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DEVELOPMENT OF A BIOREACTOR BASED ON LIPASE ENTRAPPED IN A MONOLITHIC CRYOGEL FOR ESTERIFICATION AND INTERESTERIFICATION REACTIONS

DESAROLLO DE UN BIORREACTOR COMPUESTO POR LIPASA ATRAPADA EN UN CRIOGEL MONOLÍTICO PARA LAS REACCIONES DE ESTERIFICACIÓN E INTERESTERIFICACIÓN

L.A.A. Veríssimo[1], P.C.G. Mól[2], W.C.L. Soares[3], V.P.R. Minim[3], M.C. Hespanhol[4], L.A. Minim[3]*
[1]*Federal University of Lavras, Department of Food Science, Lavras, Brazil.*
[2]*São Paulo State University, Department of Food Engineering and Technology, São José do Rio Preto, SP, Brazil.*
[3]*Federal University of Viçosa, Department of Food Technology, Viçosa, MG, Brazil.*
[4]*Federal University of Viçosa, Department of Chemistry, Viçosa, MG, Brazil.*
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Abstract

In this work, it was developed a novel bioreactor consisting of *Aspergillus niger* lipase (ANL) entrapped in a supermacroporous polyacrylamide cryogel. Bioreactor performance in catalyzing the flavor ester butyl butyrate was optimized as a function of temperature (°C), substrate molar ratio (butyric acid: n-butanol) and added water (% (v/v)). The bioreactor was also applied to interesterification reactions. The maximum esterification yield (46%) for butyl butyrate synthesis was found at 40 °C, with a butyric acid:n-butanol molar ratio of 1:1.43 and added water of 65% (v/v). The bioreactor showed to be reusable with four consecutive reuses in the esterification reaction, with a volumetric activity of 31.6 mmol/mL·h. An interesterification yield of 17.35% was achieved when the substrate concentrations of tripalmitin and triolein were both 15 mmol/L, 35% added water (v/v) and temperature of 40 °C. These results showed that the bioreactor is a promising biocatalyst for biotechnological applications.
Keywords: cryogel, lipase, entrapment, butyl butyrate, tripalmitin.

Resumen

En este trabajo se desarrolló un nuevo biorreactor compuesto por lipasa de *Aspergillus niger* (ANL) atrapada en un criogel monolítico supermacroporoso basado en poliacrilamida. El rendimiento del biorreactor en la catalización del éster aromatizante butirato de butilo se optimizó en función de la temperatura (°C), de la relación molar del sustrato (ácido butírico: n-butanol) y del agua añadida (% (v/v)). El biorreactor también se aplicó a reacciones de interesterificación. El rendimiento máximo de esterificación (46%) para la síntesis de butirato de butilo se encontró a 40 °C, con una relación molar de ácido butírico: n-butanol de 1: 1,43 y agua añadida de 65% (v/v). El biorreactor demostró ser reutilizable con cuatro reutilizaciones consecutivas en la reacción de esterificación, con un rendimiento de esterificación residual del 64,6% y una actividad volumétrica de 31,6 mmol/mL·h. Se logró un alto rendimiento de interesterificación (17,35% en 24 hrs) cuando ambas concentraciones, la de sustrato de tripalmitina y la de trioleína, fueron de 15 mmol/L, con 35% de agua añadida (v/v) y a una temperatura de 40 ºC. Estos resultados mostraron que el biorreactor es un biocatalizador prometedor para aplicaciones biotecnológicas.
Palabras clave: criogel, lipasa, atrapamiento, butirato de butilo, tripalmitina.

1 Introduction

The demand for "natural products" with functional or pharmaceutical applications has made the enzyme technology an attractive alternative for wide use in reactions in at industrial scale, promoting major advances in this technology (Lopes *et al*., 2011; Damnjanović *et al*., 2012; Martins *et al*., 2013).

In this way, lipases (glycerol ester hydrolases, EC 3.1.1.3) have received significant attention because of their versatility in catalyzing different reactions and specificity in producing products such as flavor esters, structured lipids, biodiesel, diesel additives, surfactants, chiral compounds and others (Lopes *et*

* *Corresponding author. E-mail*: lminim@ufv.br
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al., 2011; Goldbeck and Filho, 2013, Veríssimo *et al*., 2015; Orozco-Hernandéz *et al*., 2016).

The lipase is used in its immobilized form for practical and economic reasons, since this form retains the enzyme in the support and the cost of acquiring new biocatalysts is minimized. Additionally, the immobilization process allows the immobilized system to be used in continuous or semi-continuous processes (Damnjanović *et al*., 2012; Martins *et al*., 2013; Velasco-Bucheli *et al*. 2017).

One of the challenges involved in developing a new protocol for lipase immobilization is the choice of support material, since these enzymes can act simultaneously on the hydrolysis and synthesis reactions, which commonly occur in macro and micro-aqueous systems (Jaeger and Eggert, 2002; Milašinović *et al*., 2012). This decision is even more complex in the case of enzymatic reactions involving substrates such as viscous oils and fats, which impede fluid flow and mass transfer (Milašinović *et al*., 2012; Soumanou *et al*., 2013).

The polyacrylamide-based supermacroporous monolithic cryogel is a potential support for this biotechnological application because of its excellent biocompatibility with biomolecules, low cost of materials and highly porous structure, which results in a lower pressure drop, facilitated diffusion and reduced clogging problems (Arvidsson *et al*., 2003; Plieva *et al*., 2004; Uygun *et al*., 2012).

Other features that make cryogel an interesting support material for lipase immobilization is its natural water content and hydrophilic character (Arvidsson *et al*., 2003; Plieva *et al*., 2004; Uygun *et al*., 2012). Although the water content appears to shift the equilibrium reaction from synthesis to hydrolysis, in the literature it is well documented that a proper enzyme hydration state is necessary for its stability and activity (Monot *et al*., 1991; Chen, 1996, Milašinović *et al*., 2012).

