence of biocompatible solvents.

2.2. Methods

2.2.1. Water activity assay

Before starting the glycerolysis reaction, the water activity (a_w) of the biocatalysts was measured at 25°C in a Rotronic Hygroskop DT humidity sensor (DMS-100 H).

2.2.2. Aquaphilicity of the immobilization supports

The aquaphilicity (Aq) of the supports was proposed by Reslow et al. [25] as an indicator of their affinity for water. In the present work, Aq was estimated as a way to evaluate possible differences in hydrophilicity between the supports used in the immobilized lipases tested.

Aq was estimated for both immobilized biocatalysts as follows: 1.5 g of "Lipozyme IM" and "Novozym 430" were soaked in 100 cm^3 of a solution of acetone and ethanol (1:1, v:v) for 24 h and thus inactivated [26]. The supports were subse-

quently dried for 1 h at 40°C and 10 kPa absolute pressure and soaked in water saturated diisopropylether, according to the original procedure [25]. Both the initial and the final water content of the solvent were assayed, in aliquots of 100 μl, with a Metrohm 684 Karl Fischer Coulometer. The difference between these two values corresponds to the amount of water that migrated to the supports. Aq is not a true partition coefficient and depends on the ratio (dried support:saturated diisopropylether). A ratio of 1:5 (w/w), as proposed by Reslow et al. [25], was used in these assays.

2.2.3. Glycerolysis reaction

The immobilized lipase was added to a biphasic system consisting of an organic phase (12 cm^3 of a solution of refined olive residue oil in *n*-hexane (30%, w/v)) and glycerol at various molar ratios. The concentration of oil used is the usual concentration of the miscella, i.e., the solution of crude oil in hexane after extraction at industrial scale. The molar ratio of Gly/TG and the amount of biocatalyst used (L) varied according to the experimental design followed (cf., Section 2.2.7).

The olive residue oil was previously treated with alumina to remove DG, MG, FFA, oxidation products and traces of water which might be present. The reaction was carried out in a thermostated cylindrical glass vessel closed with a rubber stopper, at 30°C, under magnetic stirring. After 1-h reaction time, samples were taken and TG and products analyzed.

2.2.4. Correction for solvent evaporation

Since *n*-hexane is a rather volatile solvent (vapor pressure of 26.7 kPa at 31.6°C), its evaporation during the time course of a reaction has usually to be considered, even in vessels closed with rubber stoppers. Thus, a correction factor was calculated from blank experiments with each biocatalyst. A mass of 0.8 g of "Lipozyme IM" and "Novozym 430" (corresponding to a biocatalyst load of 19 g/100 g of TG) was soaked in 50 ml of a solution of acetone and ethanol (1:1, v:v) [26]. Then, the inactivated biocatalyst was added to the biphasic system (cf., Section 2.2.3.) containing oleic acid (1.5%, w/v) and glycerol at different molar ratios (Gly/TG) (1:1, 2:1 and 3:1), under the conditions used in the glycerolysis reactions. Aliquots were taken for 2 h and assayed for their content in FFA. Experiments were run in duplicate and it was assumed that the solvent was the sole compound to evaporate.

2.2.5. Assay for the water content of the reaction medium during glycerolysis

The water content of the reaction medium was assayed in aliquots of 1 ml, with a Metrohm 684 Karl Fischer Coulometer along the time course of glycerolysis for each of the biocatalysts tested. In these experiments, a biocatalyst load of 19 g/100 g of TG and a Gly/TG molar ratio of 2:1 were used. Experiments were run in duplicate.

2.2.6. Analytical methods

After separation by thin layer chromatography and methylation, TG and DG were assayed as fatty acid methyl esters (FAME), by gas chromatography [7]. The procedure was similar to the one previously described [7], except that the concentration of trimyristin (precursor of internal standard) in *n*-hexane was 1.0% (w/v). In addition, calibration curves for TG were established by using the mixture of TG present in the olive residue oil used, instead of the triolein standard. In olive oil, triolein is 40–59% of the total TG [27]. The olive residue oil composition is similar to olive oil. The purification of the TG fraction was achieved by passing a solution of refined olive residue oil in *n*-hexane (10%, w/v) through a column filled with 30 g of alumina.

