Journal of Molecular Catalysis B: Enzymatic 72 (2011) 13–19

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Application of response surface design to solvent, temperature and lipase selection for optimal monoglyceride production

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ARTICLE INFO

Article history:
Received 24 January 2011
Received in revised form 12 April 2011
Accepted 21 April 2011
Available online 30 April 2011

Keywords:
Monoglycerides
Immobilized lipase
Organic medium
Response surface methodology

ABSTRACT

This work focuses on the use of a temperature and solvent lipase stability procedure as a practical approach for selection of the most favorable conditions for the enzyme catalysis production of monoglycerides from glycerin and triolein. Two lipases were selected for analysis: a lipase from *Candida rugosa* immobilized on chitosan and a lipase from *Mucor miehei* immobilized on a macroporous anionic exchange resin of the phenolic type. Using a 3^2 factorial experimental design, the effects of temperature within the range of 35–45 °C and solvent ratios of acetone:isooctane between 0.25:0.75 and 0.75:0.25 (v/v) were evaluated on the activity of the lipase. Lipase from *M. miehei* revealed a higher residual activity (91%) following a 24 h incubation with the solvent acetone:isooctane at a ratio of 0.25:0.75 (v/v) at 35 °C while *C. rugosa* lipase reached a maximum residual activity of approximately 56% after a 24 h incubation with a solvent acetone:isooctane ratio of 0.25:0.75 (v/v) between 35 and 42 °C. For the *M. miehei* lipase, these results were evaluated experimentally by testing glycerolysis of triolein (biocatalyst initial water activity (a_w) 0.534, molar ratio glycerin:triolein 3:1, amount of protein 90 mg, 24 h). Using the best (35 °C, 0.75 Ac) and the worst (45 °C, 0.75 Ac) conditions for residual activity in stability assays, it was confirmed that when the predicted optimum conditions were applied, a monoolein yield of over 68% and a total conversion of triolein of approximately 89% were reached.

1. Introduction

Monoglycerides (MGs) are the most widely used emulsifiers in the food, pharmaceutical and other industries. MGs are also considered to be potentially health-beneficial emulsifiers [1].

Currently, MGs are obtained at industrial scale using nonselective inorganic catalysts at high temperatures (200–250 °C), starting with fatty acids, followed by direct esterification or reaction with pure triglycerides and by interesterification with glycerine [3]. These chemically catalyzed processes have several drawbacks, such as the generation of dark colored by-products with an undesirable flavor and low yields (30–40% MGs); therefore, to concentrate MGs up to more than 90%, purification by molecular distillation is usually required [2,4]. Using lipases instead of inorganic catalysts to produce MGs and DGs prevents side product formation, can be accomplished at lower temperatures, saves energy and generates light-colored by-products [5,6]. To make this process financially competitive, it is necessary to immobilize the lipase for re-use [3].

The synthesis of MGs by direct lipase-catalyzed esterification between glycerol and a fatty acid in organic solvents, which improves the poor solubility of the substrates in water, has been proposed in various studies [2–7]. Bellot et al. [7] investigated MGs synthesis by *Rhizomucor miehei* lipase via direct esterification between glycerol and oleic acid in organic solvents and proposed that an increase in solvent polarity using mixtures of two solvents drastically improves the selectivity toward MGs formation. Li and Ward proposed an enzymatic method for synthesis of MGs from 1,2-isopropylidene glycerol and n-3 polyunsaturated fatty acid concentrate using lipase IM-60 from *Mucor miehei* in isooctane and hexane as organic solvents [8]. Kaewthong and H-Kittikun studied the effects of various organic solvents on MG production from theglycerolysis of palm olein with immobilized *Pseudomonas* sp. lipase.

Abbreviations: a_w, water activity; MGs, monoglycerides; DGs, diglycerides; *P*, octanol–water partition coefficient; BSA, bovine serum albumin; pNPP, p-nitrophenyl palmitate; pNP, p-nitrophenol; RSM, response surface methodology; RA, residual activity; *T*, temperature; Ac, acetone volume fraction; R^2, determination coefficient (quadratic correlation coefficient); R^2_{adj}, adjusted R^2.

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doi:10.1016/j.molcatb.2011.04.017

The optimal condition for MG production was determined to be in acetone/isooctane mixtures [4].

