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Response surface modeling of glycerolysis catalyzed by *Candida rugosa* lipase immobilized in different polyurethane foams for the production of partial glycerides

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Abstract

Monoglycerides (MG) and diglycerides (DG) are the most widely used emulsifiers in food and pharmaceutical industries. In this study, MG and DG were produced by inter-esterification of refined olive residue oil with glycerol (glycerolysis), in *n*-hexane, catalyzed by *Candida rugosa* lipase immobilized in different biocompatible hydrophilic polyurethane foams, A and B. These foams, with aquaphilicities of 3.7 and 2.8, were prepared with a toluene diisocyanate ("Hypol FHP 2002^{TM}") and a diphenylmethane diisocyanate ("Hypol FHP X4300^{TM}") pre-polymer, respectively.

Response surface methodology was used for modeling the reaction, as a function of the molar ratio glycerol/triglycerides (Gly/TG, 0.5–2.0) and the initial water activity (a_w) of the biocatalyst (A, 0.24–0.91; B, 0.37–0.91). Experiments were carried out following a central composite rotatable design. With lipase in foam A, production of MG and DG could be described by first order polynomials. With foam B, MG and DG production could be fitted to concave and flat surfaces, described by a second and a first orders polynomials, respectively.

The best productions of MG and DG were achieved with the lipase in the less hydrophilic foam, B: at 24 h reaction time, 32% (w/w) MG and 18% (w/w) DG were obtained, when the initial a_w of the biocatalyst was 0.83, with a Gly/TG of 1.

Keywords: Glycerides; Glycerolysis; Lipase; Polyurethane foam; Response surface design

1. Introduction

Monoglycerides (MG) and diglycerides (DG) are the most widely used emulsifiers in food and pharmaceutical industries. Current processes for MG and DG production consist on the inter-esterification of triglycerides (TG) with glycerol (glycerolysis) in the presence of nonselective inorganic catalysts at high temperatures (200–250 °C) [1].

The replacement of inorganic catalysts by lipases (E.C. 3.1.1.3.), in the synthesis of partial glycerides, avoids side product formation and is less polluting

Abbreviations: 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 of glycerol/triglycerides; MG, monoglyceride(s); Pu, polyurethane; RSM, response surface methodology; R^2, determination coefficient (quadratic correlation coefficient); R^2_{adj}, adjusted R^2; TG, triglyceride(s)

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and energy consuming because of the mild conditions used. However, lipases for food and pharmaceutical processes are expensive because they must be free of other enzymes. Thus, to make the enzymatic process competitive, they should be reusable and stable. The activity and the operational stability of an immobilized lipase are known to depend on the molecular structure of the lipase, on the type of support and immobilization method, as well as on the reaction medium conditions [2–4]. They also seem to be related to the water activity of the system [5,6].

Lipase immobilization in polyurethane (PU) foams, where entrapment methods are coupled with chemical binding during polymer synthesis, have been reported by several authors [5,7–15].

In this study, MG and DG were produced by glycerolysis of refined olive residue oil, in *n*-hexane, catalyzed by *Candida rugosa* lipase immobilized in different biocompatible hydrophilic polyurethane foams with different hydrophobicities. The aim of this study was to investigate the effect of different immobilization supports on the kinetics of glycerolysis. For both immobilized preparations, the glycerolysis reaction was modeled, by response surface methodology (RSM), as a function of both the molar ratio glycerol/triglycerides (Gly/TG) and the initial water activity (a_w) of the biocatalyst used, and the reaction conditions optimized.

2. Materials and methods

2.1. Materials

2.1.1. Enzymes

The lyophilized lipase from *C. rugosa* (lipase AY) was a gift from Amano, UK.

2.1.2. Immobilization matrices

Hydrophilic polyurethane pre-polymers, "Hypol FHP 2002™" and "Hypol FHP X4300™", were kindly donated by Hampshire Chemical GmbH, Germany. "Hypol FHP 2002™" is a toluene diisocyanate (TDI) pre-polymer and "Hypol FHP X4300™" is composed by diphenylmethane diisocyanate (MDI) groups. "Hypol FHP 2002™" and "Hypol FHP X4300™" foams have aquaphilicities [16] of 3.7 and 2.8, respectively [15].