A Carlo Erba 6000 vega series 2 gas chromatograph equipped with a capillary column (Supelco™ 2380, 60 m × 0.25 mm i.d.; 0.20 μm film thickness, fused silica) and a flame ionization detector (FID) was used. Hydrogen was the carrier gas at a column head pressure of 60 kPa; both injector and FID were at 250°C and FAME separation was achieved under isothermal conditions (230°C).

The FFA were assayed using the Lowry and Tinsley's colorimetric method [28] with benzene replaced by *n*-heptane [7]. Since MG showed a low solubility in *n*-hexane, their quantification was achieved via an indirect method [7].

2.2.7. Experimental design

With RSM, several variables were tested simultaneously with a minimum number of trials according to special experimental designs based on factorial designs [13–16]. This methodology has the advantage of being less expensive and time-consuming than the classical methods. The response y is described by a polynomial equation as a function of the p independent variables, x_i, that is,

$$y = f(x_1, x_2, \ldots, x_p) + \varepsilon$$

where ε represents the error observed in the response y. Usually, the response is well modeled by a first or a second-order polynomial representing a $(p+1)$-dimensional surface, i.e., the *response surface*. The parameters of these equations are usually unknown and, therefore, must be estimated from the experimental data by using the statistical principle of least squares. In second-order equations, the coefficients of the squared terms influence the direction of the curvature of the response surfaces.

The designs most commonly used to fit first order models are the 2^p full factorial design. In addition to the 2^p points, a center point (repeated several times) is frequently added to the design. They are used to provide an estimation of the variance of the experimental error, which is assumed to be constant along the experimental domain [14,16]. The contribution to the error variation is not only due to the experimental errors alone, but also to the lack of fit of the estimated model.

To fit second-order models, composite designs are usually followed. They consist of augmented 2^p factorial designs with star points (also called axial points) and center points.

In our study, a central composite rotatable design (CCRD) was used. It consists of the following three sets of experimental points [14–16].

1. A factorial design with 2^p data-points (extremes), which represent the vertices of a p-dimensional cube, at a distance of $p^{1/2}$ from the origin of the coded system of reference. In the coded matrix, they correspond to the levels (-1) and $(+1)$ for each variable (factor).
2. A group of 2^p points on the axes of the system of reference, outside of the factorial matrix, but inside the experimental domain, at a distance equal to $2^{p/4}$ from the origin (star-points).
3. A third set composed by the repetition of the points at the origin of the reference system (center-points), coded as (0,0).

The choice of the center point, as well as of the star-points, result from previous studies, since the feasibility of the trials at these points must be checked, before starting the experiment [15]. The extrapolation of the responses beyond the experimental range of the independent variables may have no practical meaning. For most practical applications, the identification of the regions of independent variables corresponding to optimal responses may be directly achieved by visual examination of the response surfaces. In addition, partial differentiation is used to find the optimum of a multivariate function. These solutions are called stationary points [14].

In this work, as an attempt to model and optimize the glycerolysis of olive residue oil in *n*-hexane catalyzed by "Lipozyme IM" or "Novozym 435", a CCRD was followed as a function of both the molar ratio Gly/TG and the amount of immobilized lipase (L) used.

In the experimental designs the levels for the molar ratio (Gly/TG) and (L) tested for both biocatalysts are shown in Table 1.

2.2.8. Statistical analysis

The results of the 14 experiments were analyzed using the software "Statistica™", version 5, from Statsoft, USA. The linear and quadratic effects of

Table 1
Coded and decoded levels of the experimental factors used in experimental designs

Coded levels	Molar ratio (Gly/TG)	Lipase load (%)
(-1)	1.0	12.3
$(+1)$	3.0	25.9
$(-\sqrt{2})$	0.5	9.4
$(+\sqrt{2})$	3.5	28.3
0	2.0	18.9

(Gly/TG) and (L) and the linear interaction (Gly/TG) × (L) on glycerolysis kinetics were calculated. Their significance was evaluated by analysis of variance. A tri-dimensional surface, described by a first- or a second-order polynomial equation was fitted to each set of experimental data points (production of FFA, MG and DG and of TG converted to FFA, MG and DG). First- and second-order coefficients were generated by regression analysis. To establish the first-order models, only the factorial and center points of the CCRD were considered in the analysis. The fit of the models was evaluated by the determination coefficients (R^2) and adjusted R^2 (R^2_{adj}) [29].