A useful parameter to characterize solvent properties in relation to lipids and lipases is the octanol–water partition coefficient (P). Log P tends to be largest for compounds with extended non polar structures and smallest or negative for compounds with highly polar groups [9]. Damstrup et al. screened solvent systems of varying polarities for efficient and practical enzymatic glycerolysis of sunflower oil using Novozym® 435 lipase. They found the maximum MGs production in solvents systems with log P values in the range of 0.3–1.0 [10].

In non-aqueous media, biocatalysis loss of activity can be caused by deactivation of the enzyme overtime as a consequence of thermal and solvent effects and inhibition of reagents or products. The general method to determine the optimum reaction conditions involves experimental design sets, which are sometimes very complex, in which different solvents, reaction temperatures and reagent molar ratios are tested. The previous study of the non-aqueous residual activity evolution on different solvent and temperature conditions could be a useful approach to identify ranges of probable best (or worst) enzyme behavior. The aim of this work was to study the thermal stability of two immobilized lipases in different mixtures of organic solvents. Two binary mixtures of solvents were used: one with a high log P value (isooctane) and another with a high polarity (acetone). In this way, it was possible to simultaneously screen the influence of the hydrophobic–hydrophilic ratios of the reaction media and the temperature of the reaction on the progress of residual catalytic activity. The accuracy of this procedure in defining the enzyme performance on the immobilized lipase systems was verified by experimentally testing the glycerolysis of triolein reaction to form MGs.

2. Materials and methods

2.1. Materials

2.1.1. Enzymes

Lipase from *Candida rugosa* (Lipase AY) was supplied by Sigma–Aldrich, USA, and lipase from *Mucor miehei* (Lipozyme®) immobilized on macroporous anion-exchange resin of the phenolic type was purchased from Fluka Analytical, USA.

2.1.2. Immobilization support

Chitosan flakes (high molecular weight 602 kDa, degree of deacetylation 76.5%) were obtained from Sigma–Aldrich, USA.

2.1.3. Reagents

Glycerol (≥99%), glycerol trioleate (practical grade 65%), 4-nytrophenol and 4-nytrophenyl palmitate (spectrophotometric grade), triolein, monoolein, diolein and oleic acid (analytical grade 99%) were purchased from Sigma–Aldrich, USA. All other chemicals were of analytical grade and obtained from various sources.

2.2. Methods

2.2.1. Immobilization

The procedure for chitosan support production was described in previous works [11,12]. Briefly, chitosan flakes (4.5 g) were diluted in 150 mL of 1% (v/v) acetic acid solution and treated with glutaraldehyde until the crosslinker content was 0.0003 M. The films were cured using 30 mL of NaOH/ethanol gelling solution for 2 h. After rinsing with distilled water, gelled matrices were frozen at −45 °C for 30 min and thawed for 3 h at 4 °C. This freezing and thawing (F/T) step was carried out six consecutive times. The resulting films were cut into 1 cm × 1 cm sections. Lipase from *C. rugosa* was immobilized onto chitosan film supports according to the protocol from Orrego et al. [13]. The enzyme was dissolved and pre-incubated at 35 °C in 50 mL of phosphate buffer (pH 7.2) under gentle stirring for 2 h. The chitosan films were then submerged into the enzyme solution for 20 h at 20 °C under agitation (120 rpm). The resultant immobilized lipase on chitosan films were removed and stored at 4 °C.

2.2.2. Protein loading assay

The amount of immobilized enzyme on the membrane was determined by measuring the initial and final concentrations of protein within the enzyme solution used for the immobilization and the washing solutions, using the Biuret method at a wavelength of 540 nm using a spectrophotometer (Model 6405, Jenway, England) [14]. The protein load of lipase was expressed as the percentage of protein (%) on the chitosan membrane.

2.2.3. Water activity pre-equilibration

The initial water activity (a_w) values of immobilized enzymes were adjusted by following the gravimetric static method of equilibration as follows: *Candida rugosa* and *Mucor miehei* lipases were separately incubated in a chamber containing a saturated salt solution (NaBr), and the system was allowed to reach equilibrium at 40 °C for a_w = 0.53. The initial water activity of the pre-equilibrated immobilized enzyme system was corroborated using a water activity meter (Decagon, model 99163, Pawkit, USA).