Additionally, the cryogel can be produced in the form of monolithic columns allowing the coupling of enzymatic processes in reactors and can be operated at a continuous flow (Anuar *et al*., 2013).

In this work, we developed a novel bioreactor consisting of *Aspergillus niger* lipase (ANL) entrapped in a polyacrylamide-based supermacroporous cryogel. The bioreactor was applied in esterification reactions such as butyl butyrate synthesis, an important pineapple flavor ester for the food and beverages industries (Martins *et al*., 2013) and in interesterification reactions of triolein with tripalmitin as a model for an interesterification reaction system (Paula *et al*., 2010).

2 Materials and methods

2.1 Chemicals

Lipase from *Aspergillus niger* (ANL) was kindly provided by Prozyn BioSolution (São Paulo, Brazil). Bovine serum albumin (BSA, 98%), N,N,N',N' - Tetra-methyl-ethylenediamine (TEMED, 99%), acrylamide (AAm, 99%), N,N' - methylene-bis(acrylamide) (MBAAm, 99%), allylglycidyl ether (AGE, 99%), ammonium persulfate (APS, 98%), p-nitrophenyl palmitate (p-NPP), p-nitrophenol, butyl butyrate, butyric acid, n-butanol, hexane, polyethylene glycol with average molar mass of 1500 g/mol (PEG 1500), Triton X-100 and Tween 80 were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals used were of analytical grade.

2.2 Preparation of the cryogel column containing entrapped lipase

A supermacroporous monolithic cryogel containing entrapped lipase was produced by cryocopolymerization of AAm and MBAAm (10). The monomers (1.185 g of AAm, 0.3175 g of MBAAm) were dissolved in 17 mL of deionized water containing 0.25 mL of AGE. An 8 mL volume of lipase solution (3.45 $mg_{protein}$/mL) was added to the mixture and subsequently cooled in an ice bath. The enzyme solution contained Triton X-100 (0.34 mg/mL), PEG 1500 (0.64 mg/mL) or Tween 80 (0.64 mg/mL) as an additive. A control was made using the enzyme solution with no additive.

Free radical polymerization was initiated by adding TEMED (23.8 μL) followed by APS (27.5 mg) (10). The reaction mixture was poured into a glass column (I.D. 5 mm, height 100 mm) and frozen at -12 °C for 24 h in a refrigerated bath (QUIMIS, Brazil) containing ethanol. Pure cryogel was prepared in the same way as described above, but with deionized water instead of the enzyme solution. The column was thawed at refrigeration temperature, washed with 300 mL of water at a flow rate of 0.25 mL/min and stored at 4 °C.

2.3 *Determination of immobilized protein in the cryogel matrix*

The total protein content of the bioreactor was determined indirectly from the difference between the amount of protein added into the polymerization solution and the protein content in the washing solution. The amount of free protein was measured by the Bradford method (Bradford, 1976).

2.4 *Hydrolytic activity assay*

The hydrolytic activity of the bioreactor was measured by monitoring the hydrolysis of p-nitrophenyl palmitate (p-NPP) in p-nitrophenol (Gupta *et al*., 2002). A 6 mL volume of isopropanol solution containing p-NPP (7.95 mm/L) was mixed with 54 mL of Tris HCl buffer (50 mmol/L, pH 8.0, containing 0.4 % w/v of Triton X-100 and 0.1 % w/v of arabic gum). This solution was pumped through the column containing the immobilized lipase cryogel at a flow rate of 0.25 mL/min for 1 h at 45 °C. Samples were collected at the outlet of the bioreactor and the released p-nitrophenol content was quantified by a UV-Vis spectrophotometer (Biomate 3, Thermo Scientific, USA) at 410 nm. Under these conditions, the control sample corresponded to the initial substrate solution before passing through the bioreactor. The activity was expressed as international units (U), where 1 U was defined as the amount of enzyme required to release 1 μmol of p-nitrophenol per minute under assay conditions. Results were presented as apparent hydrolytic activity obtained from the ratio of the bioreactor hydrolytic activity and the mass of dry cryogel (U/$g_{cryogel}$).

2.5 *Determination of immobilization parameters*

Immobilization efficiency was evaluated in terms of recovery hydrolytic activity (R_{HA}) and loading efficiency (η), as follows (Milašinović *et al*., 2012):

$$R_{HA}\% = \left(\frac{U_{bioreactor}}{P_I \times HA_0}\right) \times 100 \tag{1}$$

$$\eta = \frac{C - IV_i - C_f V_f}{C_i V_i} \times 100 \tag{2}$$

where HA_0 is the specific hydrolytic activity of free lipase (U/$mg_{protein}$), P_I is the protein content immobilized per gram of cryogel ($mg_{protein}/g_{cryogel}$), $U_{bioreactor}$ is the apparent hydrolytic activity of bioreactor (U/$g_{cryogel}$), C_i is the initial protein concentration added to the column, V_i is the initial volume of the enzyme solution, C_f is the protein concentration of the washing solution and V_f is the total volume of the washing solution.

2.6 *Porosity, water fraction and swelling capacity*

The porosity (φ) of pure cryogel and cryogel containing entrapped lipase was estimated by measuring the free water content and cryogel volume of a given sample. A cryogel sample saturated with deionized water was immersed in a measuring cylinder containing deionized water of a given volume V_1, and the final volume V_2 was measured. The cryogel volume V_0 was calculated by the volume difference, i.e., $V_0 = V_2 - V_1$. The mass of the wet sample saturated with water, m_w, was weighed. The cryogel was squeezed to remove the free water within the large pores and the mass of the sample without free water (m_s) was weighed. Porosity was calculated by Eq. (3) (Plieva *et al*., 2004):

$$\varphi = \left(\frac{m_w - m_s}{\rho_w V_0}\right) \times 100 \tag{3}$$

where ρ_w is the deionized water density.

