Journal of Molecular Catalysis B: Enzymatic 76 (2012) 106–115

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Production of structured lipids by acidolysis with immobilized *Rhizomucor miehei* lipases: Selection of suitable reaction conditions

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ARTICLE INFO

Article history:
Received 25 September 2011
Received in revised form
30 November 2011
Accepted 30 November 2011
Available online 8 December 2011

Keywords:
Acidolysis
Structured lipids
Sunflower oil
Saturated fatty acids
Rhizomucor miehei lipase

ABSTRACT

Enzymatic modification of vegetable oils can be used to produce plastic fat or structured lipids (SL) avoiding the generation of *trans* fatty acids. Biocatalysts previously prepared by immobilization of *Rhizomucor miehei* lipases onto alkylated chitosan microspheres were used to synthesize SL by acidolysis reaction of sunflower oil and palmitic–stearic acids mixture. Effects of some reaction parameters, substrate molar ratio (SR), amount of biocatalyst (*E*) and amount of solvent (*H*), over selected response variables were analyzed by applying an incomplete factorial design of three factors with three levels (Box–Behnken). Responses were selected to evaluate not only the development of desired TAG but also the yield and quality of obtained products, through by-products and undesired trisaturated lipids quantification. The analysis of variable effects showed that *E* was the most significant factor on each analyzed response. Multiresponse optimization with restrictions from practical considerations revealed that maximum saturated fatty acids composition in glycerides of the SL (38.6%) is obtained when SR = 3, *E* = 0.34 g and *H* = 3.2 ml are used. Under these conditions, a change in the composition of the desired TAG group (monounsaturated TAG) from 2.5% in the original oil to 33.9% in the final product was achieved. On the other hand, time and temperature behavior studies showed that generation of trisaturated triacylglycerols and by-products were favored mainly by increasing temperature. After 12 h of reaction, overall hydrolysis and esterification reactions rates were comparable. However, TAG distribution continued changing.

1. Introduction

Nowadays, fats and oils modification is one of the main areas in food processing industry that demands novel economical and green technologies. In this respect, modification of lipids by enzymatic catalysis has become an attractive option. Lipase mediated modifications are specific and can be carried out at moderate reaction conditions with fewer side products. More specifically, the selectivity and specificity of lipases make it possible to obtain structured lipids (SL), which are tailor-made lipids with desired characteristics, like certain physical or chemical properties and/or nutritional benefits. Important structured triacylglycerols, like cocoa butter substitutes, low calorie fats, PUFA-enriched oils, and oleic acid enriched oils, have been synthetized with such technologies [1].

Likewise, processes to obtain SL can be employed as alternative methods to hydrogenation, used to convert vegetable liquid oil into semi-solid fats without the formation of the undesired *trans* fatty acids. It has been shown that *trans* fats can extend shelf life and flavor stability of products, but it can increase the risk of cardiovascular disease by raising the low – density lipoprotein cholesterol (LDL) and decreasing the high – density lipoprotein cholesterol (HDL) [2]. Among other processes, semi-solid fats can be synthesized by acidolysis reactions catalyzed by lipases, where it is possible to incorporate a desired acyl group onto a specific position of the triacylglycerol through the hydrolysis and reesterification reaction steps. In particular, by the use of *sn*-1,3-specific lipase as *Rhizomucor miehei*, free fatty acids (FFA) which are present in a reaction medium could be incorporated in *sn*-1 and *sn*-3 positions of triglycerides while original fatty acids are ideally kept in *sn*-2 position. Following this route for the synthesis of SL, oils and saturated FFA have been used in order to obtain semi-solid fats which are beneficial for human nutrition due to the fact that they preserve unsaturated or polyunsaturated long-chain fatty acids in *sn*-2 position [3–6].

Additionally, undesired reactions could take place because of the presence of diacylglycerols (DAG) produced in the hydrolysis

Abbreviations: AP, Adeq precision; BP, by-products; *D*, desirability function; DAG, diacylglycerols; *E*, amount of biocatalyst; FAME, fatty acid methyl esters; FA, fatty acid; FFA, free fatty acids; *H*, amount of solvent (hexane); LOF, lack of fit; MAG, monoacylglycerols; P + St, palmitic and stearic acids; PUFA, polyunsaturated fatty acids; R^2, *R*-squared; R^2adj, adjusted *R*-squared; R^2pred, predicted *R*-squared; S, saturated fatty acid; SL, structured lipids; SO, refined sunflower oil; SPFA, palmitic–stearic acids blend; SR, substrate molar ratio; SSS, trisaturated triacylglycerols; SUS, monounsaturated triacylglycerols; TAG, triacylglycerol; U, unsaturated fatty acid.

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doi:10.1016/j.molcatb.2011.11.022

step. Acyl migration of the long-chain acyl groups, from position *sn*-2 to *sn*-1 or *sn*-3 caused by a certain amount of DAG in the reaction mixture, has been reported in acidolysis, decreasing the yield and the purity of the desired TAG [7,8].

The potential of enzymatic processes to obtain SL has been subject of numerous researches, in which commercial biocatalysts were mainly used [5,9–12]. Likewise, the high cost and the easy attrition of the commercially available immobilized lipases restrict its use in large scale processes. As a possible solution, in a previous work we prepared 8 biological catalysts suitable to produce SL using modified chitosan microspheres as support for immobilization of R. miehei lipases [13]. It is worth mentioning, that the development of this support was based on the well-known properties of chitosan [14] and the need of changing its hydrophilic nature to a hydrophobic one in order to stabilize the open conformation of lipases and promotes their hyperactivation after their immobilization [15]. The obtained biocatalysts were used in acidolysis of sunflower oil and palmitic and stearic FFA at specific conditions. The most active biocatalyst achieved a change in the composition of palmitic and stearic acid from a value of 9.6% in the original oil to 49.1% in the final SL, representing an almost 3-fold enzyme hyperactivation. Additionally, they proved to be mechanically resistant even after several hours of use [13]. Even so, in order to scale this procedure, a wider knowledge of process variables is necessary. In this contribution, the influence of specific parameters of the reaction – the amount of biocatalyst (*E*), the substrate mole ratio (SR) and the amount of solvent (*H*) – has been studied on selected response variables. These were: % P + St, palmitic and stearic acids composition respect to total fatty acids in glycerides, % SUS, monounsaturated triglycerides (desired TAG) respect to total triglycerides, % BP, by-products (mono and diglycerides) respect to total glycerides and % SSS, trisaturated triglycerides respect to total triglycerides, as indicative of the possible acyl migration. In order to evaluate the effect of experimental factors on the responses, with a minimum number of trials, an incomplete factorial design of three factors with three levels (Box–Behnken) was applied. A fitting model for each response was obtained and a multi-response optimization was made to obtain optimal working conditions. Independent supplementary experiments were carried out at the optimum condition in order to determine the validity of fitting models in the explored domain. Additionally, the time-course of acidolysis reaction assayed in the optimum condition at two different reaction temperatures (50–60 °C) was also studied.

To sum up, this contribution intends to achieve a full understanding of the effect of main reaction parameters when a novel biocatalyst, specially prepared for modifying lipids, is used. Likewise, it wants to find the most suitable reaction conditions in order to produce the greatest change in the sunflower oil composition based on practical considerations.