2.2.4. Measurement of lipase synthetic activity in organic media

Determination of lipase synthetic activity was based on a lipase-catalyzed transesterification reaction between p-nitrophenyl palmitate (pNPP) and ethanol in n-heptane [15]. The yield of p-nitrophenol (pNP) was determined by a spectrophotometric method (Model 6405, Jenway, England) at a wavelength of 410 nm. The lipase synthetic activity was expressed as mmol of pNP liberated per minute and gram of protein.

2.2.5. Enzymatic stability

For lipase-catalyzed monoglyceride production, Damstrup et al. found that a maximum yield for solvents was accomplished when log P values ranged between 0.3 and1.0. In this work, we chose a broader range of log P values (0.46–2.85) using acetone and isooctane mixtures. The log P value of the mixture of solvents was calculated using a semiempirical formula for related-solvent engineering approaches as suggested by Hilhorst et al. [16–19]:

$$\log P_{\text{Mix}} = X_{\text{Acetone}} \times \log P_{\text{Acetone}} + X_{\text{Isooctane}} \times \log P_{\text{Isooctane}} \quad (1)$$

where X_i = component molar fraction, i = acetone or isooctane.

Initial and final enzymatic activities of immobilized lipases were measured before and after *Candida rugosa* and *Mucor miehei* lipases were incubated for 24 h in three different concentrations (v/v) of acetone:isooctane (0.25:0.75, $\log P_{\text{Mix}}$ = 2.85; 0.5:0.5, $\log P_{\text{Mix}}$ = 1.43; 0.75:0.25, $\log P_{\text{Mix}}$ = 0.46) at different temperatures (35, 40, 45 °C).

2.2.6. Experimental design

Response surface methodology (RSM) was used to model the residual activity (RA) of the immobilized enzymes (*Candida Rugosa* lipase on chitosan and *Mucor miehei* lipase on macroporous resin). For each lipase assayed, a total of 11 experiments to assess enzymatic stability were carried out following a central composite face centered design as a function of both the temperature (T) and the acetone fraction volume (Ac) in the reaction mixture of acetone and isooctane. The levels considered in both assessed variables are shown in Table 1.

Table 1
Codified levels and real values of the experimental factors used in experimental design.

	Codified levels		
	−1	0	+1
T (°C)	35	40	45
Ac (v)	0.25	0.50	0.75

2.2.7. Statistical analysis

The results of the experimental design were analyzed using "Design Expert" software version 8 from State-Easy Inc., USA. The linear and quadratic effects of T and Ac and the linear interaction $(T) \times (\mathrm{Ac})$ on the enzymatic stability of lipases were calculated, and their significance was evaluated by analysis of variance. A 3D surface, as described by a second order polynomial equation, was fitted to each set of experimental data points of residual activity. First and second order coefficients were generated by regression analysis.

2.2.8. Glycerolysis reaction

Glycerolysis reactions were performed in 25 mL screw cap flasks placed in a thermostated bath (Model 2870, Thermo Electron Corporation, USA) at the desired temperature with shaking at 200 rpm. The reaction mixtures consisted of triolein (4.85 mL, 0.005 gmol), glycerin (1.09 mL, 0.015 gmol) and fresh catalyst (162.60 mg equivalent to 90.00 mg of protein) in acetone:isooctane media. The molar ratio of glycerin to triolein was 3:1.

2.2.9. Analytical methods

The course of glycerolysis was monitored by intermittent sampling (200 μL) of each reaction sample over 6 h. The organic solvent was completely evaporated in a thermostated bath at 95 °C, and samples were re-diluted in hexane. The samples were analyzed to quantify mono-, di- and tri-olein concentrations using an HPLC system (Hitachi, Tokio) equipped with a C-18 reverse phase LiChroCART column and UV detector (215 nm). The levels of free fatty acids (FFA) were determined using the Lowry and Tinsley's colorimetric method [22].

3. Results and discussion

3.1. Protein load and protein load efficiency

Protein load and protein load efficiency values obtained for *Candida rugosa* lipase on chitosan membranes were 12.82 mg g^{-1} chitosan and 22.06%, respectively. These values are similar to those obtained in previous works [12]. The protein load of the *Mucor miehei* lipase was estimated as the protein content in the commercial lipase measured by Kjeldahl analysis (54.44%).

