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Response Surface Methodology as an Approach to Determine Optimal Activities of Lipase Entrapped in Sol–Gel Matrix Using Different Vegetable Oils

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Abstract The conditions for maximization of the enzymatic activity of lipase entrapped in sol–gel matrix were determined for different vegetable oils using an experimental design. The effects of pH, temperature, and biocatalyst loading on lipase activity were verified using a central composite experimental design leading to a set of 13 assays and the surface response analysis. For canola oil and entrapped lipase, statistical analyses showed significant effects for pH and temperature and also the interactions between pH and temperature and temperature and biocatalyst loading. For the olive oil and entrapped lipase, it was verified that the pH was the only variable statistically significant. This study demonstrated that response surface analysis is a methodology appropriate for the maximization of the percentage of hydrolysis, as a function of pH, temperature, and lipase loading.

Keywords Lipase · Canola oil · Soybean oil · Olive oil · Sol–gel encapsulation

Introduction

Recently, Reetz and others have published a number of studies showing that adding organically modified silanes (precursors), such as methyltrimethoxysilane or poly (dimethylsiloxane), to tetraethoxysilane (TEOS) during hydrolysis can produce a protein-doped organicinorganic hybrid material that is hydrophobic [1–7]. Systems already tested

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for lipases from either microbial or animal sources include the utilization of different precursors, alternative stabilizing additives (polyvinyl alcohol, albumin, gelatin, and others), and several solvents (methanol, ethanol, and others) [1, 3, 7]. The behavior of the sol–gel-encapsulated lipase systems depends on the physical and structural properties of the support and the physical and chemical properties of the lipase used [7–9]. We recently developed a bioactive organically modified silicate through sol–gel processing, starting with silica matrices produced by acid- and base-catalyzed hydrolysis of TEOS, in the presence of the additive polyethylene glycol (PEG) [6]. This is a modified methodology previously established for a chemical catalyst [6, 8], and to our knowledge, it has not been used by other groups for encapsulating the enzyme lipase. The encapsulation of *Candida rugosa* lipase (CRL) in sol–gel prepared by the hydrolysis of alkyl-substituted silanes like TEOS, in the presence of PEG, showed considerably high hydrolytic and esterification activities. This result was attributed to the interactions mediated by the hydrophobic–hydrophilic nature of CRL [6]. The hydrolysis of triglyceride esters to yield free fatty acids and glycerol represents an important group of chemical reactions relevant to the industrial processing of natural oils and fats. Hydrolysis is the primary reaction for production of free fatty acids that may then be interesterified, transesterified, or converted into high-value fatty alcohols. The mainstream current technologies for hydrolysis are based on high-temperature, high-pressure contacting processes with steam or superheated liquid water, involving high temperatures and high-pressure equipment requirements. The best known process is the Colgate Emery process [10], which typically requires operating temperatures of 250 °C and a reaction pressure of 50 bar. Enzymic fat splitting (hydrolysis) has been studied extensively using enzymes immobilized on hydrophobic polymeric supports [11, 12] and also using enzymes freely attached at a liquid–liquid interface [13]. In this latter technique, one of the primary physical requirements is the provision of a free interface at which the lipase can catalyze the reaction, and the overall rate of reaction can be enhanced using dispersions of aqueous phase in oil substrate and vice versa. Another requirement is the enhancement of mass transfer processes necessary for the supply of reaction substrate from the corresponding bulk phases. In this study, we examined the hydrolysis of vegetable oil and focused upon phenomena that affect the process feasibility of using lipolytic hydrolysis as a viable alternative to conventional techniques. The objective was also to establish operational conditions that allow a desirable working region where a high hydrolysis percentage of olive, canola, and soybean oils can be attained. The high hydrolysis percentage is important for the biotechnological process of biodiesel and fatty acids. The response surface methodology (RSM) was used as an approach for the maximization of the percentage of hydrolysis for different oils.

Experimental Procedures

Enzyme and Chemicals

Commercial CRL (Type VII—product no. L1754) was purchased from Sigma Chemical (St Louis, MO). This lipase is substantially free of α-amylase and protease and contains lactose as an extender. Nominal-specific lipase activity was 104.94 U mg^{-1} protein. PEG (MW 1,450, Merck) was used as a stabilizing agent. The silane precursor TEOS was supplied by Across Organic (New Jersey, USA) and used without further purification. Ethanol (minimum 99%), ammonia (minimum 28%), hydrochloric acid (minimum 36%), and gum Arabic were from Synth (São Paulo, Brazil). Olive oil (low acidity), soybean oil, and

canola oil were purchased at a local market. Water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. Other chemicals were of analytical grade and used as received.