2. Materials and methods

2.1. Materials

Chitosan of low molecular weight with a degree of 75–85% of deacetylation and 12.6% of moisture was obtained from Sigma–Aldrich. Refined sunflower oil (SO) was purchased from a local grocery store and it was used as received (peroxide value (PV): 1.94 mequiv./kg). *R. miehei* lipase (>20,000 U/g, Novozymes) from *Aspergillus oryzae* conditioned in the form of liquid, dodecyl aldehyde (92%, Aldrich), sodium cyanoborohydride $NaCNBH_3$ (>95%, Fluka), palmitic–stearic acids blend (SPFA) with purity grade 49–54% and 40–51% respectively (Fluka) were purchased from Sigma–Aldrich (Germany). Fatty acid methyl esters (FAME) standards were purchased from Supelco (Bellefonte, USA). Other standards (1,2,3-trioctadecanoyl-glycerol, 1,2,3-trihexadecenoyl-glycerol, 1,2,3-trioctadecadienoyl-glycerol, 1,2,3-trioctadecenoyl-glycerol, 1,2,3-trihexadecanoyl-glycerol, 1,2,3-tridecanoyl-glycerol, 1,2-distearoyl-3-palmitoyl-rac-glycerol, 1,3-dipalmitoyl-2-oleoylglycerol, 1,3-dioleoyl-2-palmitoyl-glycerol, 1,2-dilinoleyl-3-palmitoyl-rac-glycerol, 1,2-dioleoyl-3-stearoyl-rac-glycerol, 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol, 1,2-distearoyl-3-oleoyl-rac-glycerol, 1,3-dipalmitoyl-rac-glycerol, 1-monopalmitoyl-rac-glycerol, octadecenoic acid, tetradecane) were of more than 98% purity and were obtained from Sigma Chemical Co. (St. Louis, USA). Pyridine was from J.T. Baker (Philipsburg, USA) and N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Fluka (Buchs, Switzerland). All the other reagents, gases and solvents were of analytical or chromatographic grade.

2.2. Preparation of biocatalyst

Modified chitosan microspheres were prepared following the procedure described in our previous work [13], using dodecyl aldehyde in monomolar ratio 1:1 NH_2/aldehyde and 1.5 h of contact times with aldehyde. In the same way, lipase immobilization on obtained support was carried out as reported in the aforementioned work.

2.3. Lipase-catalyzed acidolysis

Enzymatic acidolysis reactions were carried out with refined sunflower oil and a mixture of free palmitic and stearic acids (SPFA). Acidolysis was performed as follows: 110 mg of SO and the correspondent amount of SPFA, needed to obtain the desired molar ratio of substrates, were dissolved in the corresponding amount of hexane and later mixed and heated at 60 °C. The reaction began when immobilized lipase was added. Reactions were performed in a screw-capped test tube in a water bath with temperature controller and magnetic agitation at 250 rpm. After 12 h (unless another time is specified), reactions were stopped removing enzymes by filtering. The solvent from the reaction mixtures was removed by evaporation under a nitrogen atmosphere and then, the reaction mixtures were maintained at −20 °C until analysis.

2.4. Experimental design

In order to evaluate the influence of chosen reaction parameters on response variables, an incomplete design of three-level and three-factor factorial with three central points (Box–Behnken design) was used, in which experimental points have been especially selected to allow an efficient estimation of coefficients in quadratic models. The three selected factors were: the amount of biocatalyst (*E*), the substrate mole ratio (SR) and the amount of solvent, hexane (*H*). They were decided according to similar works [6,16,17] in which these variables had significant effects. The setting of the factors was determined in accordance with previous studies, in addition to practical considerations. The amount of hexane in the reaction medium was set so that both, the substrates-solvent diluted systems, commonly used in continuous operations, and free solvent green systems could be simulated. Consequently, the ranges chosen were: *E* (0.21–0.39 g), SR (3–6 mol SPFA/mol SO) and *H* (0.0–3.2 ml).

Matrix design was performed using the DESIGN EXPERT 7.0 software. Used factors and levels, together with analyzed responses, are shown in Table 1. The experiment order was completely randomized.

Likewise the mentioned software was used to find fitting models in which the coefficients of the postulated models were calculated

Table 1
Experimental variables setting and selected response variables for acidolysis at 60 °C and 12 h of reaction.

N[a]	Experimental factors			Response variables[b]			
	SR (mol SPFA/mol SO)	E (g)	H (ml)	P + St (%)	SUS (%)	BP (%)	SSS (%)
1	4.5	0.30	1.6	39.1 (39.3)	34.5 (35.8)	22.5 (22.2)	8.6 (8.5)
2	3	0.30	0	37.9 (39.0)	30.6 (33.1)	19.6 (19.7)	5.5 (5.4)
3	4.5	0.39	3.2	38.5 (38.4)	36.8 (37.1)	24.3 (24.8)	9.5 (9.4)
4	6	0.39	1.6	41.0 (41.9)	32.9 (33.3)	20.1 (20.6)	9.7 (9.2)
5	4.5	0.21	0	38.0 (37.4)	30.2 (29.8)	19.3 (19.2)	4.8 (4.9)
6	6	0.21	1.6	31.1 (32.6)	21.9 (23.5)	16.3 (16.4)	5.2 (5.6)
7	4.5	0.30	1.6	42.2 (39.3)	35.6 (35.8)	21.9 (22.2)	8.9 (8.5)
8	3	0.39	1.6	37.6 (36.8)	36.7 (35.9)	25.2 (24.8)	8.7 (8.8)
9	4.5	0.21	3.2	30.1 (29.4)	21.1 (21.0)	18.4 (18.7)	4.7 (4.3)
10	6	0.30	3.2	36.3 (35.8)	27.9 (28.0)	17.5 (16.9)	6.4 (6.8)
11	3	0.21	1.6	34.5 (34.3)	27.2 (26.1)	19.9 (20.5)	5.3 (5.2)
12	6	0.30	0	45.1 (44.5)	32.6 (30.5)	17.8 (17.7)	6.2 (5.8)
13	4.5	0.30	1.6	37.8 (39.3)	37.3 (35.8)	23.2 (22.2)	8.1 (8.5)
14	3	0.30	3.2	36.7 (37.8)	31.0 (30.6)	23.6 (23.3)	6.3 (6.4)
15	4.5	0.39	0	40.3 (40.3)	33.3 (33.3)	21.3 (21.5)	6.5 (6.9)

[a] Run number.
[b] Experimental value (predicted value by corresponding model).

on the experimental response. Multiple quadratic regression models were obtained as follows:

$$\hat{y} = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii}x_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij}x_i x_j \quad (1)$$

where $\hat{y}$ is the response in study, β_0 the intercept, β_i main effect coefficient for the ith factor, β_{ij} interaction model coefficient for the interaction between factor ith and jth, and x_i and x_j are each of the factors considered.

The good quality of the fitting models was tested with analysis of variance (ANOVA), which employs statistical coefficients to describe the models. They are: R-Squared (R^2), a measure of the amount of variation around the mean explained by the model, Adjusted R-Squared (R^2adj), a measure of the amount of variation around the mean explained by the model, adjusted for the number of terms in the model. The Predicted R-Squared (R^2pred) is a measure of the amount of variation in new data explained by the model. The difference between R^2pred and R^2adj should not be greater than 20%. Otherwise there may be a problem with either the data or the model. The lack of fit value (LOF) should not be significant. The Adeq Precision (AP) measures the signal to noise ratio. A noise ratio greater than 4 is desirable and thus the model can be used to navigate the design space. Respect to the model terms, the insignificant terms were deleted based on P value. Values of $P > F$ less than 0.05 indicate model terms are significant and values greater than 0.1 indicate the model terms are not significant. It is worth mentioning that some non-significant model terms were retained because they increased the quality of the fit.

2.5. Multiresponse optimization and verification of models

In optimization problems it is common that several response variables are of interest. In this case, determination of optimum conditions of the independent variables requires simultaneous consideration of all the responses. This is called a multiple response problem which can be solved by using a desirability function D. Individual response surfaces are determined for each response. Predicted values, obtained from each response surface, are transformed to a dimensionless scale d_i. The scale of the desirability function ranges between $D = 0$ (for an unacceptable response value) and $D = 1$ (for a completely desirable one). D is calculated combining the individual desirability values [18]. By using suitable software as DESIGN EXPERT 7.0, it is possible to determine the set of variable values that maximize D.

Based on the aforementioned, different specifications on response variables were imposed with the aim to obtain the parameter values that satisfied those requirements. Firstly, the maximization of % P + St and % SUS and the minimization of % BP and % SSS were simultaneously set, allowing the reaction parameters to take any value within of the analyzed ranges. Then, based on the obtained solution and practical considerations, other criteria were evaluated. The selected conditions as optimum were assayed in independent experiments and used for checking the fittings models.