2.1.3. Reagents

Refined olive residue oil was a gift from José Carvalho Coimbra, Avanca, Portugal. Olive residue oil is obtained from olive cake by solvent extraction (*n*-hexane), after olive oil has been extracted by physical means. Triolein, trimyristin, diolein (mixed isomers), monoolein, oleic acid and glycerol (99%) of analytical grade were purchased from Sigma, USA. All other chemicals were of analytical grade and obtained from various sources.

2.2. Methods

2.2.1. Immobilization

Lipase immobilization in hydrophilic polyurethane foams occurred simultaneously with the polymerization of the polyurethane pre-polymers, in the presence of water, as previously described [9]. "Hypol FHP 2002™" foams were prepared by mixing 0.6 g of pre-polymer with 0.6 g of phosphate buffer solution (0.020 M KH_2PO_4 + 0.027 M Na_2HPO_4; pH 7.0) and 0.35 g of lipase powder. To prepare the immobilized lipase in the "Hypol FHP X4300™" foam, 0.2 g of lipase powder, 0.4 g of pre-polymer and 0.4 ml of phosphate buffer solution were used. The lipase load was set according to the highest efficiency obtained in the hydrolysis of olive residue oil carried out with the same enzyme entrapped in these two polymers [15]. The resulting foams were cut into small cuboids (~0.07 cm^3) and introduced in the reaction medium.

Different a_w values were obtained by drying the immobilized preparations at 40 °C, under reduced pressure, for different lengths of time [15]. Before the cuboids were added to the reaction medium, their water activity (a_w) was measured at 25 °C in a ROTRONIC HYGROSKOP DT humidity sensor (DMS-100H).

2.2.2. 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. The concentration of oil used was the usual concentration in the miscella, i.e. the solution of crude oil in hexane after extraction at industrial scale. The olive residue oil was previously treated with alumina to remove DG, MG, FFA,

oxidation products and traces of water [5,17]. Both the molar ratio glycerol/triglycerides (Gly/TG) and the initial water activity (a_w) of biocatalyst used varied according to the followed experimental design (cf. Section 2.2.4).

The reaction was carried out in a thermostated cylindrical glass vessel closed with a rubber stopper at 30 °C, under magnetic stirring. After 24 h reaction time, samples were taken and residual TG and products analyzed.

2.2.3. Analytical methods

After separation by thin layer chromatography and methylation, TG and DG were assayed as fatty acid methyl esters, as previously described [17]. The FFA were assayed using the Lowry and Tinsley's colorimetric method [18] with benzene replaced by *n*-heptane [10]. Since monoglycerides showed a low solubility in *n*-hexane, their quantification was achieved via an indirect method [10].

2.2.4. Experimental design

Response surface methodology (RSM) was used for modeling the glycerolysis reaction and to optimize reaction conditions [19,20]. With RSM, several variables are tested simultaneously with a minimum number of trials according to special experimental designs based on factorial designs [19,20]. The response y is described by a polynomial equation as a function of the p independent variables. 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 addition, partial differentiation of polynomial equations is used to find the optimum of a multivariate function. These solutions are called stationary points [19,20].

For each immobilized preparation, the experiments were carried out following a central composite rotatable design (CCRD) as a function of both the initial molar ratio glycerol/triglyceride (Gly/TG) and the initial water activity (a_w) of the biocatalyst. Thus, for each immobilized lipase preparation, a total of 11 experiments was carried out in each CCRD: four factorial points, coded levels as (+1) and (–1); four star points, coded as $(+\sqrt{2})$ and $(-\sqrt{2})$; and three center points coded as 0. The levels considered in both the CCRD are as shown in Table 1. Concerning the initial a_w of the biocatalyst used, the levels $(+\sqrt{2})$ and

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

Coded levels	Molar ratio (Gly/TG)	Water activity (a_w) of the biocatalyst	
		"Hypol FHP 2002™"	"Hypol FHP X4300™"
(–1)	1.0	0.339	0.453
(+1)	3.0	0.815	0.829
$(-\sqrt{2})$	0.5	0.240	0.375
$(+\sqrt{2})$	3.5	0.914	0.907
0	2.0	0.577	0.641