The sample was lyophilized and the dried mass md was determined, which was used to calculate the total water fraction (φ_w), according to Eq. (4):

$$\varphi_w = \left(\frac{m_w - m_d}{\rho_w V_0}\right) \times 100 \tag{4}$$

The swelling capacity of the cryogel, S ($g_{water}/g_{\text{dry cryogel}}$) was determined according to Eq. (5) (Plieva *et al*., 2004):

$$S = \frac{(m_w - m_d)}{m_d} \tag{5}$$

Results of immobilization parameters and morphological characterization (porosity, water fraction and swelling capacity) were subjected to analysis of variance (ANOVA) and means were compared by the Tukey test at 95 % significance using SAS v.9 software. Each experiment was done in triplicate and data were expressed as means ± standard deviation.

2.7 Bioreactor performance on esterification and interesterification reactions

2.7.1 Esterification reaction

The synthesis reaction of butyl butyrate was carried out using a substrate solution with hexane as the reaction medium and n-butanol and butyric acid as substrates. Temperature (°C), substrate molar ratio (butyric acid: n-butanol) and added water (% (v/v)) were selected according to each defined experimental condition (Table 1). In all experiments the butyric acid concentration remained constant at 0.120 mol/L while the n-butanol concentration varied from 0.116 mol/L to 0.330 mol/L.

The substrate solution was pumped through the bioreactor at a continuous flow rate of 0.1 mL/min for 24 h at different experimental conditions (Table 1). Samples were collected at the outlet of the bioreactor and the content of butyl butyrate and butyric acid was determined by high performance liquid chromatography (HPLC). The esterification yield (Y%) in butyl butyrate synthesis was determined according to Eq. (6) (Martins *et al*., 2013):

$$Y\% = \frac{(C_0 - C_f)}{C_0} \times 100 \qquad (6)$$

where C_0 is the initial molar concentration of butyric acid (mol/L) and C_f is the final molar concentration of butyric acid (mol/L).

A central composite rotatable design (CCRD) with three independent variables, temperature (°C) (X_1), substrate molar ratio (butyric acid:n-butanol) (X_2) and added water (% v/v) (X_3) was applied in order to obtain the optimal conditions for the esterification reaction catalyzed by the bioreactor.

The experimental results obtained from the CCRD were analyzed with the response surface regression procedure (RSREG, SAS Institute Inc., v. 9.0, Cary, NC, USA) using the second order polynomial equation (Eq. (7)):

$$Y = \beta_0 + \sum \beta_i X_i \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + e \qquad (7)$$

where Y is the response variable (esterification yield, $Y\%$), β_0 is the model intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient, X_i and X_j are the levels of the independent variables.

The statistical significance of the model was evaluated by ANOVA and the suitability of the model was assessed using Fisher's test (F-test) by testing for significant differences between sources of variation in the experimental results, e.g. the significance of the regression, the lack of fit, and the coefficient of multiple determination (R^2). Parameters with less than 95 % significance ($p > 0.05$) were excluded and aggregated to the error term. All statistical analyses were carried out using SAS v.9 software.

Table 1. Experimental design and results of central composite rotatable design

Assay	Temperature X_1	Substrate molar ratio X_2	Added water X_3	Esterification yield (%)
1	-1 (35.0)[a]	-1 (1:2.0)	-1 (60.0)	9.21
2	-1 (35.0)	-1 (1:2.0)	+1 (70.0)	23.94
3	-1 (35.0)	+1 (1:1.11)	-1 (60.0)	45.62
4	-1 (35.0)	+1 (1:1.11)	+1 (70.0)	2.63
5	+1 (45.0)	-1 (1:2.0)	-1 (60.0)	7.26
6	+1 (45.0)	-1 (1:2.0)	+1 (70.0)	17.34
7	+1 (45.0)	+1 (1:1.11)	-1 (60.0)	34.43
8	+1 (45.0)	+1 (1:1.11)	+1 (70.0)	6.48
9	-1.682 (31.59)	0 (1:1.43)	0 (65.0)	24.01
10	+1.682 (48.41)	0 (1:1.43)	0 (65.0)	7.87
11	0 (40.0)	-1.682 (1:2.75)	0 (65.0)	16.81
12	0 (40.0)	+1.682 (1:0.97)	0 (65.0)	16.87
13	0 (40.0)	0 (1:1.43)	-1.682 (56.59)	6.56
14	0 (40.0)	0 (1:1.43)	+1.682 (73.41)	12.49
15	0 (40.0)	0 (1:1.43)	0 (65.0)	48.7
16	0 (40.0)	0 (1:1.43)	0 (65.0)	46.27
17	0 (40.0)	0 (1:1.43)	0 (65.0)	49.25
18	0 (40.0)	0 (1:1.43)	0 (65.0)	40.16

[a]Numbers in parenthesis represents the uncoded experimental values.

Table 2. Influence of different additives on immobilization and morphological parameters of lipase entrapped in cryogel.