2.6. Time course of acidolysis products and temperature effect

Generally, enzymatic reactions and therefore, reaction products are influenced not only by the analyzed variables but also by reaction time and temperature [11]. So, in this specific reaction (acidolysis) the incorporation of fatty acids, the amount of by-products and the acyl migration, among others, could change with these parameters. On this account, acidolysis reactions were carried out, in the previously selected optimum condition for the parameters, at different times (2, 4, 8, 12, and 24 h) and at two different temperatures (50 and 60 °C) of reaction. The additional value of temperature, 50 °C, was selected based on the melting point of palmitic and stearic mixture (lowest limit) and on the boiling point of hexane at atmospheric pressure (highest limit).

2.7. Acidolysis products analysis

2.7.1. Fatty acid composition

Acidolysis reaction products were purified by alkaline deacidification in order to removed free fatty acids, as suggested Carrín and Crapiste [10]. FAME from SL were prepared by cold transesterification with methanolic KOH according to the Official Method Ce 2-66 [19], and were analyzed by gas–liquid chromatography (GLC) with a 4890D series gas chromatograph (Agilent, Hewlett-Packard) and a fused-silica capillary column (SP-2380, 30 m × 0.25 mm × 0.2 μm film thickness; Supelco Inc.). The carrier gas was hydrogen with a linear velocity of 18 cm/s. The injector was used in split mode with a ratio of 1:50. The oven temperature was programmed to be at 170 °C for 15 min, further to increase to 210 °C at a rate of 4 °C/min, and held for 10 min. The injector and detector temperatures were 220 °C. FAME were identified by comparing their retention times with authentic standards. Data acquisition and peak integration were performed using HP 3398A GC Chemstation Software (Hewlett-Packard, 1998).

2.7.2. Triacylglycerol (TAG) profiles

TAG quantification of the non-deacidified reaction product was performed by GLC by means of a 4890D series gas chromatograph (Agilent, Hewlett-Packard) equipped with a FID (adapted from IRMM Method EUR 20831 EN). A metallic capillary column (MXT-65TG, 30 m × 0.25 mm × 0.1 μm film thickness; Restek, Bellefonte, USA) was used. The injector was used in split mode (split ratio of 1:70) and held at 360 °C. The detector temperature was constant and equal to 380 °C. The oven temperature was programmed to be at 40 °C for 4 min, then increased first from 40 °C to 350 °C at the rate of 15 °C/min and then to 360 °C at the rate of 0.2 °C/min. Hydrogen was used as the carrier gas at a linear velocity of 33.6 cm/s. Internal standard method was used to quantify TAG using tripalmitolein as standard. Relative response factors of all available standard TAG were correlated with their relative residence time in order to quantify TAG whose standards were not available. Data acquisition and peak integration were performed using HP 3398A GC Chemstation Software (Hewlett-Packard, 1998). The analysis was carried out identifying each TAG and then grouping them in four different categories according to the number of saturated (S) and unsaturated (U) fatty acids in the molecule (without distinguish positional isomers). Consequently, content of TAG in products is reported as SSS, SUS, UUS, and UUU (% g/100 g on total TAG).

2.7.3. By-products analysis

FFA, monoacylglycerols (MAG) and DAG were analyzed in simultaneous mode with TAG analysis by GLC. This technique also allowed us to identify the presence of free glycerol in the analyzed sample. The internal standard method was used to quantify each group of by-products (FFA, MAG, and DAG) with a calibration curve for each one, being tetradecane the internal standard for FFA and glyceryl tridecanoate for MAG and DAG. Calibration curves were constructed using oleic acid, monopalmitin, and dipalmitin as standards of FFA, MAG, and DAG, respectively. Data acquisition and peak integration were carried out using HP 3398A GC Chemstation Software (Hewlett-Packard, 1998).

3. Results and discussion

3.1. Acidolysis products analysis

3.1.1. Fatty acid composition

The approximate fatty acids composition of the original SO, obtained by GLC analysis as fatty acid methyl esters (FAME), was: 56.6% C18:2 (linoleic acid), 31.0% C18:1 (oleic acid), 6.3% C16:0 (palmitic acid) and 3.2% C18:0 (stearic acid). As expected, the SO was abundant in unsaturated fatty acids and the FA composition was in agreement with other studies [20–22]. The palmitic and stearic acid composition in the obtained SL is shown in Table 1. As it can be observed, all the products of reaction show a much greater composition than SO in P + St and a pertinent decreasing of oleic and linoleic acids (data not shown). For the proposed design, the obtained SL reached a composition of P + St between 30.1–45.1%, which represent a 225–372% increase respect to original oil (9.6%).

3.1.2. Triacylglycerol (TAG) profiles

Table 2 shows the TAG profiles of SO and a certain structured lipid of the experimental design, SL Run 12. The predominant TAG species in SO were: OLL (L = linoleic, O = oleic), LLL, OOL, PLL (P = palmitic) and OOO. The TAG profile for SO was in agreement with other studies [20,22]. Whereas the major TAG species in SL Run12 were: PLSt (P = palmitic, St = stearic), POL, PLP, PLL, POSt and StOL. Comparing with SO, new species were found: PPP, PPSt and StStL. Besides, TAG species with saturated fatty acid increased and TAG species with unsaturated fatty acid decreased, as it was expected, considering that the acidolysis reaction was performed with saturated FA (P + St) as acyl donors. The analysis of TAG groups (SSS, SUS, UUS and UUU) also evidenced the change of the TAG composition, which meant a sharp decrease of the UUU group and an increase of the remaining groups.

Table 2
Triacylglycerol composition of sunflower oil and the SL obtained by multiresponse optimization (wt%).

TAG[a]	Type of TAG[b]	Sunflower oil	SL O1[d] (Run 12)	SL O2[e]
PPP	SSS	ND[c]	1.72	1.58
PPSt	SSS	ND	3.27	3.03
POP	SUS	0.30	4.42	3.47
PLP	SUS	0.98	8.54	8.09
StStP	SSS	0.07	2.47	2.28
POSt	SUS	0.29	6.87	5.57
POO	UUS	2.80	4.25	3.36
PLSt	SUS	0.85	10.94	10.87
POL	UUS	6.54	8.95	9.10
PLL	UUS	8.30	7.07	9.10
StStSt	SSS	0.04	0.68	0.73
StStO	SUS	0.13	2.26	1.96
OOS	UUS	1.26	3.05	2.37
StStL	SUS	ND	4.28	3.93
OOO	UUU	8.12	3.23	2.47
StOL	UUS	2.59	6.80	7.01
OOL	UUU	15.23	5.39	4.76
StLL	UUS	3.06	4.65	6.35
OLL	UUU	27.36	6.61	7.88
LLL	UUU	21.81	3.54	5.55
NI[f]		0.29	1.01	0.54
$\sum$ SSS		0.11	8.13	7.62
$\sum$ SUS		2.51	37.31	33.88
$\sum$ UUS		24.22	34.78	37.29
$\sum$ UUU		71.57	18.77	20.67
% By-product		0.01	17.8	24.90

[a] P: palmitic acid; St: stearic acid; O: oleic acid; L: linoleic acid.
[b] S: saturated acid; U: unsaturated acid.
[c] Not detected.
[d] Structured lipid obtained by optimization resulting from maximization of % P + St and % SUS responses, and minimization of % BP and % SSS responses.
[e] Structured lipid obtained by optimization resulting from maximization of % P + St with SR and H fixed to 3 and 3.2, respectively.
[f] Not identified.

Regarding the SUS group, we wanted it to be the major reaction product, since these lipids contain two saturated fatty acids that give features of solid or semisolid fats. Furthermore, the SUS group preserves the unsaturated fatty acid in *sn*-2 position, provided that the enzyme is specific and migration on the original FA in *sn*-2 position does not occur. On the other hand, the fact that the UUS group increased, revealed that the group became a reaction intermediary between UUU and SUS. It was also found a major amount of the SSS group in the obtained SL. This shows that acyl migration occurred, implying the migration of saturated FA from *sn*-1 or *sn*-3 position to *sn*-2 position. This non-enzymatic reaction was undesirable because it reduces the selectivity to the desired TAG.