3.2. RSM experiments and model fitting

The initial enzymatic activity of the evaluated lipases was measured in triplicate before incubating them with the organic solvents. *Mucor miehei* lipase had the highest initial enzymatic activity, with a value of 35.96 ± 0.63 μmol min^{-1} $g_{prot}{}^{-1}$, while *Candida rugosa* lipase had an enzymatic activity of 16.43 ± 0.27 μmol min^{-1} $g_{prot}{}^{-1}$.

Assays were used to determine the enzymatic activity of each lipase after 24 h incubation with the organic solvents at a fixed temperature. The residual activity (RA) was defined as follows:

$$\text{Residual activity}\,(\%) = \frac{\text{final activity}}{\text{initial activity}} \times 100 \qquad (2)$$

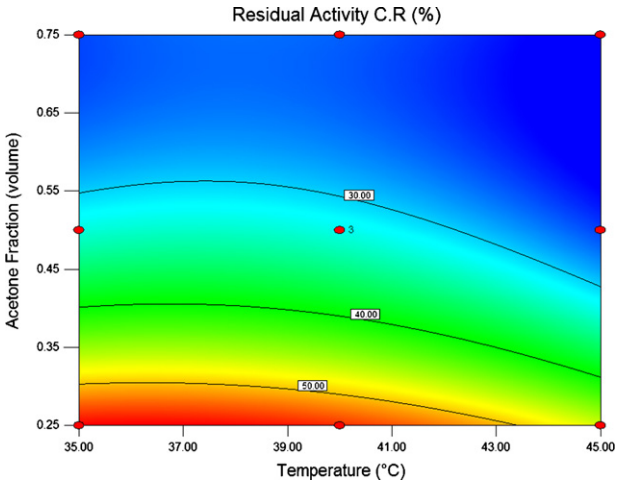

Fig. 1. Effect of temperature and acetone fraction on the percentage RA of the *Mucor miehei* lipase.

Table 2 shows the results of final and residual activities for both lipases. It should be noted that the coded values of each factor (shown in brackets) corresponds to the real value of the factor level.

Data in Table 2 were analyzed by RSM to construct an empirical model for the representation of the residual activity of each lipase in terms of the temperature and the acetone fraction in the reaction medium. Based on regression analysis at a 95% confidence interval, a quadratic model was used to fit the observed data by least squares analysis, and the significance of the lack of fit error and p-values of the parameter estimations were determined. ANOVA results are shown in Table 3.

The F-value of the models were 47.56 and 81.00, with a corresponding p-value of 0.0003 and <0.0001, for *Mucor miehei* and *Candida rugosa* lipases, respectively, implying that the model was significant at the 95% confidence level. The effects of the two synthesis variables as well as their interactions can be evaluated based on their p-values. The p-value at which every term in the selected model should be significant was set to 0.05. While the interaction term T(Ac) was the most significant factor affecting the percentage of RA of the *Mucor miehei* lipase, the independent variable temperature (T) and the quadratic term of temperature (T^2) also influenced the response. The independent variable and the quadratic term of the acetone fraction in the reaction medium did not cause a significant effect on the percentage of RA within the designed intervals. The residual activity of the *Candida rugosa* lipase was more greatly influenced by the independent variable acetone fraction (Ac) than that of the *Mucor miehei*. The independent variable acetone fraction (Ac) and the quadratic terms of the temperature and the acetone fraction (T^2 and Ac^2) also significantly affected the residual activity of *Candida rugosa*, while the response for the *Candida rugosa* lipase was not affected by the interaction term T(Ac).

The empirical models were obtained for predicting RA for both lipases (Table 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.

The effects of temperature (T) and acetone fraction (Ac) on the residual activity of the *Mucor miehei* and *Candida rugosa* lipases are shown in Figs. 1 and 2, respectively.

Catalytic activity and stability in non-aqueous media are directly correlated with solvent hydrophobicity (high log P) because nonpolar and non-water miscible media, as opposed to polar and miscible media, do not withdraw the bound water crucial for the enzyme structure and activity [23].

In some cases, the addition of small amounts of water-miscible solvents such as acetone results in enhanced enzyme activity

Table 2
Experimental conditions of central composite design (CCD) runs of Design-Expert 8.0 and corresponding results (the response).