Table 2
Effects (L, linear; Q, quadratic) of the tested variables and interactions, and respective significance levels (α), on the production of FFA, MG and DG during glycerolysis of refined olive residue oil in *n*-hexane, catalyzed by *C. rugosa* lipase immobilized in different PU foams

Response	Variable	Effects on	
		"Hypol FHP 2002™"	"Hypol FHP X4300™"
FFA (%)	a_w (L)	11.50[a]	8.86[a]
	a_w (Q)	−6.21[a]	4.14[b]
	Gly/TG (L)	−2.95[c]	−5.98[c]
	Gly/TG (Q)	0.97 n.s.	3.93[b]
	Interaction	−0.57 n.s.	0.07 n.s.
MG (%)	a_w (L)	−3.44 n.s	9.39[a]
	a_w (Q)	–	14.98[a]
	Gly/TG (L)	6.69[b]	−6.05[c]
	Gly/TG (Q)	–	1.89 n.s.
	Interaction	−2.50 n.s.	−7.39[c]
DG (%)	a_w (L)	7.01[b]	11.29[c]
	a_w (Q)	–	–
	Gly/TG (L)	−1.65 n.s.	−6.70[b]
	Gly/TG (Q)	–	–
	Interaction	−4.53 n.s.	−2.45 n.s.
Converted TG (%)	a_w (L)	14.89[a]	22.31[c]
	a_w (Q)	−9.79[b]	11.19 n.s.
	Gly/TG (L)	−2.03 n.s.	−18.65[c]
	Gly/TG (Q)	−2.80 n.s.	11.03[b]
	Interaction	−7.16[b]	−2.05 n.s.

n.s., Not significant effects.
[a] At $\alpha < 0.001$.
[b] At $\alpha < 0.05$.
[c] At $\alpha < 0.01$.

$(-\sqrt{2})$ correspond to: (i) the a_w of the biocatalyst immediately after immobilization, and (ii) the final equilibrium a_w value attained by drying [15], respectively.

2.2.5. Statistical analysis

The results of each CCRD were analyzed using the software "Statistica™", version 5, from Statsoft, USA. The linear and quadratic effects of (Gly/TG) and (a_w) and the linear interaction (Gly/TG) × (a_w) on the kinetics of glycerolysis were calculated. Their significance was evaluated by analysis of variance. A 3D 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 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}) [21].

3. Results and discussion

Significant effects, either linear or quadratic, of the molar ratio (Gly/TG) and of the initial a_w of the biocatalyst on the production of FFA, MG, DG and on the TG conversion (i.e. TG converted to FFA, MG and DG), during the glycerolysis reaction catalyzed by each of the biocatalysts tested, are as shown in Table 2. Positive effects of the factors (Gly/TG) or (a_w) or of their interaction (Gly/TG) × (a_w) indicate that the response increases with the increase in these factors.

Also, a multiple regression analysis was performed to fit first or second order polynomial equations to the experimental data points (Table 3), which can be described by 3D response surfaces (Figs. 1–4). 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 [21].

After 24 h glycerolysis, the production of FFA can be well-fitted to second order models, as a function of Gly/TG and a_w, representing a convex or a concave

Table 3
Model equations for the response surfaces fitted to the experimental data points from glycerolysis reaction, as a function of the molar ratio (Gly/TG) and the initial a_w of the immobilized lipase in different PU foams, and respective R^2 and R^2_{adj}