Additive	Immobilization parameters			
	Protein content ($mg_{protein}/g_{cryogel}$)	Apparent hydrolytic activity ($U/g_{cryogel}$)	R_{HA} (%)	η (%)
Control (without additive)	7.74b ± 0.31	0.047d ± 0.002	20.64a ± 2.2	43.52b ± 0.31
Triton X-100	16.17a ± 0.32	0.114a ± 0.0005	20.58a ± 2.5	59.05a ± 0.79
PEG 1500	16.77a ± 0.79	0.086b ± 0.001	19.55a ± 2.0	54.59a ± 2.48
Tween 80	12.53c ± 0.34	0.062c ± 0.001	16.78a ± 0.3	39.08b ± 0.29
	Morphological parameters			
	φ	φ_w	S ($g_{water}/g_{\text{dry cryogel}}$)	
Pure cryogel	77.8a ± 0.9%	89.2a ± 3.7%	18.175a ± 0.034	
Control (without additive)	77.5a ± 1.2%	85.2a ± 1.2%	16.257a ± 0.073	
Triton X-100	76.1a ± 0.7%	88.6a ± 1.7%	15.755a ± 0.845	
PEG 1500	76.7a ± 2.8%	89.1a ± 3.7%	15.00a ± 0.802	
Tween 80	76.5a ± 2.4%	88.7a ± 1.4%	15.691a ± 0.033	

* Mean values with the same letter do not statistically differ from each other by the ANOVA Tukey test (p= 0.05).

Table 3. Experimental results of interesterification reactions catalyzed by the bioreactor

Assay	Substrate concentration (mmol/L)	Added water % (v/v)	Protein content (mgprotein/gcryogel)	H (%)	I (%)
1	15:15	35	14.057	6.69	17.35
2	15:15	65	14.07	2.23	4.51
3	30:30:00	35	14.848	2.92	0.38
4	30:30:00	65	13.304	0.017	0.39

2.7.2 *Quantification of butyric acid and butyl butyrate*

The butyric acid and butyl butyrate content were determined with a liquid chromatograph (Shimadzu, Japan) using a diode array detector (Shimadzu, Japan) at a wavelength of 210 nm. A reverse phase column (C18 apHera^{TM}, 250 mm × 4.6 mm, Supelco Analytical) was used for chromatographic separation. The mobile phase was composed of acetonitrile/water (75:25, v/v) at a flow rate of 0.8 mL/min. A sample volume of 20 μL was injected into the chromatograph and the esters were eluted from the column using an isocratic method (Chen, 1996). Sample blank corresponded to the substrate solution.

2.7.3 *Interesterification reaction*

The interesterification reaction of tripalmitin (PPP) with triolein (OOO) was performed using hexane as the reaction medium. The effect of the substrate concentration (15 mmol/L and 30 mmol/L) and added water (35 % and 65 % (v/v)) on interesterification yield (%) was evaluated and the tested the experimental were listed in Table 3. In all experiments the substrate molar ratio (tripalmitin:triolein) was maintained at 1:1.

The substrate solution was pumped through the bioreactor at a continuous flow rate of 0.1 mL/min for 24 h at a fixed temperature of 40 °C. Samples were collected at the bioreactor outlet and the triglycerides and free fatty acids contents (palmitic and oleic acid) were analyzed by HPLC. The interesterification yield (I %) was calculated according to Eq. (8) (Paula *et al.*, 2010).

$$I\% = \frac{(C_{C_{50}} + C_{C_{52}})}{(C_{C_{48}} + C_{C_{54}})} \times 100 \tag{8}$$

where $C_{C_{50}}$ and $C_{C_{52}}$ are the concentrations (mmol/L) of triglycerides having 50 and 52 carbons in the residues of fatty acids $C_{C_{48}}$ and and $C_{C_{54}}$ are the concentrations (mmol/L) of tripalmitin and triolein.

The indexes "0" and "t" represent the concentrations at time zero and at a given time after the reaction began.

The hydrolysis degree (H %) was calculated according to Eq. (9).

$$H\% = \frac{(FA_f)_t}{(FA_t)_0} \times 100 \quad (9)$$

where FA_f is the total free fatty acids concentration (mmol/L) released in the reaction at time "t" and FA_t is the theoretical total concentration of fatty acids that would be released, based on total hydrolysis of the triglycerides.

2.7.4 *Quantification of free fatty acids and triglycerides*

The free fatty acids and triglycerides contents were determined using a liquid chromatograph (Shimadzu, Japan) at a wavelength set at 205 nm. A reverse phase column (C18 apHera^{TM}, 250 mm × 4.6 mm, Supelco Analytical) was used for chromatographic separation, with the mobile phase composed of pure acetonitrile and water (97:3 v/v) at a flow rate of 1 mL/min and a temperature of 40 °C. The triglycerides, palmitic acid (P) and oleic acid (O) were eluted from the column using an isocratic method (Borch, 1975).

Product concentrations were determined by detecting all triglycerides present in the reaction medium where the sum of the carbon numbers in the fatty acid residues resulted in 50 (PPO, POP, and OPP) and 52 (POO, OPO, and OOP).

2.8 *Operational stability of the bioreactor*

The operational stability of the bioreactor was evaluated by successive esterification reactions of butyric acid with n-butanol according to the procedure described previously. The reactions were performed over 24 h in hexane with a substrate molar ratio of butyric acid:n-butanol of 1:1.43, 65 % (v/v) added water and a temperature of 40 °C. After each run the bioreactor was rinsed with 100 mL of water at a flow rate of 1 mL/min and stored for 12 h at room temperature. The bioreactor was then used in a new run with fresh substrates. The residual esterification yield (%) of bioreactor was calculated in terms of esterification yield (%) of the bioreactor measured after each cycle compared with the $Y\%$ of the bioreactor before the first cycle. Volumetric productivity of the bioreactor was calculated as the number of mmol of butyl butyrate synthesized based on the reaction over 24 h, per sample volume (mL).