Run	Level variables		Response value			
	T (°C)	Ac (v)[a]	Final activity (μmol min^{-1} g$_{prot}^{-1}$)		Residual activity (%)	
			M.M[b]	C.R[c]	M.M[b]	C.R[c]
1	35 (−1)	0.25 (−1)	17.01	9.05	47.28	55.06
2	45 (1)	0.25 (−1)	28.17	7.64	78.33	46.52
3	35 (−1)	0.75 (1)	32.69	4.34	91.06	26.40
4	45 (1)	0.75 (1)	16.35	3.80	45.54	23.11
5	40 (0)	0.5 (0)	28.36	5.29	78.99	32.26
6	40 (0)	0.5 (0)	28.55	5.23	79.53	31.89
7	40 (0)	0.5 (0)	28.13	5.27	78.36	32.13
8	35 (−1)	0.5 (0)	26.38	5.47	73.36	33.35
9	45 (1)	0.5 (0)	20.09	3.96	55.85	24.15
10	40 (0)	0.25 (−1)	28.47	9.26	79.16	56.46
11	40 (0)	0.75 (1)	28.52	3.96	79.29	24.15

[a] Acetone fraction (volume).
[b] *Mucor miehei* lipase.
[c] *Candida rugosa* lipase immobilized in chitosan.

Table 3
ANOVA results for the selected models of Design-Expert 8.0 for the response of each lipase.

Lipase	Source	Sum of squares	Degree of freedom	Mean square	F-Value	p-Value Prob > F
Mucor miehei	Model	2179.15	5	435.83	47.56	0.0003
	T	170.49	1	170.49	18.61	0.0076
	Ac	20.61	1	2.25	20.61	0.1940
	T(Ac)	1465.46	1	1465.46	159.93	<0.0001
	T^2	496.26	1	496.26	54.16	0.0007
	(Ac)^2	0.99	1	0.99	0.11	0.7558
	Lack of fit	45.13	3	15.04	43.90	0.0224
	Pure error	0.69	2	0.34		
Candida rugosa	Model	1451.61	5	290.32	81.00	<0.0001
	T	73.76	1	73.76	20.58	0.0062
	Ac	1186.95	1	1186.95	331.14	<0.0001
	T(Ac)	6.87	1	6.87	1.92	0.2247
	T^2	23.93	1	23.93	6.68	0.0492
	(Ac)^2	182.23	1	182.23	50.84	0.0008
	Lack of fit	17.59	3	5.86	35.59	0.0275
	Pure error	0.33	2	0.16		

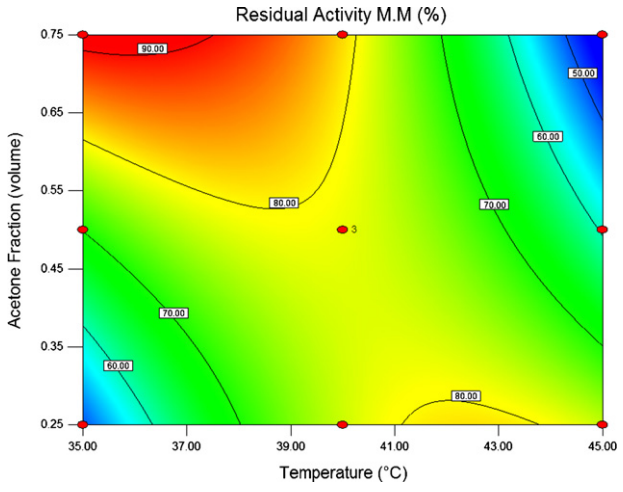

Fig. 2. Effect of temperature and acetone fraction on the percentage RA of the *Candida rugosa* lipase.

and stability [24]; however, when the concentration is increased, most water-miscible solvents have an inhibitory effect on the biocatalyst mainly due to changes in the affinity of the enzyme for the substrate [25]. In the present work, the *Candida rugosa* lipase immobilized on chitosan displayed this behavior. There was a noticeable effect of increasing the acetone concentration on the residual activity (RA), which was reduced to 23.11% when the volume fraction of acetone in reaction media was 0.75. In contrast, a maximum residual activity of 56.46% was found for the 0.25 acetone volume fraction. For this enzyme, the modeled RA was influenced by both temperature and solvent concentration, with the latter having the primary effect on the response ($p < 0.0001$). The model also predicted a more favorable outcome for RA within the temperature range 35–40 °C. Similar results for RA were obtained in a study with *Candida Rugosa* lipase immobilized on chitosan for esterification in hexane. This catalyst had an RA up to 80% after 12 h incubation in the solvent [26].