Compounds	Model equations	R^2	R^2_{adj}
Free fatty acids (%)	"Hypol FHP 2002™": $FFA = -14.71 + 89.80a_w - 54.81a_w^2 - 2.73(Gly/TG) + 0.49(Gly/TG)^2 - 1.20(Gly/TG) \times (a_w)$	0.995	0.989
	"Hypol FHP X4300™": $FFA = 13.66 - 30.65a_w + 58.49a_w^2 - 4.19(Gly/TG) + 1.97(Gly/TG)^2 - 10.39(Gly/TG) \times (a_w)$	0.938	0.885
Monoglycerides (%)	"Hypol FHP 2002™": $MG = -0.995 + 3.26a_w + 6.37(Gly/TG) - 5.24(Gly/TG) \times (a_w)$	0.940	0.850
Diglycerides (%)	"Hypol FHP X4300™": $MG = 61.53 - 207.44a_w + 211.93a_w^2 + 5.79(Gly/TG) + 0.95(Gly/TG)^2 - 19.64(Gly/TG) \times (a_w)$	0.973	0.949
Diglycerides (%)	"Hypol FHP 2002™": $DG = -10.35 + 33.79a_w + 4.67(Gly/TG) - 9.52(Gly/TG) \times (a_w)$	0.925	0.812
	"Hypol FHP X4300™": $DG = -9.97 + 43.07a_w + 0.83(Gly/TG) - 6.52(Gly/TG) \times (a_w)$	0.911	0.845
Converted triglycerides (%)	"Hypol FHP 2002™": $TG_{conv} = -37.71 + 161.12a_w - 86.46a_w^2 + 13.26(Gly/TG) - 1.40(Gly/TG)^2 - 15.04(Gly/TG) \times (a_w)$	0.965	0.921
	"Hypol FHP X4300™": $TG_{conv} = 44.46 - 74.29a_w + 158.36a_w^2 - 9.99(Gly/TG) + 5.51(Gly/TG)^2 - 33.37(Gly/TG) \times (a_w)$	0.912	0.839