The obtained SL reached a composition of SUS between 21.1–37.3% (Table 1), representing a 740–1346% increase respect to the original oil (2.51%).

3.1.3. By-products analysis

As mentioned above, the acidolysis mechanism is initiated by hydrolysis of original fatty acids which allows subsequent esterification with new acyl chains. The hydrolysis products, free fatty acids, monoacylglycerols and diacylglycerols, are necessary intermediaries. However, their presence in the final product decrease the yield of the SL.

The by-products analysis was performed on non-deacidified reaction products. Therefore, the quantified free fatty acids were not only side products but also remaining reagents. On this account,

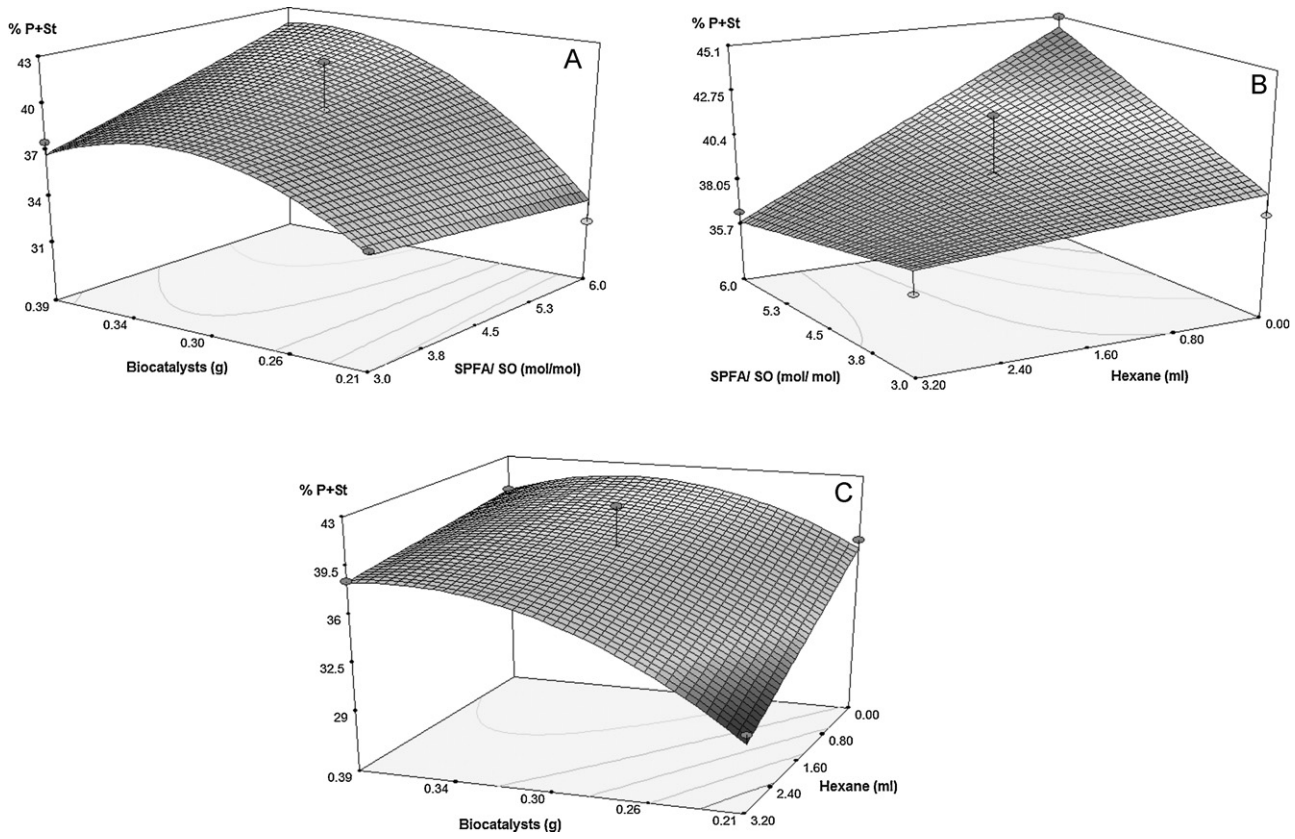

Fig. 1. Response surface plot of the palmitic and stearic acid composition respect to total fatty acids in glycerides (% P + St). Effect of (A) amount of biocatalyst and the substrate mole ratio at amount of hexane fixed at 1.6 ml (central point); (B) amount of hexane and the substrate mole ratio at amount of biocatalyst fixed at 0.3 g (central point); (C) amount of biocatalyst and amount hexane at substrate mole ratio fixed at 4.5 (central point).

in order to compare the different experiences, the percentage of by-products (% BP) was calculated as percentage of monoglycerides and diglycerides respect to total glycerides. While SO had a content of 0.01% of BP (Table 2), the SL had a considerably higher content between 16.3–25.2% (Table 1). This % BP range was found as result of assayed reaction conditions. So, a detailed analysis of the effect of reaction parameters will be made in Section 3.2.3.

It is worth mentioning, that glycerol was not detected in any of the samples, meaning that hydrolysis reaction could not be completed in the analyzed range of experimental variables.

3.2. Model fitting

All the selected models presented AP values higher than 12.7, indicating adequate signals. LOF values were not significative (see Section 2.4 for statistical coefficients description).

3.2.1. Palmitic and stearic acids composition

The effects of the experimental factors on palmitic and stearic acids composition respect to total fatty acids in glycerides (% P + St), considering a quadratic model and the interactions between the independent variables, are shown in Fig. 1. According to R^2 this refined model explains 91.24% of the variability in % P + St which indicates that it is suitable to represent the real relationship among reaction parameters. The R^2adj and R^2pred were 82.49% and 66.20% respectively, which were in reasonable agreement. The fitting model, expressed in actual factors, was:

$$\%\,\mathbf{P}+\mathbf{St} = 13.524 - 1.978 \times \mathrm{SR} + 174.629 \times E - 1.215 \times H + 12.683 \times SR \times E - 0.783 \times \mathrm{SR} \times H + 10.621 \times E \times H - 359.507E^2 \quad (2)$$

Testing the model coefficients with the *t*-test, linear and quadratic terms of the amount of biocatalyst (E) together with the amount of hexane (H) showed to be the most significant factors to determine % P + St according to their *P*-values, E ($P = 0.0013$), E^2 ($P = 0.0105$) and H ($P = 0.0035$). Substrate mole ratio (SR) and interaction $E \times H$ were not significant ($P > 0.1$). *P*-Values of interactions SR × E ($P = 0.0731$) and SR × H ($P = 0.0540$) were relatively low to reject their effects.

The highest levels of incorporation of palmitic and stearic acids were achieved, for all the amounts of hexane, using the highest amount of enzyme (biocatalysts) and the highest ratio of substrates in the reaction media (Fig. 1A). The fact that increasing the molar ratio of substrates increased the enzyme activity would indicate that inhibition of biocatalyst, by acidification of the microenvironment due to an excess of free fatty acids, did not occur [23]. Moreover, enzymatic reaction could have been restricted by low availability of free fatty acids in the reaction medium.