3. Results and discussion

3.1. Aq and water activity of immobilized biocatalysts

The estimated Aq values were 0.52 and 3.29, respectively, for "Novozym 435" and "Lipozyme IM", suggesting a higher hydrophilicity of the immobilization support used in "Lipozyme IM".

The initial a_w of "Novozym 435" was 0.32 (average of 5 measurements, standard deviation = 0.023) and 0.12 (average of 5 measurements, standard deviation = 0.012) for "Lipozyme IM". These results indicate that the water present in the biocatalyst is less available for hydrolysis reactions in the case of "Lipozyme IM" as compared to "Novozym 435".

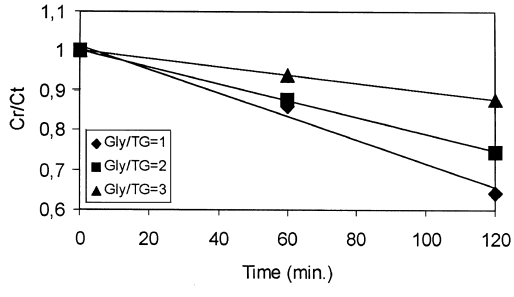

Fig. 1. Evaporation curves for oleic acid, under different molar ratios (Gly/TG), during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Novozym 435". C_r / C_t is the ratio between the real and the apparent concentrations of oleic acid at time t.

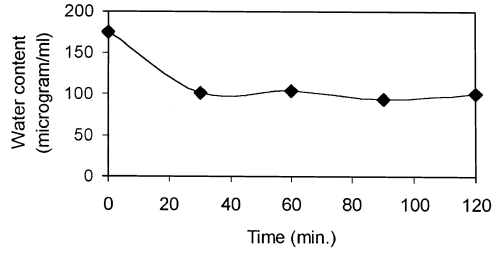

Fig. 2. Water content of the reaction medium during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Novozym 435" (see text for details).

3.2. Correction of solvent evaporation

The ratio of C_r, the real concentration if no solvent evaporation had occurred, to C_t, the observed concentration of oleic acid at time t (min) is shown in Fig. 1 as a function of reaction time. The catalyst was "Novozym 435" and different (Gly/TG) molar ratios were used. The increase in the glycerol ratio prevents the evaporation of the solvent. This may probably be ascribed to the emulsification of glycerol in the organic medium acting as a barrier against evaporation. A similar profile was observed for "Lipozyme IM" (data not shown). Thus, the evaporation of *n*-hexane has to be taken into account in these systems, even in short reaction times.

3.3. Assay for the water content of the reaction medium during glycerolysis

The water content of the reaction medium measured during the time course of the glycerolysis of olive residue oil in *n*-hexane catalyzed by "Novozym 435", when a Gly/TG molar ratio of 2:1 was used, is shown in Fig. 2. A fast decrease in the water content of the reaction medium was observed. This was probably due to water partitioning between the organic medium, the glycerol phase and the immobilization matrix. After equilibrium was attained, the water content was constant along the remaining time course of the reaction. Similar results were obtained with "Lipozyme IM" catalyzed glycerolysis (data not shown). These results are in agreement with glycerolysis kinetics [9]. They also suggest that both

Table 2
Effects and respective significance levels (α) on the production of FFA during glycerolysis of refined olive residue oil in *n*-hexane

Factor	"Novozym 435"	"Lipozyme IM"
Gly/TG (linear term)	−1.468 (n.s.)	−4.125***
Gly/TG (quadratic term)	−0.611 (n.s.)	1.709*
Biocatalyst load (linear term)	−1.113 (n.s.)	3.547***
Biocatalyst load (quadratic term)	−3.671*	1.119 (n.s.)
(Gly/TG)× (biocatalyst load)	−3.603*	−1.641 (n.s.)

(n.s.) not significant effects.
*At $\alpha < 0.05$.
***At $\alpha < 0.001$.