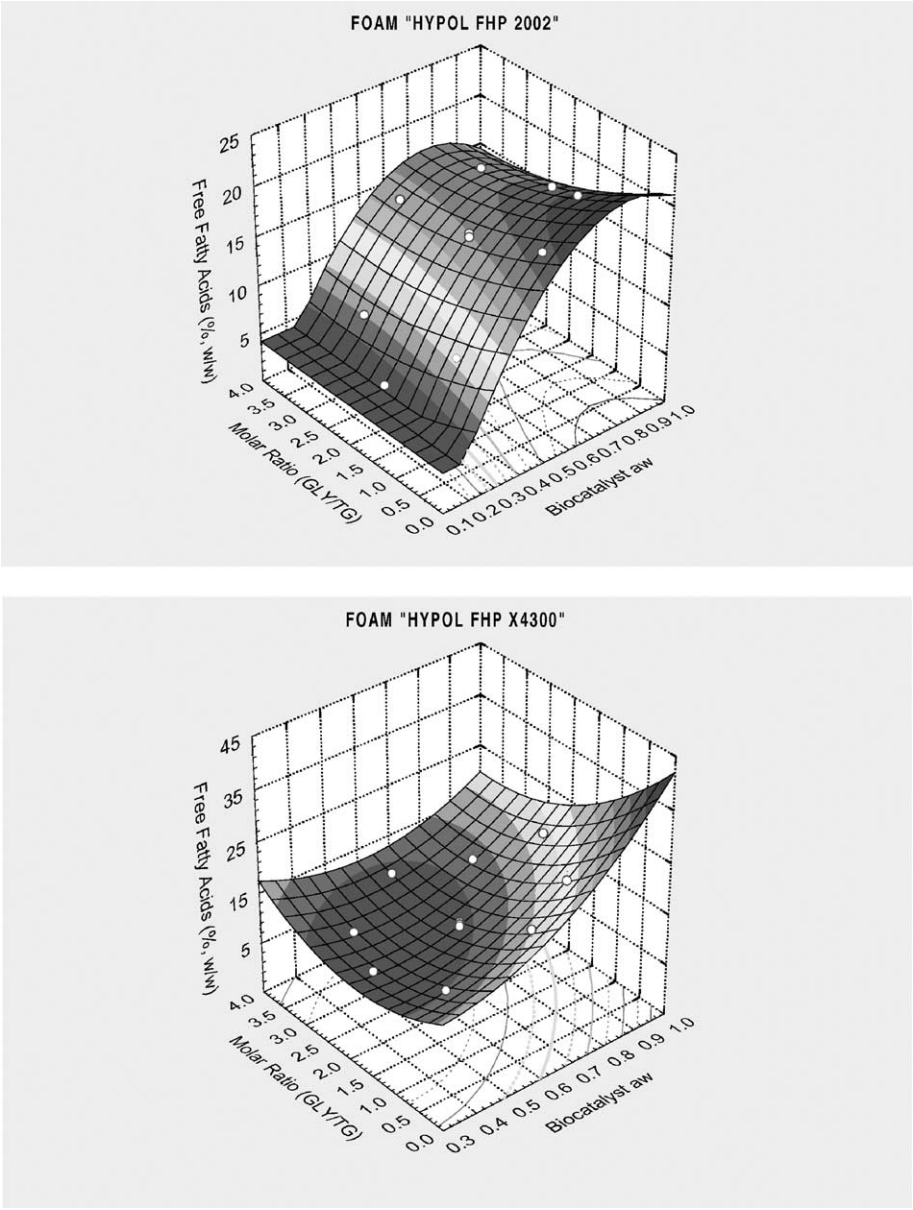

Fig. 1. Response surface fitted to the experimental data points corresponding to the production of free fatty acids, during glycerolysis of olive residue oil in *n*-hexane catalyzed by *C. rugosa* lipase immobilized in PU foams ("FHP 2002" and "FHP X4300"), as a function of the initial a_w of biocatalyst and the molar ratio (Gly/TG).

surface when the lipase in "FHP 2002" or in "FHP X4300" foam is used (Fig. 1), respectively. The FFA are produced during the first step of lipase-catalyzed glycerolysis, where fatty acids are released from glycerides to the reaction medium [5,17]. In both the cases, higher FFA levels are observed at higher a_w values. This may be ascribed to the competing hydrolysis reaction of glycerides [22–25].