3 Results and discussion

3.1 *Effect of additives on immobilization parameters and morphological properties*

The effects of the additives Triton X-100, PEG 1500 and Tween 80 on the protein content, apparent hydrolytic activity, R_{HA} and η of the bioreactors were evaluated and the experimental results were subjected to ANOVA at 95% significance (Table 2). The presence of additives significantly affected (p =0.05) the apparent hydrolytic activity (U/g$_{cryogel}$) and protein content immobilized (mg$_{protein}$/g$_{cryogel}$), with Triton X-100 being the additive that provided the greatest increases in these parameters.

According to Palomo *et al*. (2002) and Rodrigues *et al*. (2008), free ANL exhibit the tendency to form aggregates in solution, which can negatively influence its activity. The addition of surfactants such as Triton X-100 can minimize aggregate formation, leading to increased dispersion of ANL in the solution and consequent increase in bioreactor activity.

The presence of additives did not significantly affect the morphological parameters of the cryogel containing entrapped lipase and pure cryogel (Table 2). Therefore, the morphological structure of cryogel was maintained and the bioreactor also had high apparent hydrolytic activity.

3.2 *Bioreactor performance on ester synthesis*

A CCRD was applied in order to optimize the esterification yield of the butyl butyrate synthesis catalyzed by the bioreactor containing entrapped ANL with the additive Triton X-100. The experimental results obtained from CCRD (Table 1) were subjected to ANOVA using a Fisher's test. The results of the ANOVA are shown in a Pareto chart (Figure 1), where the standardized estimated effect of each factor is plotted in decreasing order and compared to the minimum magnitude of a statistically significant factor with a 95% confidence level, represented by the dashed vertical line.

The quadratic effect of added water had the highest influence on esterification yield, followed by its interaction with substrate molar ratio (Figure 1).

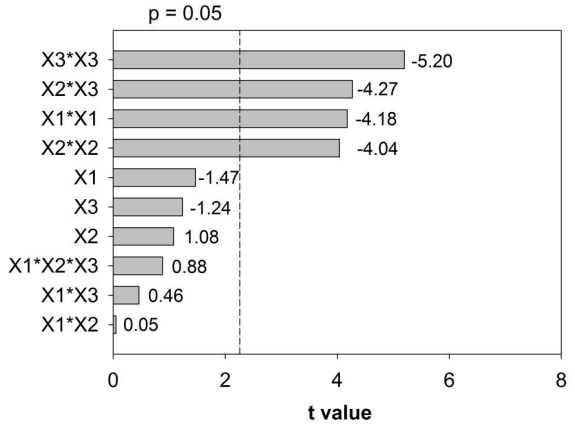

Fig. 1. Pareto chart for the standardized effects of the coded variables temperature (X_1), substrate molar ratio (X_2) and added water (X_3) on the esterification yield.

This result suggests that the esterification reaction was primarily governed by the total water content in the system.

The ANOVA performed for significant factors indicated that the model is statistically valid (p =0.0004) with a coefficient of determination (R^2) of 0.89, with a non-significant lack of fit (p >0.15). This result showed the model was adequate to represent the real relationship among the reaction variables. This reduced model can be described by Eq. (10), in terms of coded values.

$$Y = 45.80 - 3.15X_1 + 2.31X_2 - 2.65X_3 - 9.32X_1^2 - 9.00X_2^2 - 11.59X_3^2 - 11.97X_2X_3 \quad (10)$$

where Y is the percentage of esterification yield and X_1, X_2 and X_3 are the coded values of temperature, substrate molar ratio and added water.

The relationship between the reaction parameters and the esterification yield can be examined by the contour plots depicted in Figure 2 (a), (b) and (c), which were generated from the predicted model.

From the contour plots (Figure 2), it may be observed that the esterification yield increased until reaching a maximum value (approximately 45 %) when the substrate molar ratio was around 1:1.43 (acid:alcohol) and with the increase in temperature from 35 °C to 40 °C and the quantity of added water from 60 % to 65 % (v/v).

The initial effect of added water and substrate molar ratio on esterification yield is intrinsically correlated to the partition effects of the relatively hydrophilic substrates butyric acid and n-butanol to the aqueous phase (Monot *et al*., 1991; Castro *et al*., 1999). In this context, the negative effect of the interaction substrate molar ratio (X_2) and added water (X_3) can be explained based on the fact that at low coded levels of substrate molar ratio from 1:2.75 to 1:1.8 (acid:alcohol) and added water (around 60 % v/v), both the butyric acid and n-butanol were almost fully solubilized in the aqueous phase. As a result, a high substrate molar concentration and the alcohol excess may have become toxic to the enzyme microenvironment or induced some inhibitory effect (Romero *et al*., 2005; Mhetras *et al*., 2010; Guillén *et al*., 2012).

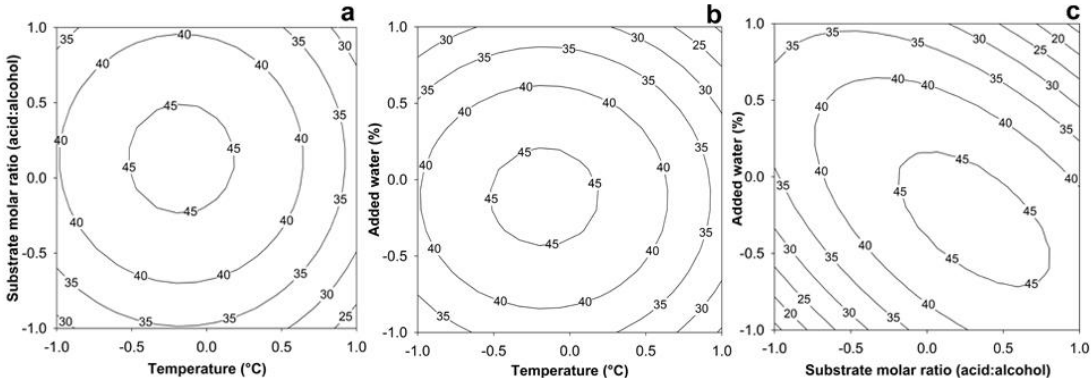

Fig. 2. Effects of reaction parameters on esterification yield: (a) temperature (°C) and substrate molar ratio (acid:alcohol); (b) temperature (°C) and added water (%); (c) substrate molar ratio (acid:alcohol) and added water (%). The numbers inside the contour plots indicate the esterification yield (%) at given reaction conditions. In each figure, the missing variable was fixed at the central point.