The performance with respect to RA for immobilized *Mucor miehei* lipase showed contrary results. In this case, the most advantageous combination of temperature and solvent mixture was a

Table 4
Model equations for the response surfaces fitted to the experimental data points from percentage of residual activity, as a function of the temperature (T) and acetone fraction (Ac) of *Mucor miehei* and *Candida rugosa* lipases, and respective R^2 and R^2_{adj}.

Responses	Model equations	R^2	R^2_{adj}
Residual activity M.M (%)	$RA_{M.M} = -1081.75 + 51.38T - 15.31T(Ac) - 0.56T^2$	0.98	0.96
Residual activity C.R (%)	$RA_{C.R} = -53.44 + 8.61T - 233.91Ac - 0.13T^2 + 135.70(Ac)^2$	0.99	0.97

low temperature (35 °C) and a high acetone concentration (0.75-low log *P*), with 91% of RA after 24 h. In a comparative study on the effect of solvent media on *Candida antarctica* lipase-catalyzed glycerolysis, superior monoglyceride production was found to occur in high content acetone solvent [27]; however, in this study, the *Mucor miehei* lipase demonstrated high RA performance (84% of RA) at 42–43 °C and a 0.25 volume fraction of acetone in the solvent mixture. It should be noted that the immobilized *Mucor miehei* lipase in the solvent systems analyzed in this work is an example of an enzymatic reaction system for which there is no correlation between activity retention and the log *P* value as cited above.

The effects of organic solvents on enzyme activity are primarily due to the impact of the solvent on interactions with the essential enzyme–bound layer of water rather than with the enzyme itself [28]. In non-aqueous catalysis, immobilized enzymes may compete not only with the solvent but also with the support, as preserving their essential monolayer of water on the surface is required for their biocatalytic function. The hydrophilicity/hydrophobicity characteristics of the solvent and the support play important roles in the preservation of the water bound to the enzyme. In some cases, the type of catalyzed reaction also could affect the availability of water to the enzyme. A total loss of activity for Lipozyme®-(the same lipase used in this work) catalyzed esterification was detected after its first use. The water produced by the reaction was accumulated on the support and facilitated the enzyme denaturalization [29]. In this type of system, it was suggested that the presence of a polar solvent in the reaction media could remove the water overload and consequently preserve the enzyme in its active state [30,31].

Chitosan has higher water capacity (aquaphilicity) than the Lipozyme® support. The presence of acetone in the reaction system could configure a water-competitive environment in which an enzyme (in the present work, *Candida rugosa* lipase) is unable to preserve its bound water. This interaction with the available water could explain the poor results of this biocatalyst in media containing a high concentration of acetone solvent.

3.3. Glycerolysis of triolein

Due to the superior residual activity (RA) of *Mucor miehei* lipase in most of the assays after 24 h incubation, the glycerolysis reaction was conducted using this biocatalyst. There were two zones of high RA observedfor *Mucor miehei* lipase: 35 °C/0.75 Ac and 45 °C/0.25 Ac, with 91% and 84% of RA, respectively. There were also two zones of low RA observed for the same lipase: 35 °C/0.25 Ac and 45 °C/0.75 Ac, with 47% and 45% of RA, respectively. In this work, a non-aqueous enzymatic activity method based in a transesterification reaction was used for the stability study (instead of the commonly used measurement of lipase activity by hydrolysis). Therefore, it is possible that the predictions of the RSM stability analysis can also forecast the catalytic behavior of the enzyme in the glycerolysis reaction in organic medium. In order to test this hypothesis, the glycerolysis of triolein reaction was conducted in duplicate in the above mentioned four zones: two for high RA and two for low RA. The initial water activity of the biocatalyst was controlled to 0.53, the molar ratio of glycerin:triolein was 3:1 and the amount of enzyme in the reaction media was 90 mg.

Figs. 3–6 show the reaction evolution. Monoolein, diolein, and oleic acid production and non-reacted triolein concentration were plotted against time for two reaction trials. The ability to superimpose these graphs demonstrates the reproducibility of these assays.