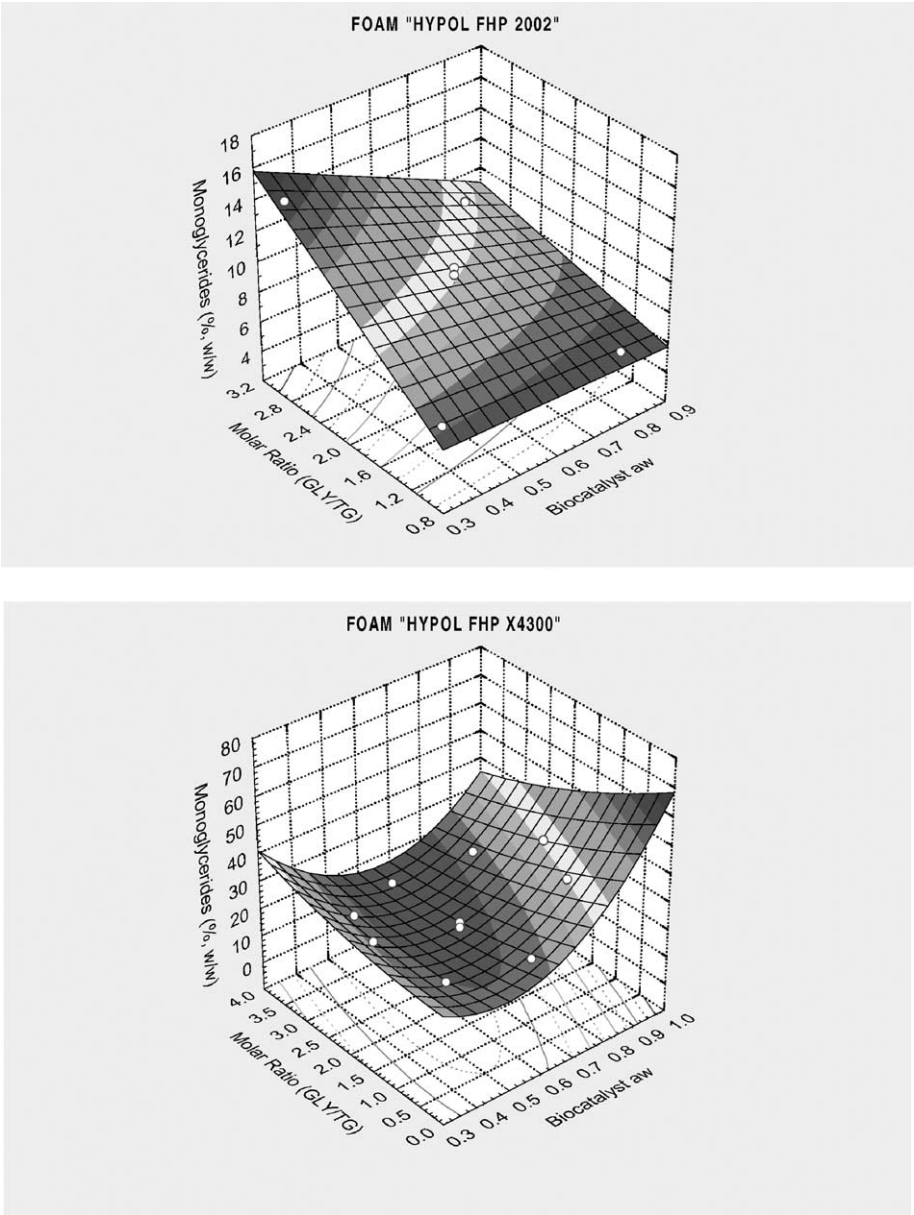

Fig. 2. Response surface fitted to the experimental data points corresponding to the production of monoglycerides, during glycerolysis of olive residue oil in *n*-hexane catalysed by *C. rugosa* lipase immobilized in PU foams ("FHP 2002" and "FHP X4300"), as a function of the initial a_w of biocatalyst and the molar ratio (Gly/TG).

When the *C. rugosa* lipase in "FHP 2002" foam is used, the produced MG can be fitted to a flat surface, described by a first order polynomial (Fig. 2, Table 3): the highest MG levels are obtained under low initial a_w values and high Gly/TG molar ratios. In fact, glycerol is a highly hydrophilic compound and a powerful water binder [26]. Thus, it tends to migrate into the hydrophilic foam, promoting a further a_w decrease in