The interaction SR × H is shown in Fig. 1B. As it could be noticed, at the lowest amount of SR and independently of the amount of H, and at the highest amount of H and independently of the SR, the concentration of P + St did not change significantly. On the other hand, this response was maximized using the highest level of SR and without hexane. Although $E \times H$ interaction turned to be insignificant as a general result, it was observed a particular behavior at the central point of SR (Fig. 1C). Working at the lowest level of solvent (hexane: 0 ml), it was found that the composition of P + St increased slowly with the increase of the enzyme concentration up to about 0.30 g. Further increase in enzyme concentration did not result in any increase in % P + St. At the highest level of hexane (3.2 ml), the composition of palmitic and stearic acids was found to increase rapidly as the amount of biocatalyst increased up to about

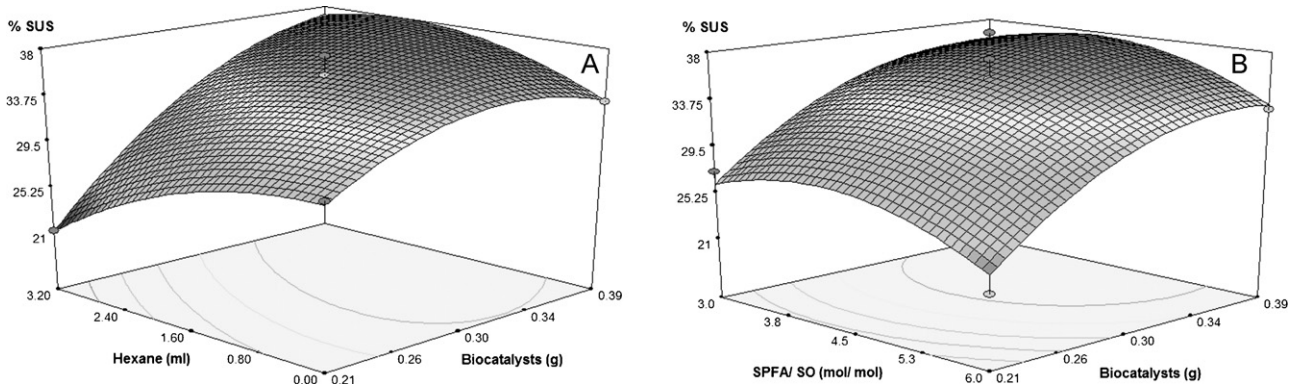

Fig. 2. Response surface plot of monounsaturated triglycerides respect to total triglycerides (% SUS). Effect of (A) amount of biocatalyst and amount of hexane at substrate mole ratio fixed at 4.5 (central point); (B) amount of biocatalyst and the substrate mole ratio at amount of hexane fixed at 1.6 ml (central point).

0.33 g. After that, it remained approximately constant. On the other hand, at the highest level of enzyme, the composition of palmitic and stearic acids was almost the same for all the amounts of hexane, whereas at the lowest level of enzyme, the increase in the amount of hexane resulted in a rapid decrease in the incorporation of P + St. These results could reflect a different behavior of the system with and without solvent, since the incorporation of hexane to the reaction medium not only lowered the viscosity of the system and favored the solubility of substrates, but also diluted them.

According to the results of the statistical analysis, biocatalyst load was the most significant factor affecting the incorporation of P + St, which evidences that no biocatalysts agglomeration takes place in the range of *E* used. The use of an immobilized form of the biocatalyst surely contributed to prevent its aggregation [24]. This result contributes to demonstrate that the used support of immobilization was appropriate to be used with lipases in these working conditions.

3.2.2. Monounsaturated triglycerides as desired TAG

The effects of the experimental factors on monounsaturated triglycerides (desired TAG) respect to total triglycerides (% SUS) are shown in Fig. 2. The analysis of variance (ANOVA) indicated that the model represented by Eq. (3), quadratic in SR, *E* and *H* was the most adequate to represent the actual relationship between the response and the significant factors, with a $R^2 = 94.49\%$. The R^2adj and R^2pred were 88.99% and 79.15% respectively, which were in reasonable agreement. The best predicted model for % SUS in terms of the factors was:

$$\%\,\mathrm{SUS} = -29.079 + 10.965 \times \mathrm{SR} + 254.1984 \times E - 4.44289 \times H + 21.835 \times E \times H - 1.313 \times SR^2 - 391.081 \times E^2 - 0.900 \times H^2 \quad (3)$$

Related to the SUS model, the most significant factor proved to be the amount of biocatalyst in the reaction medium ($P < 0.0001$). Other significant terms were: SR^2 ($P = 0.0114$), E^2 ($P = 0.0082$), H^2 ($P = 0.0328$) and the interaction $E \times H$ ($P = 0.007$). The lineal terms SR and *H* had relatively low *P*-values ($P = 0.0664$ and $P = 0.0744$, respectively) to reject their effects.

Fig. 2A shows the effect of the amount of biocatalyst, the amount of hexane and their mutual interaction on % SUS. At the lowest level of hexane, the % SUS was found to rise with an increase in the amount of biocatalyst up to a certain extent (0.33 g), but further increase in *E* resulted in a small decrease in the analyzed response. At the highest level of hexane, the % SUS continued rising with an increase in the amount of biocatalyst until reaching the maximum value. Furthermore, it can be noticed that, at the lowest amount of enzyme, the % SUS decreased with the increase of the amount of hexane whereas at the highest level of enzyme, the % SUS rose with the increase of the amount of solvent up to a certain point (2.4 ml), decreasing slightly when greater amounts were used. That could indicate that adding hexane to the reaction medium with a small amount of biocatalyst, causes a dilution effect, which possibly makes substrates not to be so available for the enzyme.

The effect of substrate mole ratio on % SUS was very weak, showing for each level of biocatalyst almost the same response value for the range of SR (3–5.3). That shows the insignificant interaction $E \times \mathrm{SR}$. Higher values of free fatty acids in the medium resulted in a small decreasing in the % SUS (Fig. 2B).

3.2.3. By-products: mono and diglycerides

The effects of the experimental factors on by-products (mono and diglycerides) respect to total glycerides (% BP) are shown in Fig. 3. The analysis of variance (ANOVA) indicated that the model represented by Eq. (4), quadratic in SR and *H* was the most adequate for representing the actual relationship between the response and the significant factors, with a $R^2 = 97.26\%$. The R^2adj and R^2pred were 94.52% and 86.64% respectively, which were in reasonable agreement. The predicted model for % BP in terms of the factors was:

$$\%\,\mathbf{BP} = 4.681 + 5.884 \times \mathrm{SR} + 13.144 \times E + 1.949 \times H - 0.445 \times \mathrm{SR} \times H + 6.590 \times E \times H - 0.729 \times \mathrm{SR}^2 - 0.463 \times H^2 \quad (4)$$

According to the *P*-value linear terms of the substrate mole ratio (SR) together with the amount of biocatalyst (*E*) showed to be highly significant factors ($P < 0.0001$) to determine % BP. Remaining terms of the model proved to be significant were: *H* ($P = 0.0155$), SR^2 ($P = 0.0015$), H^2 ($P = 0.0082$) and the interactions $\mathrm{SR} \times H$ ($P = 0.0113$) and $E \times H$ ($P = 0.0192$).

As it can be observed in Fig. 3A, for all the levels of amount of hexane (0–3.2 ml), the % BP was found to decrease with an increase in substrate mole ratio. At low SR values the increment of *H* resulted in the increment of % BP. However, as soon as SR values were higher, the effect of *H* over % BP turned to be insignificant. Similarly, Fig. 3B shows that for all quantities of biocatalyst (*E*), the % BP decreased with increasing SR.

Regardless of the amount of hexane, an increment in the amount of biocatalyst incremented the amount of by-products. Probably, the use of more enzymes involved the addition of more water to the system and favored the hydrolysis step [4]. On the other hand, an increment in substrate mole ratio decreased this response. That could be explained based on the fact that an increment in the amount of palmitic and stearic free fatty acids in the acidolysis medium makes possible that intermediate compounds can