Table 4
Effects and respective significance levels (α) on the production of DG during glycerolysis of refined olive residue oil in *n*-hexane

Factor	"Novozym 435"	"Lipozyme IM"
Gly/TG (linear term)	0.251 (n.s.)	−15.620**
Biocatalyst load (linear term)	5.366*	3.471 (n.s.)
(Gly/TG)× (biocatalyst load)	0.314 (n.s.)	−3.735 (n.s.)

(n.s.) not significant effects.
*At $\alpha < 0.05$.
**At $\alpha < 0.01$.

hydrolysis and esterification reactions occur in a reduced extension, since no significant amount of water is consumed or released to the reaction medium.

3.4. Glycerolysis experiments

Glycerolysis experiments were carried out according to a full factorial design 2^2 and a CCRD, as a function of both the molar ratio (Gly/TG) and the biocatalyst load (L). The obtained results (production of FFA, MG, DG and total conversion) were used to calculate the significant effects, either linear or quadratic, of the molar ratio (Gly/TG) and of the biocatalyst load (L), on the glycerolysis reaction catalyzed by each of the biocatalysts tested. Therefore, positive effects of the factors (Gly/TG or L) or of their interaction (Gly/TG) × (L) indicate that the response increases with the increase in these factors. Significant effects of both factors on the production of FFA, MG, DG and on the TG conversion (i.e., TG converted to FFA, MG and DG) are shown in Tables 2–5, for each lipase preparation assayed.

In addition, a multiple regression analysis was performed to fit first- or second-order polynomial equations to the experimental data points, respectively for "Novozym 435" (Table 6) and "Lipozyme IM" catalyzed glycerolysis (Table 7). The high values of R^2 and R^2_{adj} show a close agreement between the experimental results and the theoretical values predicted by the models [29].

The FFA are produced during the first step of lipase-catalyzed glycerolysis, where fatty acids are released from glycerides to the reaction medium [9].

Table 3
Effects and respective significance levels (α) on the production of MG during glycerolysis of refined olive residue oil in *n*-hexane

Factor	"Novozym 435"	"Lipozyme IM"
Gly/TG (linear term)	−9.407**	−0.467 (n.s.)
Biocatalyst load (linear term)	−3.604 (n.s.)	19.576*
(Gly/TG)× (biocatalyst load)	5.708*	−9.720 (n.s.)

(n.s.) not significant effects.
*At $\alpha < 0.05$.
**At $\alpha < 0.01$.

Table 5
Effects and respective significance levels (α) on the TG converted to FFA, MG and DG during glycerolysis of refined olive residue oil in *n*-hexane

Factor	"Novozym 435"	"Lipozyme IM"
Gly/TG (linear term)	−2.589*	20.572**
Biocatalyst load (linear term)	1.034 (n.s.)	−22.546**
(Gly/TG)×(biocatalyst load)	0.430 (n.s.)	13.328**

(n.s.) not significant effects.
*At $\alpha < 0.05$.
**At $\alpha < 0.01$.

Table 6
Model equations for the response surfaces fitted to the experimental data points as a function of the molar ratio (Gly/TG) and the percentage of "Novozym 435" used (L) in the glycerolysis reaction and respective R^2 and R^2_{adj}

Compounds	Model equations	R^2	R^2_{adj}
FFA	$\%FFA = -16.20 + 5.52(Gly/TG) - 0.31(Gly/TG)^2 + 2.11L - 0.04\ L^2 - 0.26(Gly/TG) \times (L)$	0.811	0.622
MG	$\%MG = 65.14 - 12.67(Gly/TG) - 1.10\ L + 0.42(Gly/TG) \times (L)$	0.925	0.868
DG	$\%DG = 9.69 - 0.31(Gly/TG) + 0.35\ L + 0.02(Gly/TG) \times (L)$	0.863	0.727
Converted triglycerides	$\%TG_{conv.} = 61.71 - 6.30(Gly/TG) + 0.04\ L + 0.10(Gly/TG) \times (L)$	0.738	0.542

It has been reported that they may also result from hydrolysis, occurring even in low water activity media [30–32].

The response surface plots of the production of FFA, as a function of the biocatalyst load and the (Gly/TG) molar ratio, are shown in Figs. 3 and 4.