Another aspect observed refers to the fact that the butyl butyrate presented a high partition coefficient in water - hexane system (13), which explains why it was possible to have continuous synthesis of this ester in spite of the presence of elevated water content in the biphasic systems, since the butyl butyrate produced was completely transferred to the hexane rich phase (Monot *et al*., 1991; Castro *et al*., 1999; Klibanov *et al*., 2000). At high substrate concentrations (obtained at low coded levels of substrate molar ratio around 1:2.75 to 1:1.8) however the polarity of the hexane rich phase may have increased and led to a decrease in the partition coefficient of butyl butyrate, so the reaction equilibrium may have shifted because of a favorable partition coefficient of the product to the water rich phase since a low esterification yield was observed (20 % to 30 %) (Klibanov *et al*., 2000; Romero *et al*., 2005).

Thus, the increasing of added water up to 65 % (v/v) and the decreasing of the substrate molar ratio from 1:2.75 to approximately 1:1.43 (Figure 2) resulted in an increase of the percentage of esterification until reaching a maximum value of around 45 %.

Similar behavior was verified by Ben Salah *et al*. (2007) who obtained maximum esterification yields when 45 % (w/w) of water (in relation the total amount of the reaction mixture) was used in butyl acetate synthesis catalyzed by *Rhizopus oryzae* lipase immobilized on Celite 545. Nevertheless, the continuous increase of water added in the substrate solution over 65 % (v/v) resulted in a reduced esterification yield that may have been caused by a shifting of the equilibrium reaction for hydrolysis of the product, butyl butyrate (Chowdary and Prapulla, 2002; Ben Salah *et al*., 2007; Mhetras *et al*., 2010). The water content released during the synthesis reaction and the high initial content of added water in the substrate solution may also have contributed to the shifting of the equilibrium reaction since this will accumulate on the support due to the excellent swelling properties of the cryogel (Chowdary and Prapulla, 2002; Pires-Cabral *et al*., 2007).

As shown in Figure 2 a and b, the bioreactor reached the best esterification yield at a constant temperature of 40 °C, suggesting the immobilized system was most stable at this temperature. Above this value, yields decreased, probably due to a negative effect on enzyme stability or thermal inactivation of the lipases (Monot *et al*., 1991; Ben Salah *et al*., 2007).

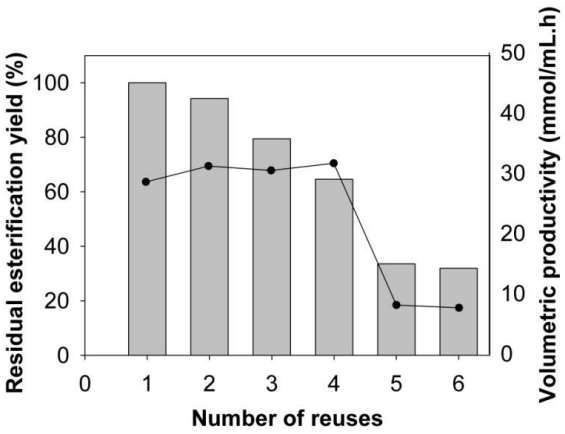

Fig. 3. Residual esterification yield (bars chart) and volumetric productivity (-●-) of the bioreactor as a function of the number of reuses.

3.3 Operational stability of biocatalysts

The bioreactor was used repeatedly in the esterification of butyl butyrate at temperature of 40 °C, substrate molar ratio of 1:1.43 and 65 % (v/v) of added water and the residual esterification yield as a function of reuse number was assessed (Figure 3). For the first two reuses, only 6 % of loss of residual esterification yield was observed. After six reuses, the bioreactor retained 31.9 % of esterification yield. This result was lower than those reported by Santos *et al*. (2007) (45 %) using a *Candida rugosa* lipase adsorbed in poly (N-methylolacrylamide) after six batch process of butyl butyrate synthesis. On the other hand, our results for volumetric productivity (7.64 mmol/mL.h) after the 6th cycle were higher than the result (4.75 mmol/mL.h) found by Milašinović *et al*. (2012) after 48 h of reaction of n-amyl isobutyrate in a batch process, using a *Candida rugosa* lipase entrapped into hydrogels.

3.4 Bioreactor performance on interesterification reactions

The interesterification reactions in the bioreactor occurred only at systems with a lower substrate concentration (15 mmol/L). Moreover, the highest value of *I* (%) was obtained when the lowest water content (35 % v/v) was added to the substrate solution (Table 3). Thus, at low levels of substrate concentration the added water negatively influenced the interesterification yield and the excess water (65 % v/v) favored the equilibrium of the reaction to triglycerides hydrolysis instead of its re-esterification.

According to Soumanou *et al*. (2013), the interesterification reaction catalyzed by lipases is a special case of fatty acids transference and involves, at molecular level, sequential reactions of hydrolysis and acyl exchange.

The initial step of interesterification reactions involves triacylglycerol hydrolysis' of to produce diacylglycerols, monoacylglycerols and free fatty acids. Hydrolysis products are produced during interesterification until equilibrium is achieved (Marangoni, 2002). However, when the water content is low the re-esterification is favored and the interesterification will occur (Marangoni, 2002; Pérignon *et al*., 2013).