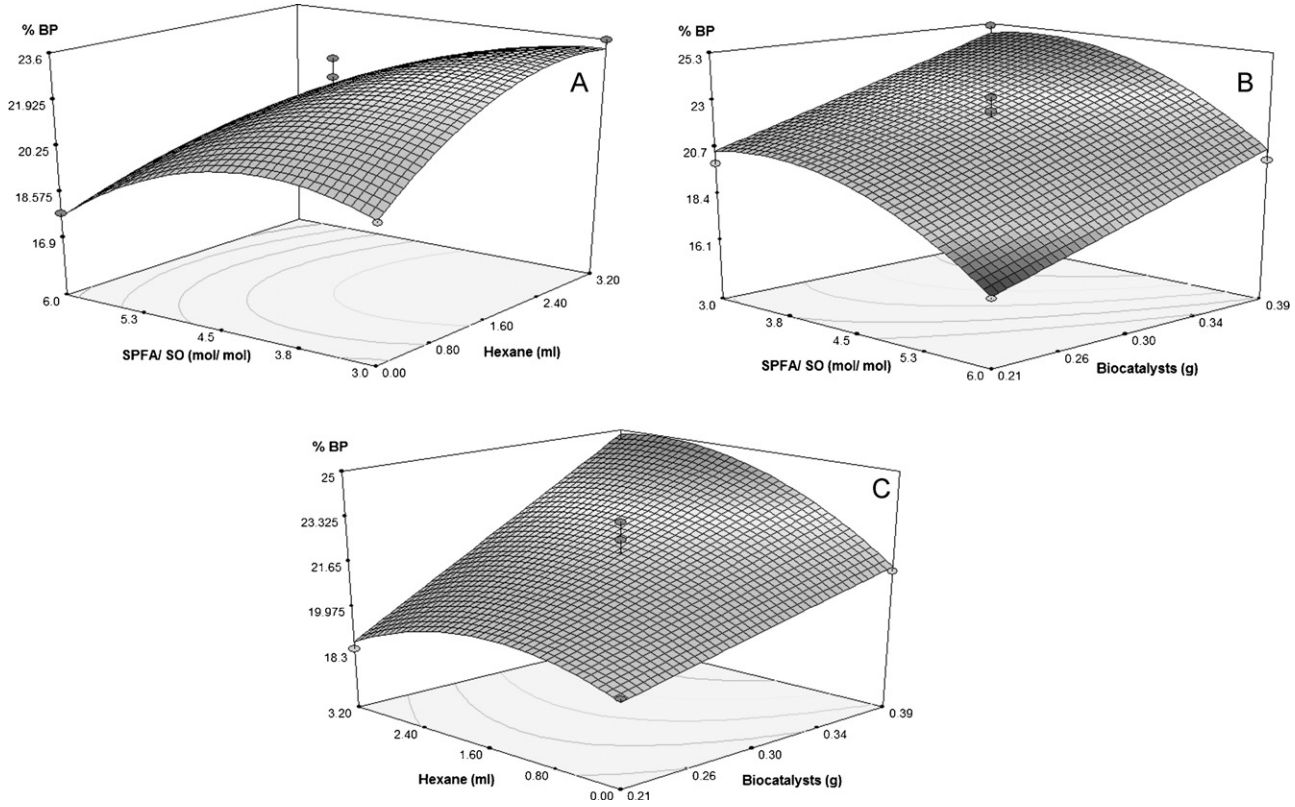

Fig. 3. Response surface plot of the by-products (monoglycerides and diglycerides) respect to total glycerides. Effect of (A) amount of hexane and the substrate mole ratio at amount of biocatalyst fixed at 0.3 g (central point); (B) amount of biocatalyst and the substrate mole ratio at amount of hexane fixed at 1.6 ml (central point); (C) amount of biocatalyst and amount of hexane at substrate mole ratio fixed at 4.5 (central point).

be reesterificated faster by lipases action. Agreement results have been previously reported [25] in acidolysis between triolein and palmitic acid, finding that DAG content decreased with larger ratio palmitic/triolein. Similarly, Xu et al. [4] showed that DAG formation increased with the increase of substrate ratio defined as molar ratio oil/capric acid in the acidolysis between them. This fact could be true at high E values since in those conditions high % P + St was obtained. However, at low E values not only % BP decreased with the increase in SR but also % P + St and % SUS. So it could be thought that acidification of the reaction medium influenced over the hydrolysis lipase activity when it is in the range of E dependency.

Regarding Fig. 3C, for all quantities of hexane (H), the % BP was found to increase with an increase of the amount of biocatalyst. Moreover, it could be observed that the higher the level of H with the increment of E, the more significant the increment of % BP was. Without solvent in the reaction medium, substrates are more viscous. So, any step of the reactions of hydrolysis or esterification could be limited by mass transfer mechanisms.

3.2.4. Trisaturated triglycerides: acyl migration indicative

The presence of trisaturated triglycerides in the reaction product was due to acyl migration as side reaction. For this reason, trisaturated TAG content can be used as an indicator of this undesirable reaction and the factors effects on it can be inferred.

The effects of the experimental factors on trisaturated triglycerides, respect to total triglycerides (% SSS), are shown in Fig. 4. The analysis of variance (ANOVA) indicated that the model represented by Eq. (5), quadratic in SR, E and H, was the most adequate for representing the actual relationship between the response and the significant factors, with a $R^2 = 96.87\%$. The R^2adj and R^2pred were 93.74% and 79.04% respectively, which were in reasonable agreement. The predicted model for % SSS in terms of the factors was:

$$\%\,\mathbf{SSS} = -10.345 + 3.286 \times \mathrm{SR} + 49.397 \times E + 0.674 \times H + 5.581 \times E \times H - 0.350 \times \mathrm{SR}^2 - 64.239 \times E^2 - 0.639 \times H^2 \quad (5)$$

Once again the amount of biocatalyst proved to be the most significant factor ($P < 0.0001$). Other factors that resulted significant were: H ($P = 0.0166$), SR^2 ($P = 0.011$), E^2 ($P = 0.0576$), H^2 ($P = 0.0002$) and the interaction $E \times H$ ($P = 0.0082$). The linear term SR did not result significant ($P > 0.1$).

An examination of Fig. 4A suggests that for all the amounts of hexane, the percentage of trisaturated triglycerides rose when the amount of biocatalyst increased in the media. This effect was more remarkable at high amounts of hexane (significant $E \times H$ interaction). Similarly, for all the ratios of substrate, it was found that the % SSS quickly increased when the amount of enzyme was increased (Fig. 4B). However, in this case the interaction $E \times$ SR was not significant. It is to say, the effect of E over % SSS was similar for each SR analyzed and vice versa.

On the other hand, the combined effects, amount of substrate and amount of hexane, are shown in Fig. 4C. This shows that for all the ratios of substrates the % SSS increased with an increase in the amount of hexane up to 1.9 ml value. Beyond this point, it decreased gradually with an increase in the amount of the mentioned solvent. A similar behavior was observed in all the amounts of hexane when the ratio of substrates was varied. The % SSS rose with the increase of substrate mole ratio to a certain point (4.5 mol/mol). Later, it decreased at the same rate, at greatest amounts.

The amount of biocatalyst in the reaction media was the most influential factor. The highest amounts of E produced the highest contents of SSS in the obtained SL. This result was in agreement with the results found in by-products analysis since greater amounts of MAG and DAG promoted the acyl migration [4]. Thus,