For the "Novozym 435" system, a saddle-shaped response surface is fitted to the experimental data (Fig. 3). The plot illustrates the dominant contribution of the quadratic effect of "Novozym 435" load, as well as of the interaction term, to the model (both negatively) (Table 2). It is a complex response surface with maximum and minimum values encountered at various combinations of the independent variables [14]. In fact, for "Novozym 435", the production of FFA seems to depend more on the amount of biocatalyst than on the (Gly/TG) ratio.

For the "Lipozyme IM" system, the FFA production is significantly dependent on the amount of lipase (linear effect) and on the molar ratio (Gly/TG) (linear and quadratic effects) (Table 2) and a concave response surface is obtained (Fig. 4). In fact, higher FFA levels are observed when low (Gly/TG) ratios and high loads of "Lipozyme IM" are used. Glycerol is a potent water binder and has a very low a_w of approximately 0.17 [33]. Thus, the increase in glycerol content leads to a reduction in the a_w [34] of the reaction medium and, therefore, restrains the competing hydrolysis reaction.

The observed FFA surface for "Novozym 435" (Fig. 3) suggests that this biocatalyst may be less sensitive to the a_w value in the glycerolysis medium than "Lipozyme IM" (Fig. 4). In fact, during the transesterification of palm oil stearin (POS) with soybean oil and ω–3 polyunsaturated fatty acids concentrate catalyzed by "Novozym 435", in solvent-free media, no significant effect of the initial a_w of the biocatalyst was observed in the range 0.28–0.62 [35]. In contrast, during the transesterification of POS with palm kernel oil catalyzed by "Lipozyme IM", an increase in the initial a_w of the biocatalyst was followed by an increase in the amount of FFA, which were probably produced in the hydrolysis reaction [21].

For both immobilized lipases, a second-order polynomial model, established via a CCRD, is well

Table 7
Model equations for the response surfaces fitted to the experimental data points, as a function of the molar ratio (Gly/TG) and the percentage of "Lipozyme IM" used (L) in the glycerolysis reaction and respective R^2 and R^2_{adj}

Compounds	Model equations	R^2	R^2_{adj}
FFA	$\%FFA = 4.71 - 3.19(Gly/TG) + 0.85(Gly/TG)^2 + 0.04L + 0.01\ L^2 - 0.12(Gly/TG) \times (L)$	0.971	0.942
MG	$\%MG = -27.49 + 13.34(Gly/TG) + 2.85L - 0.71(Gly/TG) \times (L)$	0.915	0.830
DG	$\%DG = 9.27 - 2.59(Gly/TG) + 0.80L - 0.27(Gly/TG) \times (L)$	0.970	0.939
Converted triglycerides	$\%TG_{conv.} = 114.14 - 8.33(Gly/TG) - 3.60\ L + 0.97(Gly/TG) \times (L)$	0.991	0.982

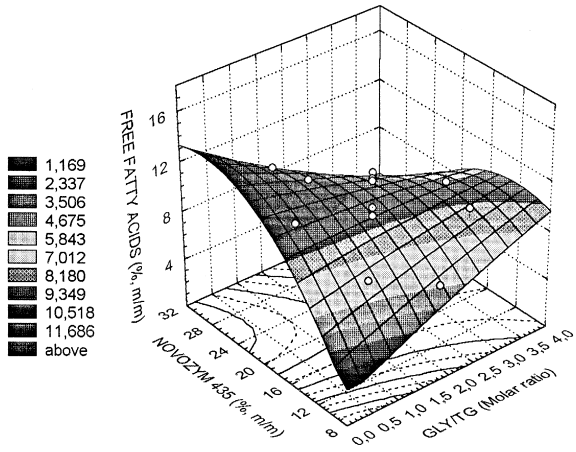

Fig. 3. Response surface fitted to the experimental data points corresponding to the production of FFA during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Novozym 435", as a function of the amount of biocatalyst and the molar ratio (Gly/TG).

adjusted to the experimental data points (Tables 6 and 7).

The production of MG and DG can be described by flat surfaces (Figs. 5–8), fitted to the experimental points and established via full factorial designs with 3 center points, for both catalyzed systems used.