As reported by Pérignon *et al*. (2013), the water content is the limiting step in the interesterification reaction and its content in the reaction medium modulates the course of the reaction.

For the system containing 30 mmol/L of tripalmitin and triolein, a negligible formation of triglyceride C_{50} and C_{52} was noticed even when using low levels of water (35 % v/v). This behavior may be related to the influence of substrate concentrations in the interesterification reactions. The negative effect of substrate concentration may have resulted from the accumulation of free fatty acids in the reaction medium because of triglyceride hydrolysis, leading to acidification of the aqueous phase surrounding the enzyme and consequently in a loss of its catalytic activity (Paula *et al*., 2010; Pérignon *et al*., 2013).

The best interesterification yield (17.35 %) was achieved at the lower equimolar concentration of substrate (15 mmol/L), added water of 35 % (v/v) and constant temperature of 40 °C.

This result was higher than those obtained by Tecelão at al. (2010) using *C. parapsilosis* lipase immobilized on Accurel MP 1000 (8.5 %) and those obtained by Paula *et al*. (2010) who obtained a maximum interesterification yield of 10 % using tripalmitin (60 mmol/L) and triolein (40 mmol/L) as substrate and catalyzed by ANL covalently immobilized on polysiloxane-polyvinyl alcohol.

Conclusions

The lipase entrapped in polyacrylamide cryogel offered an easy and low-cost way to immobilize lipases, since the application of bioreactor on hydrolysis and esterification reactions was effective. The addition of non-ionic surfactant Triton X-100 to the lipases solution during the entrapment process significantly increased the apparent hydrolytic activity recovered of the bioreactor, while maintaining its morphological structure. The use of the bioreactor in esterification reactions in the presence of organic solvents was efficient, achieving a maximum esterification yield of 46%. Moreover, the bioreactor was shown to be effective in interesterification reactions and has potential for future industrial applications. The possibility of reusing the bioreactor four consecutive times makes this system very attractive for industrial applications.

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References

Anuar, S.T., Zhao, Y-Y., Mugo, S.M., Curtis, J.M. (2013). The development of a capillary microreactor for transesterification reactions using lipase immobilized onto a silica monolith. *Journal of Molecular Catalysis B: Enzymatic 92*, 62-70.

Arvidsson, P., Plieva, F.M., Lozinsky, V.I., Galaev, I.Y., Mattiasson, B. (2003). Direct chromatographic capture of enzyme from crude homogenate using immobilized metal affinity chromatography on a continuous supermacroporous adsorbent. *Journal of Chromatography A 986*, 275-290.

Ben Salah, R., Ghamghui, H., Miled, N., Mejdoub, H., Gargouri, Y. (2007). Production of butyl acetate ester by lipase from novel strain of *Rhizopus oryzae. Journal of Bioscience and Bioengineering 103*, 368-372.

Borch, R.F. (1975). Separation of long-chain fatty acids as phenacyl esters by high-pressure liquid chromatography. *Analytical Chemistry 47*, 2437-2439.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry 72*, 248-254.

Castro, H., Oliveira, P., Soares, C.F., Zanin, G. (1999). Immobilization of porcine pancreatic lipase on celite for application in the synthesis of butyl butyrate in a nonaqueous system. *Journal of the American Oil Chemists' Society 76*, 147-152.

Chen J-P. (1996). Production of ethyl butyrate using gel-entrapped *Candida cylindracea* lipase. *Journal of Fermentation and Bioengineering 82*, 404-407

Chowdary, G.V. and Prapulla, S.G. (2002). The influence of water activity on the lipase catalyzed synthesis of butyl butyrate by transesterification. *Process Biochemistry 38*, 393-397.

Damnjanović, J.J., Žuža, M.G., Savanović, J.K., Bezbradica, D.I., Mijin, D.Ž., Bošković-Vragolović, N., Knežević-Jugović, Z.D. (2012). Covalently immobilized lipase catalyzing high-yielding optimized geranyl butyrate synthesis in a batch and fluidized bed reactor. *Journal of Molecular Catalysis B: Enzymatic 75*, 50-59.

García-Dávila, J., Ocaranza-Sánchez, E., Sánchez, C., Ortega-Sánchez, E., Tlecuitl-Beristaín, S., Martínez-Ayala, A.L. (2017). FTIR analysis of hydrotreated Jatropha curcas L. seed oil over Ni-Mo catalyst for biofuel production. *Revista Mexicana de Ingeniería Química 16*, 337-345.

Goldbeck, R. and Filho, F.M. (2013). Screening, characterization, and biocatalytic capacity of lipases producing wild yeasts from Brazil biomes. *Food Science and Biotechnology 22*, 79-87.

Guillén, M., Benaiges, M.D., Valero, F. (2012). Biosynthesis of ethyl butyrate by immobilized recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris. Biochemical Engineering Journal 65*, 1-9.

Gupta, N., Rathi, P., Gupta, R. (2002). Simplified para-nitrophenyl palmitate assay for lipases and esterases. *Analytical Biochemistry 311*, 98-99.

Jaeger, K-E. and Eggert, T. (2000). Lipases for biotechnology. *Current Opinion in Biotechnology 13*, 390-397.

Klibanov, A.M., Samokhin, G.P., Martinek, K., Berezin, I.V. (2000). A new approach to preparative enzymatic synthesis. *Biotechnology and Bioengineering 67*, 737-747.

Lopes, D.B., Duarte, M.C.T., Macedo, G.A. (2011). Biosynthesis of oleyl oleate wax ester by non-commercial lipase. *Food Science and Biotechnology 20*, 1203.