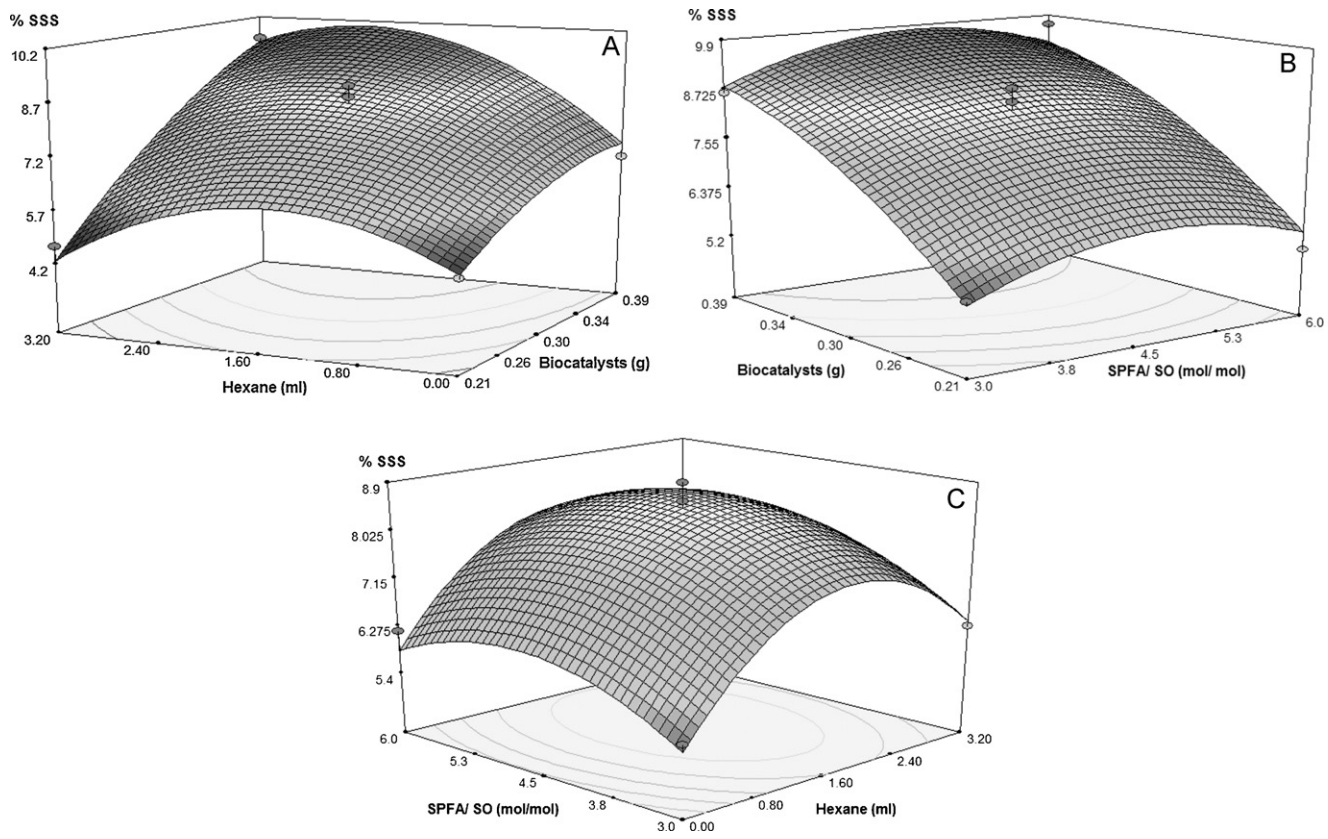

Fig. 4. Response surface plot of trisaturated triglycerides respect to total triglycerides (% SSS). Effect of (A) amount of biocatalyst and amount of hexane at substrate mole ratio fixed at 4.5 (central point); (B) amount of biocatalyst and the substrate mole ratio at amount of hexane fixed at 1.6 ml (central point); (C) amount of hexane and the substrate mole ratio at amount of biocatalyst fixed at 0.3 g (central point).

unsaturated fatty acids in *sn*-2 position could migrate allowing the enzyme removed them from the triglycerides. Subsequent migration of saturated fatty acids could take place allowing the formation of SSS compounds.

With regard to the hexane effect, some works have shown that it reduces the rate of acyl migration [7], though in this study this phenomenon was observed after a certain hexane value.

As it can be noticed in Table 1, for all the response variables, the predicted values were very close to the experimental values, meaning that the experimental models were adequately fitted.

3.3. Multiresponse optimization and verification of models

The goal of multiresponse optimization was to find a good set of conditions that met all the requirements imposed on response variables. Firstly, the maximization of % P + St and % SUS and the minimization of % BP and % SSS were simultaneously set allowing the reaction parameters to take any value within the analyzed ranges. The maximum value of desirability function D ($D = 0.7$) is found when the acidolysis reaction is carried out in the following conditions: SR = 6 mol/mol, $E = 0.3$ g and $H = 0$ ml, in which the predicted response values are: % P + St = 44.49, % SUS = 30.48, % BP = 17.66 and % SSS = 5.82. This combination of parameter values that coincided with one of the runs of experimental design (Run 12), fulfilled at a large extent the imposed criteria and showed that free solvent green systems were the most effective. However, from a practical standpoint, it turns out easier to carry out the reactions in presence of solvent, preferably in diluted medium to avoid blockage of system lines, if the work is done in continuous or semicontinuous systems. In consequence, another optimization was performed, setting as criteria the higher amount of hexane, the lower mol substrate ratio and the maximum % P + St. The optimum found result was: SR = 3 mol/mol, $E = 0.34$ g and $H = 3.2$ ml, in which the predicted response values were: % P + St = 38.50, % SUS = 33.71, % BP = 24.73 and % SSS = 7.50, and the corresponding D value was 0.83. These optimum conditions were used in four independent assays in order to check the validity of the predictive models. The experimental response values found in those conditions were (mean value ± standard deviation): % P + St = 38.60 ± 0.09, % SUS = 33.88 ± 0.75, % BP = 24.97 ± 0.37 and % SSS = 7.62 ± 0.17, which were very closed to the predicted values. The TAG profile of obtained SL (SL O2 in Table 2) showed that major TAG species were PLSt, POL, PLL, PLP, StOL and StLL. Comparing SL O2 with SL O1, these species were almost the same but with a different distribution. So, a higher proportion of UUS group and a lower proportion of SUS group were found in SL O2, which is directly related to the combined effect of the lower SR used with the dilution generated by hexane presence. Furthermore, it could be noticed a higher amount of by-products, meaning that the hydrolysis step was faster than the esterification one. It is worth mentioning that this study was focused on chemical features of SL, but knowledge of their physical properties would be performed in order to determine their final application.

3.4. Time course of acidolysis product and temperature effect

Fig. 5 shows the evolution in time of triacylglycerol groups, by-products and the palmitic and stearic acids composition in the acidolysis carried out in the selected optimum condition for the parameters, at different reaction temperatures, 50 and 60 °C. With regards to the P + St composition (Fig. 5A), significant differences in temperature were not found during the first 2–4 h of reaction.

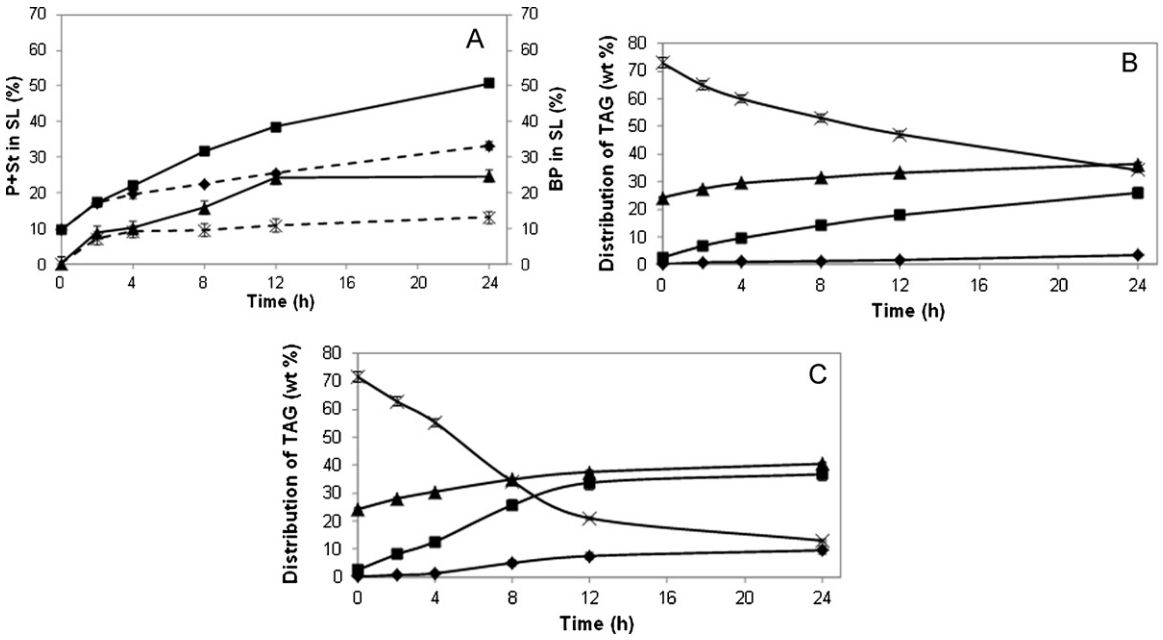

Fig. 5. Time course of acidolysis of SO and SPFA, at optimization conditions (SR = 3, E = 0.34 g and H = 3.2 ml); (A) composition of palmitic and stearic acids at 50 °C (♦) and 60 °C (■), and composition of by-products at 50 °C (×) and 60 °C (▲); evolution of triacylglycerols groups (UUU = ×, UUS = ▲, SUS = ■ and SSS = ♦), (B) at T = 50 °C and (C) at T = 60 °C.