With "Novozym 435", the amount of MG increased with the decrease in (Gly/TG) and a positive interaction effect was also observed (Table 3, Fig. 5). In "Lipozyme IM"-catalyzed glycerolysis, MG production increased with the amount of biocatalyst used (Table 3, Fig. 6). For both biocatalysts, the highest MG productions were obtained when the

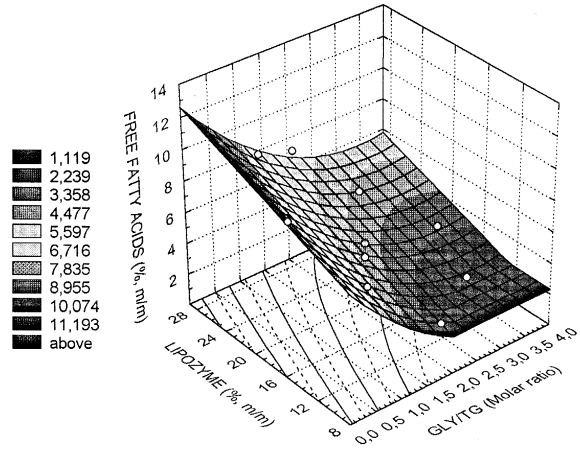

Fig. 4. Response surface fitted to the experimental data points corresponding to the production of FFA during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Lipozyme IM", as a function of the amount of biocatalyst and the molar ratio (Gly/TG).

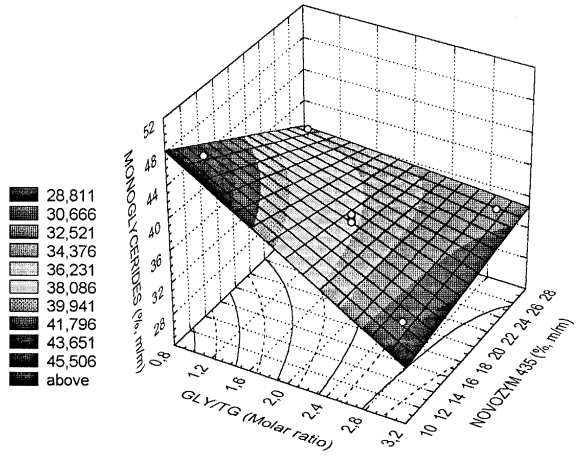

Fig. 5. Response surface fitted to the experimental data points corresponding to the production of MG, during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Novozym 435", as a function of the amount of biocatalyst and the molar ratio (Gly/TG).

(Gly/TG) molar ratio was used at the lowest level tested (Gly/TG = 1). By the law of mass action, and since the stoichiometric value (Gly/TG) in glycerolysis is equal to 2, an increase in MG production was to be expected at higher (Gly/TG) ratios.

The observed results may be explained by a different affinity of glycerol (hydrophilic compound) for each of the two immobilization matrices. This may be particularly important with "Lipozyme IM", since the Aq of its support is about 6 times higher than that of "Novozym 435", leading to higher glycerol concentrations in the microenvironment. When high (Gly/TG) ratios are used (i) an inactivation effect of glycerol on the lipases [10,36,37]

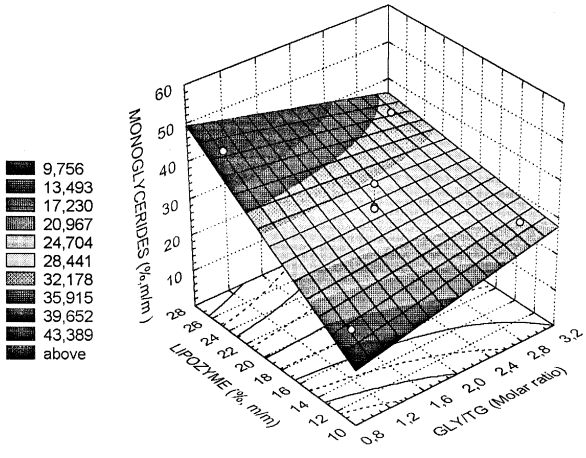

Fig. 6. Response surface fitted to the experimental data points corresponding to the production of MG during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Lipozyme IM", as a function of the amount of biocatalyst and the molar ratio (Gly/TG).