Martins, A.B., Friedrich, J.L.R., Cavalheiro, J.C., Garcia-Galan, C., Barbosa, O., Ayub, M.A.Z., Fernandez-Lafuente, R., Rodrigues, R.C. (2013). Improved production of butyl butyrate with lipase from *Thermomyces lanuginosus* immobilized on styrene-divinylbenzene beads. *Bioresource Technology 134*, 417-422.

Milašinović, N., Knežević-Jugović, Z., Jakovljević, Ž., Filipović, J., Kalagasidis Krušić, M. (2012). Synthesis of n-amyl isobutyrate catalyzed by *Candida rugosa* lipase immobilized into poly(N-isopropylacrylamide-co-itaconic acid) hydrogels. *Chemcal Engineering Journal 181-182*, 614-623.

Mitra, K., Kim, S-A., Lee, J-H., Choi, S-W., Lee, K-T. (2010). Production and characterization of α-linolenic acid enriched structured lipids from lipase-catalyzed interesterification. *Food Science and Biotechnology 19*, 57-62.

Monot, F., Borzeix, F., Bardin, M., Vandecasteele, J-P. (1991). Enzymatic esterification in organic media: role of water and organic solvent in kinetics and yield of butyl butyrate synthesis. *Applied Microbiology and Biotechnology 35*, 759-765.

Marangoni, A.G. (2002). Lipases: structure, function, and properties. In: *Lipid Biotechnology*, (T.M. Kuo and H.W. Gardner, eds.), Pp 65-78. Marcel Dekker, Inc., New York.

Mhetras, N., Patil, S., Gokhale, D. (2010). Lipase of *Aspergillus niger* NCIM 1207: A potential biocatalyst for synthesis of isoamyl acetate. *Indian Journal of Microbiology 50*, 432-437.

Orozco-Hernández, J.M., Romero, R., Natividad, R., Baeza-Jiménez. (2016). Improvement strategies for the enzymatic production of biodiesel in the presence of primary alcohols. *Revista Mexicana de Ingeniería Química 15*, 935-942.

Palomo, J.M., Muñoz, G., Fernández-Lorente, G., Mateo, C., Fernández-Lafuente, R., Guisán,

J.M. (2002). Interfacial adsorption of lipases on very hydrophobic support (octadecyl-Sepabeads): immobilization, hyperactivation and stabilization of the open form of lipases. *Journal of Molecular Catalysis B: Enzymatic 19-20*, 279-286.

Paula, A., Nunes, G., Silva, J., Castro, H., Santos, J. (2010). Screening of food grade lipases to be used in esterification and interesterification reactions of industrial interest. *Applied Biochemical and Biotechnology 160*, 1146-1156.

Pérignon, M., Lecomte, J., Pina, M., Renault, A., Simonneau-Deve, C., Villeneuve, P. (2013). Activity of immobilized *Thermomyces lanuginosus* and *Candida antarctica* B lipases in interesterification reactions: effect of the aqueous microenvironment. *Journal of the American Oil Chemists' Society 90*, 1151-1156.

Pires-Cabral, P., da Fonseca, M.M.R., Ferreira-Dias, S. (2007). Modelling the production of ethyl butyrate catalysed by *Candida rugosa* lipase immobilised in polyurethane foams. *Biochemical Engineering Journal 33*, 148-158.

Plieva, F.M., Andersson, J., Galaev, I.Y., Mattiasson, B. (2004). Characterization of polyacrylamide based monolithic columns. *Journal of Separation Science 27*, 828-836.

Rodrigues, D.S., Mendes, A.A., Adriano, W.S., Gonçalves, L.R.B., Giordano, R.L.C. (2008). Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. *Journal of Molecular Catalysis B: Enzymatic 51*, 100-109.

Romero, M.D., Calvo, L., Alba, C., Daneshfar, A., Ghaziaskar, H.S. (2005). Enzymatic synthesis of isoamyl acetate with immobilized *Candida antarctica* lipase in n-hexane. *Enzyme and Microbial Technology 37*, 42-48.

Santos, J.C., Nunes, G.F.M., Moreira, A.B.R., Perez, V.H., de Castro, H.F. (2007). Characterization of *Candida rugosa* lipase immobilized on poly(N-methylolacrylamide) and its application in butyl butyrate synthesis. *Chemical Engineering & Technology 30*, 1255-1261.

Soumanou, M.M., Pérignon, M., Villeneuve, P. (2013). Lipase-catalyzed interesterification reactions for human milk fat substitutes production: A review. *European Journal of Lipid Science and Technology 115*, 270-285.

Tecelão, C., Silva, J., Dubreucq, E., Ribeiro, M.H., Ferreira-Dias, S. (2010). Production of human milk fat substitutes enriched in omega-3 polyunsaturated fatty acids using immobilized commercial lipases and *Candida parapsilosis* lipase/acyltransferase. *Journal of Molecular Catalysis B: Enzymatic 65*, 122-127.

Uygun, M., Uygun, D.A., Özçalişkan, E., Akgöl, S., Denizli, A. (2012). Concanavalin A immobilized poly(ethylene glycol dimethacrylate) based affinity cryogel matrix and usability of invertase immobilization. *Journal of Chromatography B 887-888*, 73-78.

Velasco-Bucheli, R., Gil, J.H., García, C.M., Durango, D.L. (2017). Transformation of cinnamyl alcohol using immobilized *Colletotrichum acutatum* cells as catalyst. *Revista Mexicana de Ingeniería Química 16*, 55-65.

Veríssimo, L.A.A., Soares, W.C.L., Mól, P.C.G., Minim, V.P.R., Silva, M.C.H., Minim, L.A. (2015). Optimization of flavor ester synthesis catalysed by *Aspergillus niger* lipase. *African Journal of Microbiology Research 9*, 922-928.