The percentage of different fatty acids varies according to oil origin, and the mean molar mass of the fatty acids in the oil is required for calculating the percentage of oil hydrolysis. The fatty acid composition of each tested oil in this work is given at Table 1. It can be observed that canola and olive oils are richest in oleic acid (57.71 and 69.00%, respectively) and soybean oil has the greatest amount of linoleic and linolenic fatty acids (54.50 and 8.30, respectively). Some components present in small quantities are not analyzed and consequently the sum of percentage composition values does not add up to 100%. Then for calculating the mean molar mass of the fatty acids in the oil it is necessary to first normalize the composition according to Eq. 1:

$$x_{N_i} = \frac{x_i}{\sum_i^n x_i} \tag{1}$$

where x_i is the percentage mass fraction of component i, x_{N_i} is the normalized mass fraction of component i, and n is the number of fatty acids present in the oil.

Then, the mean molar mass of the fatty acids in the oil ($\overline{M}$) is calculated as:

$$\overline{M} = \sum_i^n M_i x_{Ni} \tag{2}$$

where M_i is the molar mass of the fatty acid i.

The oil lipids mean molar mass ($\overline{\mathrm{MO}}$) is given by Equation (3):

$$\overline{\mathrm{MO}} = G + 3\overline{M} - 3W \cong 38.05 + 3\overline{M} \tag{3}$$

where G is the molar mass of glycerol (92.0935 g/mol) and W is the molar mass of water (18.0152 g/mol).

Encapsulation of Lipase from *Candida rugosa* in Sol–Gel Matrices

The methodology previously established by Soares et al. [6] was used as briefly described: 30 mL of TEOS was dissolved in 36 mL of absolute ethanol under nitrogen-inert atmosphere.

Table 1 Fatty acids composition for different vegetable oils.

Name	Short	Molar mass (g mol^{-1})	Soybean oil (% w/w)	Canola oil (% w/w)	Olive oil (% w/w)
Palmitic	C16:0	256.42	10.50	5.11	13.85
Oleic	C18:1	282.26	22.30	57.71	69.00
Linoleic	C18:2	278.43	54.50	22.45	12.25
Linolenic	C18:3	276.43	8.30	6.28	<0.1
Palmitileic	C16:3	254.42	<0.1	<0.2	1.90
$\overline{M}$=oil fatty acids mean molar mass (g mol^{-1})[a]	–	–	276.71	279.42	277.54
$\overline{\mathrm{MO}}$=oil lipids mean molar mass (g mol^{-1})[a]	–	–	868.18	876.31	870.67

Composition Source: [14]

[a] These table values were calculated in this work using Eq. 2 for $\overline{M}$ and Eq. 3 for $\overline{\mathrm{MO}}$, after normalizing the fatty acids composition to 100% (Eq. 1).

To this, 0.22 mL of hydrochloric acid dissolved in 5 mL of ultrapure water was slowly added, and the mixture was agitated (200 rpm) for 90 min at 35 °C. Then, 10 mL of lipase solution (18.29 mg mL^{-1}), PEG solution (5 mg mL^{-1}, 8 mL added), and 1 mL of ammonium hydroxide dissolved in 6 mL of ethanol were added (hydrolysis solution), and the mixture was kept under static conditions for 4 h to obtain the chemical condensation. The bulk gel was washed with heptane and acetone and dried under vacuum at room temperature for 24 h [6].

Hydrolysis Tests

For the free lipase hydrolysis tests, the substrate was prepared by mixing 30–70 mL of the selected oil with 70–30 mL of gum Arabic solution (7% w/v) containing free CRL (Table 2) and fixing the free lipase loading values (1 mL, 5 mg mL^{-1}).

For the encapsulated lipase runs, the substrate was prepared with 50% oil to water ratio and the percentage of hydrolysis was investigated by running enzymatic hydrolysis according to an experimental design with three different temperatures (30, 37, and 44 °C), pH values (4.0, 7.0, and 10.0) and encapsulated lipase-loading values (6, 7.5, and 9 mg mL^{-1}; Table 3).