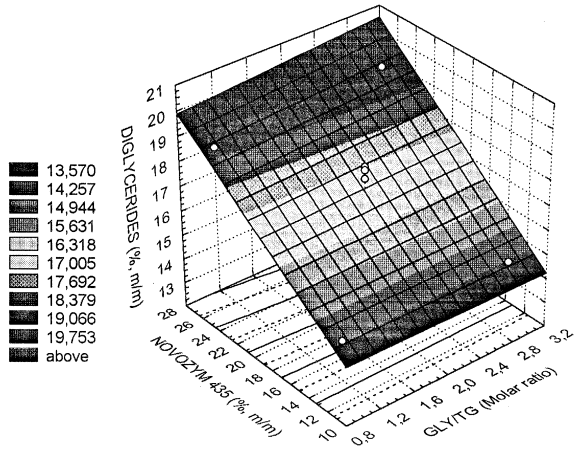

Fig. 7. Response surface fitted to the experimental data points corresponding to the production of DG during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Novozym 435", as a function of the amount of biocatalyst and the molar ratio (Gly/TG).

and/or (ii) the formation of a glycerol layer around the biocatalyst particles restraining the contact between the lipase and the hydrophobic substrates (TG and DG) may occur. In addition, in the systems under study, when high (Gly/TG) ratios are used, it is difficult to achieve good mixing in the reaction vessel. This is due to the specific gravity ($\rho = 1.26$ at 20°C) and viscosity (1490 cP at 20°C) of glycerol being considerably higher than the respective values for the organic solvent ($\rho = 0.660$; viscosity = 0.326 cP at 20°C).

The production of DG, during glycerolysis catalyzed by "Novozym 435", increases (i) strongly with the amount of biocatalyst used and (ii) slightly with the (Gly/TG) level (Table 4, Fig. 7). When "Lipozyme IM" is used, a decrease in DG formation

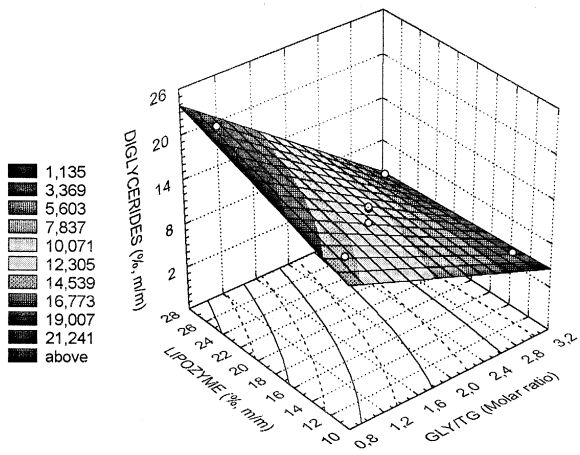

Fig. 8. Response surface fitted to the experimental data points corresponding to the production of DG during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Lipozyme IM", as a function of the amount of biocatalyst and the molar ratio (Gly/TG).

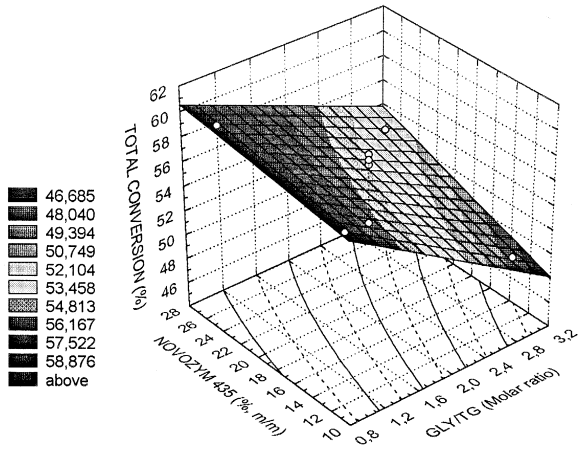

Fig. 9. Response surface fitted to the experimental data points corresponding to the total conversion of triglycerides during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Novozym 435", as a function of the amount of biocatalyst and the molar ratio (Gly/TG).

is observed with increasing (Gly/TG) ratio (Table 4, Fig. 8). The effect of glycerol on the production of DG seems to be less pronounced with "Novozym 435" than with "Lipozyme IM". This may be explained by the lower dependence of "Novozym 435"on the a_w of the system, which is also related to the amount of glycerol in the medium, as well as by the relatively hydrophobic behavior of the immobilization support ($Aq_{Novozym} = 0.52$). Thus, "Novozym 435" seems to promote the access of TG to the microenvironment, while hindering the access of glycerol.