Fig. 3 shows the glycerolysis of triolein in the first zone of high RA (35 °C, 0.75 Ac). After 24 h of reaction, the mass compositions of monoolein, diolein and oleic acid were 68.39%, 17.53% and 3.52%, respectively. The conversion of triolein was approximately 89.04%. The effects seen in these results are explained by the polarity and

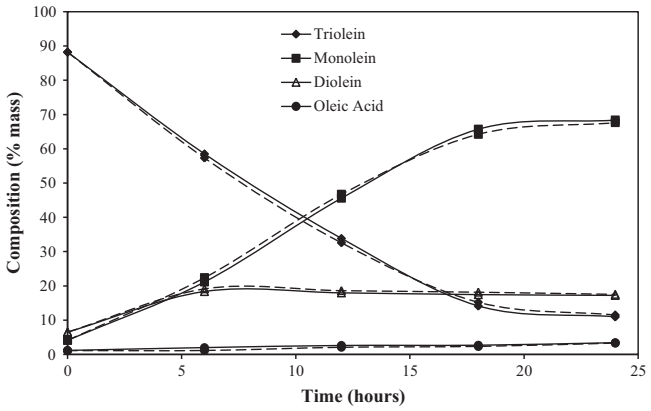

Fig. 3. Glycerolysis of triolein catalyzed by *Mucor miehei* lipase at 35 °C in acetone:isooctane (0.75:0.25, v/v). High RA zone. First (–) and second (– – –) assays.

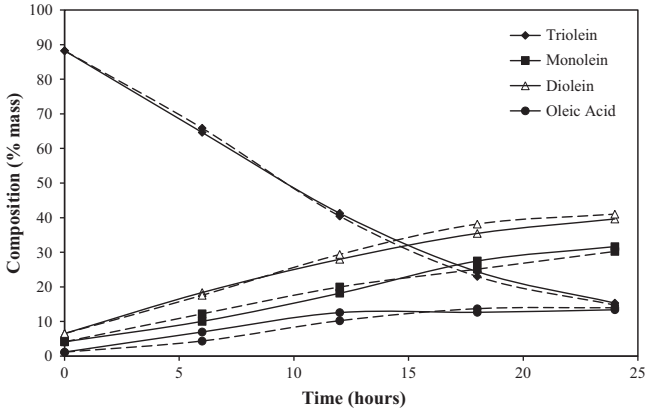

Fig. 4. Glycerolysis of triolein catalyzed by *Mucor miehei* lipase at 45 °C in acetone:isooctane (0.25:0.75, v/v). High RA zone. First (–) and second (– – –) assays.

the hydrophobicity of the organic solvents on the reaction, as it has been shown that increasing the proportion of the polar solvent shifts the equilibrium toward MG production. In fact, the transesterification of ethyl oleate with glycerol at optimized conditions up to 68% revealed that MG were formed after 12 h by lipase from *Candida antarctica* in acetone [32]. In other published works studying optimization of monoolein production [2,4,6,33], lower monoolein yields (20.12–54.38%) were reported.

Fig. 4 shows the evolution of the reaction in the second zone of high RA (45 °C, 0.25 Ac). The production of monoolein, diolein

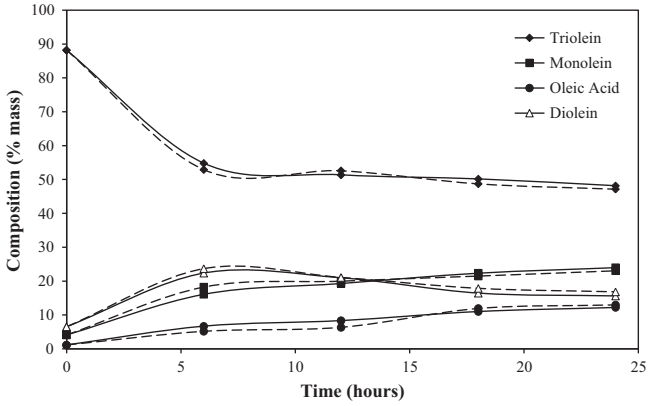

Fig. 5. Glycerolysis of triolein catalyzed by *Mucor miehei* lipase at 35 °C in acetone:isooctane (0.25:0.75, v/v). Low RA zone. First (–) and second (– – –) assays.