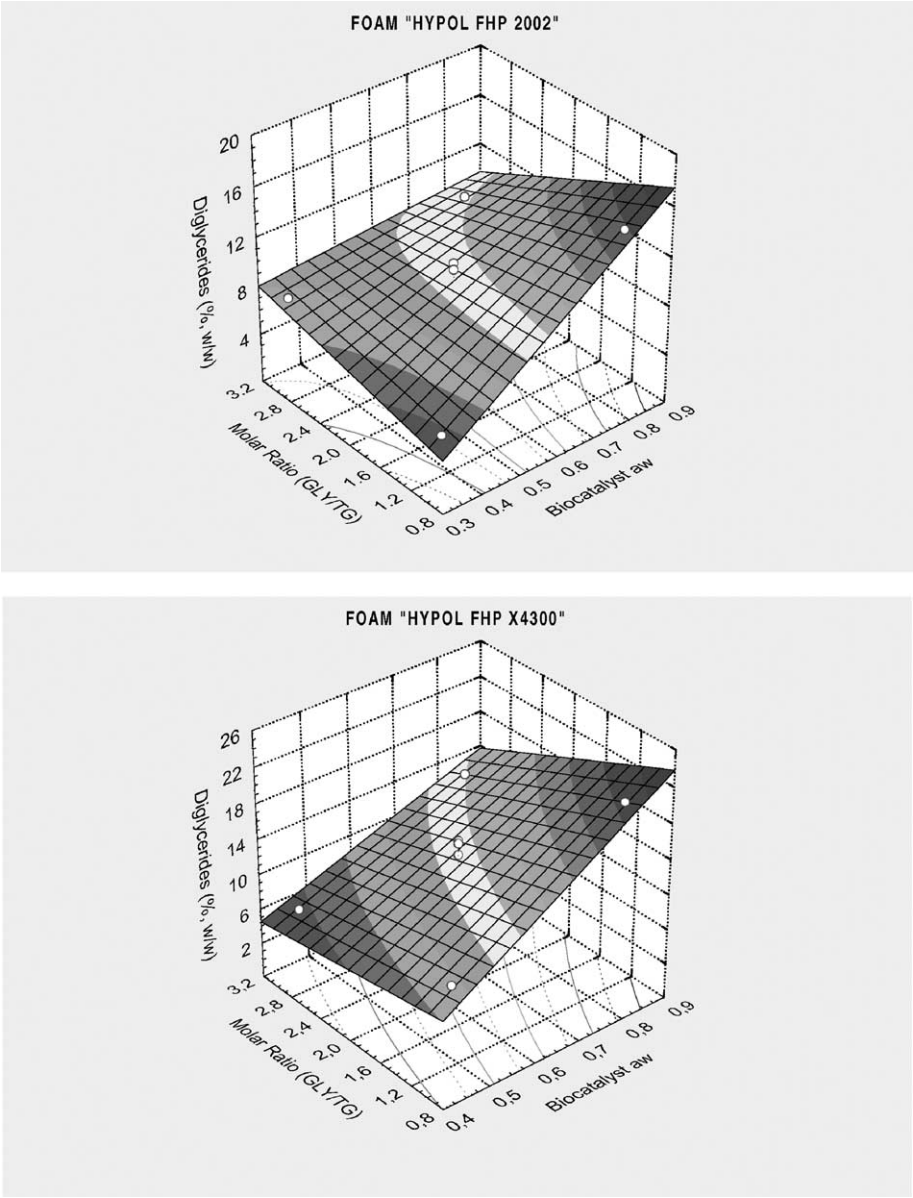

Fig. 3. Response surface fitted to the experimental data points corresponding to the production of diglycerides, during glycerolysis of olive residue oil in *n*-hexane catalysed by *C. rugosa* lipase immobilized in PU foams ("FHP 2002" and "FHP X4300"), as a function of the initial a_w of biocatalyst and the molar ratio (Gly/TG).

the microenvironment and, therefore, promoting the glycerolysis reaction [5]. However, the accumulation of glycerol inside the support may cause lipase deactivation [12]. When the reaction was catalyzed by the lipase immobilized in the less hydrophilic foam ("FHP X4300"), MG production is represented by a concave surface (Fig. 2), described by a second order polynomial with a minimum, calculated by partial differentiation, at an initial a_w value of about 0.66 for a molar Gly/TG ratio equal to 3.8 (Table 3). Higher