After this time, the P + St incorporation was markedly faster at 60 °C. At 24 h of reaction, the SL obtained at 60 °C contained about 50% of P + St, whereas the SL obtained at 50 °C contained about 30%, which shows that a major temperature had a positive effect on this response variable. Similar results have been reported in previous works for *R. miehei* lipase immobilized on another support (macroporous anion exchange phenolic resin). For instance, Carrín and Crapiste [10] reported the higher incorporation of saturated acids at 60 °C in acidolysis of sunflower oil and the same mixture of palmitic and stearic acids catalyzed by Lipozyme RM IM. In agreement, Sahin et al. [9] performed acidolysis reaction of tripalmitin, hazelnut oil fatty acids, and stearic acid with Lipozyme RM IM, and found that the highest incorporation of stearic acid was achieved at 60 °C.

By-products generation showed a similar behavior than the P + St incorporation during the first 4 h of reaction (Fig. 5A). After that time, the content of by-products in the reaction medium rose significantly with temperature, reaching different stationary states after 12 h. Comparing BP and P + St results, it could be concluded that the steps of the general enzymatic reaction (hydrolysis and esterification) progressed at the same rate after about 12 h.

In reference to the distribution of TAG groups (Fig. 5B and C), the UUU group showed an approximately linear decrease, which was sharper at 60 °C until 12 h. After this point, the decreasing was much smaller. The UUS group increased very slowly for both temperatures, rising slightly faster at 60 °C. Relating to the SUS group, the desired TAG, its increase was greater at 60 °C. The rate of rising at 50 °C was about proportional to all the reaction time, whereas at 60 °C it was linear up to 12 h and a plateau was observed after that. Last of all, it was found that the SSS group linearly rose during the time range analyzed at 50 °C. However, at 60 °C that group seemed to follow the tendency of SUS and BP, which was expected because they are obtained after the hydrolysis of SUS compounds. As it can be observed, the temperature and time had positive effect on % SSS and therefore on the acyl migration, being the temperature effect the more important. Acyl migration has a good linear relationship with time course of the enzymatic acidolysis, which indicates that the reaction is a non-enzymatic process [5].

Furthermore, as it is known, fatty acids with different chain lengths and numbers of double bonds have different migration rates. This behavior could be estimated for palmitic and stearic acids by comparison of increments between PPP and StStSt. After 12 h of reaction at 60 °C, the amounts of PPP and StStSt changed from a not detected value to 1.58% and from 0.04 to 0.73%, respectively. This suggests that a greater migration of palmitic acid occurred. A similar result was found by Sahin [9], who reported that *sn*-2 positions of his obtained SL, were predominantly occupied by palmitic acid followed by oleic, stearic, and linoleic acids. So, it could be thought that using stearic acid alone as saturated FA in the acidolysis reaction would be an aspect to take into account to improve the product quality.

4. Conclusions

In this study, biocatalyst prepared by immobilization of *R. miehei* lipases onto alkylated chitosan microspheres were used to successfully synthesize structured lipids by acidolysis reaction of sunflower oil and palmitic–stearic acids mixture. The analysis of the effects of the selected reaction parameters showed that the amount of biocatalyst was the most significant factor over each analyzed response dividing the behavior of the reaction between a high and a moderate enzyme-dependency zone. The presence of hexane contributed to highlight this behavior. Optimal working conditions, to obtain maximum incorporation of saturated fatty acids, were set and checked with and without solvent restriction. Time-behavior analysis showed that generation of trisaturated triacylglycerols and by-products was favored by increasing the temperature and, to a lesser extent, the time. Reaction yields could be stabilized after 12 h of reaction. However, TAG groups continued changing which shows that equal overall rates of hydrolysis and esterification reactions had been reached.

The present work provides knowledge of the behavior of these biocatalyst in an actual system. The evaluation of required product quality and process conditions must generate a compromise decision.

Acknowledgments

This work was financially assisted by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and the Universidad Nacional del Sur (UNS) in Argentina.

References

[1] H.T. Osborn, C.C Akoh, Comp. Rev. Food Sci. Food Saf. 1 (2002) 93–103.
[2] D. Li, P. Adhikari, J.A. Shin, J.H. Lee, Y.J. Kim, X.M. Zhu, J.N. Hu, J. Jin, C.C. Akoh, K.T. Lee, LWT - Food Sci. Technol. 43 (2010) 458–464.
[3] M.M. Jensen, M.S. Christensen, C.E. Høy, Ann. Nutr. Metab. 38 (1995) 104–116.
[4] X. Xu, H. Mu, A.R.H. Skands, C.E. Hoy, J.A. Nissen, J. Am. Oil Chem. Soc. 76 (1999) 175–181.
[5] X. Xu, L.B. Fomuso, C.C. Akoh, J. Agric. Food Chem. 48 (2000) 3–10.
[6] M.E.M.B. de Araújo, P.R.B. Campos, T.M. Noso, R.M. Alberici, I.B. da Silva Cunha, R.C. Simas, M.N. Eberlin, P.O. Carvalho, Food Chem. 127 (2011) 28–33.
[7] X. Xu, A. Skands, C. Høy, H. Mu, S. Balchen, J. Adler-Nissen, J. Am. Oil Chem. Soc. 75 (1998) 1179–1186.
[8] T. Yang, M.B. Fruekilde, X. Xu, Food Chem. 92 (2005) 101–107.
[9] N. Sahin, C.C. Akoh, A. Karaali, J. Agric. Food Chem. 53 (2005) 5779–5783.
[10] M.E. Carrín, G.H. Crapiste, J. Food Eng. 84 (2008) 243–249.
[11] C. Pacheco, G.H. Crapiste, M.E. Carrín, J. Food Eng. 98 (2010) 492–497.
[12] M. Sharma, N.K. Rastogi, B.R. Lokesh, Process Biochem. 44 (2009) 1284–1288.
[13] C.A. Palla, C. Pacheco, M.E. Carrín, Biochem. Eng. J. 55 (2011) 199–207.
[14] B. Krajewska, Enzyme Microb. Technol. 35 (2004) 126–139.
[15] R. Fernandez-Lafuente, P. Armisen, P. Sabuquillo, G. Fernandez-Lorente, J.M. Guisan, Chem. Phys. Lipids 93 (1998) 185–197.
[16] C.J. Shieh, C.C. Akoh, P. Koehler, J. Am. Oil Chem. Soc. 72 (1995) 619–623.
[17] E. Hita, A. Robles, B. Camacho, P.A. Gonzalez, L. Esteban, M.J. Jimenez, M.M. Munio, E. Molina, Biochem. Eng. J. 46 (2009) 257–264.
[18] S.L.C. Ferreira, R.E. Bruns, H.S. Ferreira, G.D. Mato, J.M. David, G.C. Brandão, E.G.P. da Silva, L.A. Portugal, P.S. dos Reis, A.S. Souza, W.N.L. dos Santos, Anal. Chim. Acta 597 (2007) 179–186.
[19] Official methods and recommended practices of the American Oil Chemists' Society, Champaign, IL, 2006.
[20] J. Reske, J. Siebrecht, J. Hazebroek, J. Am. Oil Chem. Soc. 74 (1997) 989–998.
[21] M. Serrano-Vega, E. Martínez-Force, R. Garcés, Lipids 40 (2005) 369–374.
[22] G. Perretti, E. Finotti, S. Adamuccio, R. Della Sera, L. Montanari, J. Am. Oil Chem. Soc. 81 (2004) 1119–1123.
[23] V.V. Yankah, C.C. Akoh, J. Am. Oil Chem. Soc. 77 (2008) 495–500.
[24] R.C. Rodrigues, R. Fernandez-Lafuente, J. Mol. Catal. B: Enzym. 64 (2010) 1–22.
[25] S. Bloomer, P. Adlercreutz, B. Mattiasson, Biocatal. Biotransform. 5 (1991) 145–162.