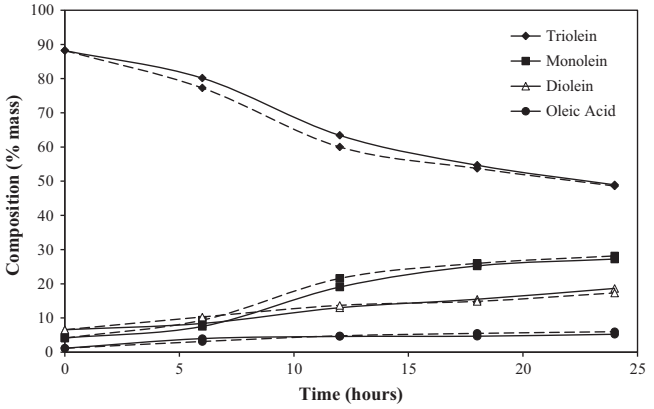

Fig. 6. Glycerolysis of triolein catalyzed by *Mucor miehei* lipase at 45 °C in acetone:isooctane (0.75:0.25, v/v). Low RA zone. First (–) and second (– – –) assays.

and oleic acid after 24 h of reaction was approximately 31.64%, 41.02% and 13.99%, respectively. The total conversion of triolein was 85.25%. In this case, despite the acceptable value of substrate conversion of the reaction, the monoolein yield was poor, and diolein was the main reaction product. This may be because the production of diolein is favored by the lower polarity of the reaction medium. These results agree with another study in which higher diglyceride synthesis took place in medium rich in non-polar organic solvents [32]. In another published work, in the direct esterification between glycerol and oleic acid catalyzed by *Rhizomucor miehei* lipase, the diolein production increased as the proportion of 2-methyl-2-butanol (polar solvent) in n-hexane decreased [7].

Fig. 5 shows the product compositions of the reaction in one zone of low RA (35 °C, 0.25 Ac). The compositions of monoolein, diolein and oleic acid after 24 h of reaction were approximately 23.98%, 16.82% and 12.98%, respectively, while the total conversion of triolein was 52.85%. These conversions to triolein and monoolein were considered low. Almost identical results were found in the second zone of low RA (45 °C, 0.75 Ac), shown in Fig. 6, as after 24 h of reaction, the compositions of monoolein, diolein and oleic acid were approximately 28.12%, 18.72% and 5.64%, respectively, and the total conversion of triolein was 51.42%. The main difference between these assays was the diolein yield profile, which was bell shaped for the first low RA zone assay and an approximately hyperbolic curve for the second assay.

In conclusion, the results obtained by the glycerolysis of triolein assays were consistent with the behavior predicted trough the preliminary experimental setup for lipase stability. In the two zones in which a high enzyme RA was predicted, the conversion of triolein was also increased with a monoolein yield considerably higher at 35 °C and 0.75 volume fraction of acetone. Conversely, in the two solvent/temperature zones that previously showed poor stability (low RA), the final compositions and the conversion for the glycerolysis reaction were very similar, and in both cases, the monoolein yield was poor.

4. Conclusions

For the selection of a suitable lipase catalytic system for monoglyceride production, we studied the catalytic residual activity of two immobilized lipases (*Candida rugosa* on chitosan and *Mucor miehei* on macroporous anion-exchange resin of the phenolic type) in three different mixtures of acetone/isooctane at different temperatures over 24 h.

The conditions that optimized the residual activity RA (%) of the *Mucor miehei* enzyme were the lowest assayed reaction temperature (35 °C) and the highest fraction of acetone in the solvent system (0.75). Conversely, for the *Candida rugosa* lipase, the maximum RA (≈56%) was achieved at the lowest acetone concentration in reaction system (0.25) and at approximately 36 °C. Surface response modeling proved to be valuable. The equations developed can be predictive of the effects of solvent ratio and temperature on the lipase RA selected response.

The use of the RSM-correctly predicted higher and lower RA log *P*/temperature conditions for the superior and inferior conversions of triolein in the glycerolysis trials. These results suggest the effectiveness of this procedure in the determination of the performance of immobilized lipase systems in non-aqueous media lipase catalysis optimization.

Acknowledgements

The authors are grateful to the Universidad Nacional de Colombia for financial support and to COLCIENCIAS and its program "Jóvenes Investigadores e Innovadores 2008".

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