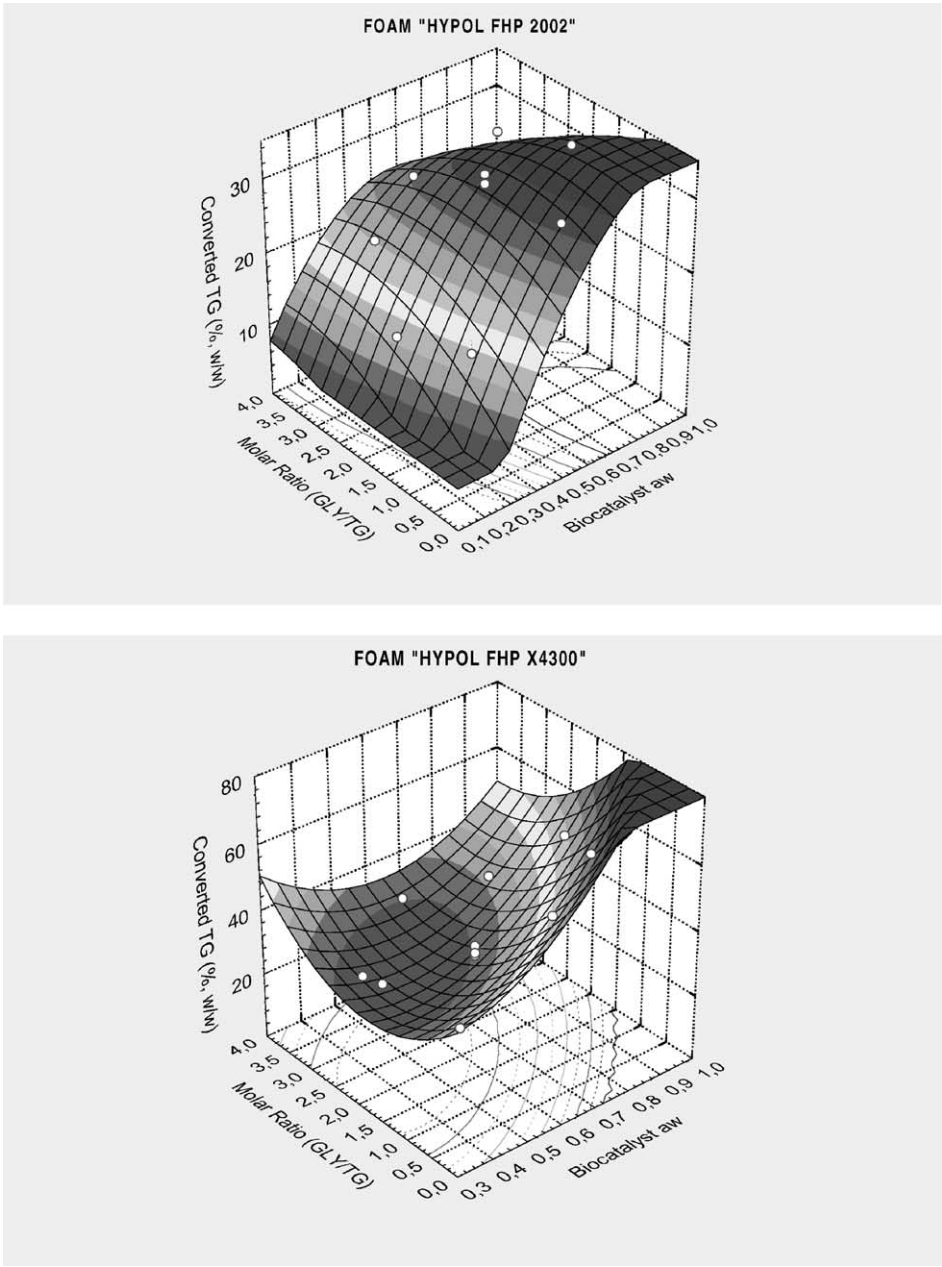

Fig. 4. Response surface fitted to the experimental data points corresponding to the conversion of triglycerides, during glycerolysis of olive residue oil in *n*-hexane catalysed by *C. rugosa* lipase immobilized in PU foams ("FHP 2002" and "FHP X4300"), as a function of the initial a_w of biocatalyst and the molar ratio (Gly/TG).

conversions at extreme a_w values indicate an increase in the hydrolytic and glycerolytic rates, at high and low a_w, respectively.

For both the immobilized preparations, an increase in DG production is observed at increased a_w. This can probably be ascribed to the hydrolysis of glycerides, since the a_w is too high to promote the glycerolysis [5,25]. A direct relationship between DG production and a_w was also found in other systems [5,22,27,28].

The TG consumption after 24 h can be fitted to a convex or a concave surface (Fig. 4), described by second order polynomials (Table 3), when the *C. rugosa* lipase is immobilized in "FHP 2002" or in "FHP X4300" foams, respectively. For both the biocatalysts, the amount of converted TG increased with the initial a_w of the immobilized preparation, probably due to the increase in TG hydrolysis. In fact, the surfaces representing the amount of converted TG (Fig. 4) show the same profile as the response surfaces for FFA (Fig. 1). Concerning the lipase in "FHP 2002" foam, a maximum of only about 35% TG conversion was attained, at high initial a_w values. The highest TG conversion (62%), corresponding to the best productions of MG (32% (w/w)) and DG (18% (w/w)) was observed with the lipase in the "FHP X4300" foam, under an initial a_w of 0.83 and a Gly/TG of 1. These values are comparable to those obtained when the commercial immobilized lipase from *Rhizomucor miehei* ("Lipozyme IM") was used as a catalyst for the same reaction, after 1 h reaction and at the same (Gly/TG) ratio. The explanation may reside on a similar affinity for water, since the aquaphilicity values [16], estimated for both the supports, are similar (3.3 for "Lipozyme IM" and 2.9 for "FHP X4300" foam) [15,17].

Higher activity was also observed with the lipase immobilized in "FHP X4300", when compared to its counterpart, in the hydrolysis of olive residue oil in a biphasic aqueous/*n*-hexane medium [15]. This is probably due to a lower inactivation during foam polymerization, which appears to be related to the strength and/or the number of covalent bonds between the enzyme and the support [15].

4. Conclusions

The immobilization support appears to be a key parameter on the modeling of glycerolysis and on the optimization of reaction conditions aimed at the production of partial glycerides. The use of polyurethane foams with different aquaphilicities leads to distinct microenvironment conditions due to different partition coefficients between the foams and the substrates and products. Higher productions of MG and DG were obtained with the lipase immobilized in the less hydrophilic foam. Probably, this type of foam, by restraining the entrance of glycerol, alleviates the risks of lipase inactivation.

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