For both biocatalysts tested, the production of MG and DG can be described and predicted by first order polynomial models (Tables 6 and 7). In addition, both immobilized lipases presented a high global

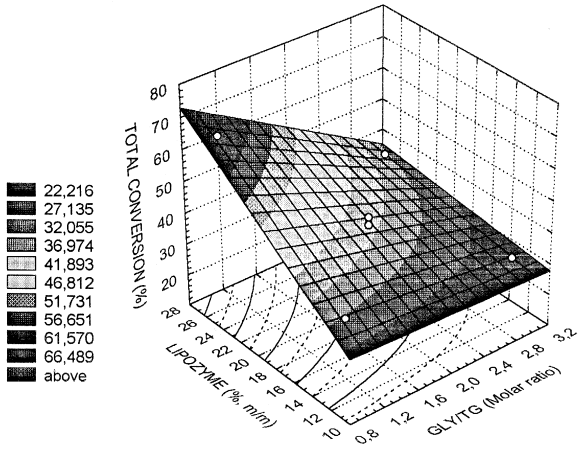

Fig. 10. Response surface fitted to the experimental data points corresponding to the total conversion of triglycerides during glycerolysis of olive residue oil in *n*-hexane catalyzed by "Lipozyme IM", as a function of the amount of biocatalyst and the molar ratio (Gly/TG).

activity, since, a total conversion of about 60% of the initial TG was obtained after 1 h (Figs. 9 and 10).

4. Conclusions

Since both flat surfaces, described by first order polynomials and saddle-like surfaces (second-order polynomials), do not present stationary points, the optimum values for the glycerolysis systems under study aimed at the production of MG and DG, cannot be estimated. However, the operational conditions inside the technological space considered in this work can be identified to obtain the highest levels of MG and DG.

The highest MG production was approximately 43–45% (w/w, on the basis of total fat) for both biocatalysts assayed at a (Gly/TG) molar ratio equal to 1 (Figs. 5 and 6). In the case of "Novozym 435", the lowest load used (12%, w/w) gave the best results, in contrast with "Lipozyme IM" with which a concentration of about 26% (w/w) was necessary to obtain the highest production. Under these conditions, the amount of FFA was approximately 2% and 10% (w/w), respectively, for "Novozym 435"- and "Lipozyme IM"-catalyzed systems (Figs. 3 and 4).

With "Lipozyme IM", the highest productions of MG (Fig. 6) were also followed by the highest production of DG (about 20%) (Fig. 8). With "Novozym 435", a similar amount of DG was obtained when a lipase concentration of 26% was used, either with a (Gly/TG) molar ratio equal to 1 or 3 (levels (−1) and (+1), respectively) (Fig. 7).

Taking into account FFA production and lipase loading, "Novozym 435" seems to be a better biocatalyst for the glycerolysis of olive residue oil in *n*-hexane, aimed at the production of MG, than "Lipozyme IM".

Higher conversions are expected if longer reaction times are used. However, due to the high viscosity and density of glycerol, the biocatalyst particles tend to stick to the bottom of the reactor and the abrasive effect of the magnetic stirrer leads to their disruption, if long reaction times are used. In future work, magnetic stirring should be avoided so as to maintain the integrity of the matrix and thus the half-life of the biocatalyst. For the purpose, different reactor configurations will be attempted.

List of symbols

Aq	Aquaphilicity
a_w	Thermodynamic activity of water
CCRD	Central composite rotatable design
DG	Diglyceride(s)
FFA	Free fatty acid(s)
Gly	Glycerol
(Gly/TG)	Molar ratio glycerol/triglycerides
(L)	Amount of biocatalyst used (g/100 g oil)
MG	Monoglyceride(s)
RSM	Response surface methodology
R^2	Determination coefficient (quadratic correlation coefficient)
R^2_{adj}	Adjusted R_2
TG	Triglyceride(s)

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