Determination of the Percentage of Oil Hydrolysis

The percentage of oil hydrolysis (POH) was determined by titration of the released fatty acids and defined as the percentage weight of free fatty acids in the sample, in comparison with the maximum theoretical amount of free fatty acids that could be produced if all the oil in the sample was hydrolyzed:

$$\mathrm{POH}\ (\%) = \frac{N_a \times 0.02 \times 10^{-3} \times MM}{W_t \times f_0 \times f_1} \times 100 \tag{4}$$

where:

f_0 fraction of oil in the sample at the start of reaction (g_{lipids}/g_{sample})

f_1 ratio of the mass of fatty acids produced at total hydrolysis to the mass of oil ($g_{fatty\ acids}/g_{lipids}$)

Table 2 Free enzyme: Effect of pH, temperature, and oil/water ratio on the hydrolysis of olive, canola, and soybean oils.

Runs	pH	Temperature (°C)	Oil/water ratio (%)	Olive oil POH (%)	Canola oil POH (%)	Soybean oil POH (%)
1	4	30	40	11.13	3.91	4.80
2	10	30	40	4.64	5.54	4.29
3	4	44	40	11.69	3.85	4.98
4	10	44	40	4.47	4.16	4.30
5	4	30	60	5.13	3.10	2.83
6	10	30	60	0.74	0.62	1.76
7	4	44	60	5.99	3.12	2.03
8	10	44	60	0.91	1.16	1.23
9	7	37	50	6.55	6.47	5.97
10	7	37	50	6.89	6.13	5.77
11	7	37	50	6.83	7.22	7.43
12	7	37	50	7.30	6.43	7.07
13	7	37	50	7.65	6.65	6.65

[a] Values reached after a reaction time of 4 h

Table 3 Encapsulated CRL: effect of factors on the hydrolysis of olive, canola, and soybean oils.

Runs	pH	Temperature (°C)	Loading Biocatalyst (mg mL^{-1})	Olive oil[a] POH (%)	Canola oil[a] POH (%)	Soybean oil[a] POH (%)
1	4	30	6.0	6.89	6.08	6.86
2	10	30	6.0	1.84	2.09	1.32
3	4	44	6.0	7.48	6.77	6.38
4	10	44	6.0	1.75	1.24	1.43
5	4	30	9.0	7.86	5.76	6.88
6	10	30	9.0	2.20	2.13	1.35
7	4	44	9.0	7.17	6.92	7.40
8	10	44	9.0	1.95	1.70	1.53
9	7	37	7.5	2.68	2.43	2.54
10	7	37	7.5	2.63	2.45	3.14
11	7	37	7.5	3.34	2.39	2.82
12	7	37	7.5	2.86	2.31	2.91
13	7	37	7.5	3.09	2.32	2.76

[a] Values reached after a reaction time of 4 h

$\overline{M}$ mean molar mass of fatty acids in the oil which was calculated from the composition data presented in Table 1, according to Eq. 2 (g mol^{-1})
N_a volume of sodium hydroxide solution required during titration of the fatty acids (mL)
W_t weight of the sample (g)

Note that Eq. 4 is similar to that found in Rooney and Weatherle [15], but it has the factor f_1 that was missing in the earlier work. Given the composition of an oil molecule and its hydrolysis reaction that requires three molecules of water, the factor f_1 can be calculated as:

$$f_1 = \frac{\text{mass of free fatty acids at total hydrolysis}}{\text{mass of oil}} = \frac{3\,\overline{M}}{\overline{\text{MO}}} \cong \frac{1}{1 + \frac{12.68}{\overline{M}}} \tag{5}$$

where $\overline{\text{MO}}$ is given by Eq. 3 and $\overline{M}$ is calculated with Eq. 2.

Experimental Design with Response Surface Methodology

The results of the experimental design were analyzed using the software STATISTICA®, USA as a function of pH, temperature, and lipase loading. The coefficients were generated by regression analysis. The quality of the fit by the models was evaluated by examining the coefficients (R^2) and p (analysis of variance).

Results and Discussion

Free Lipase

A central composite experimental design leading to a set of 13 experiments with different variable value combinations for finding the maximal region of the percentage of hydrolysis, as a function of pH, temperature, and concentration of reactants (oil-to-water ratio), is shown at Table 2 for free lipase. The percentage of hydrolysis (POH%) ranged from 0.62 to

11.69%, according to the experimental condition. When lower pH (4) and lower substrate concentration (30%) for olive oil were used, the POH was higher, whereas in the case of canola and soybean oils, better results were observed using 50% oil–water ratio at 37 °C, pH 7 (Table 2).

Tables 4 and 5 show the variables' effects and interactions for free lipase as obtained from the factorial design analysis for canola and soybean oil, respectively. It can be seen that the oil/water ratio is the most important variable, with a negative effect, which means that an increase in the oil/water ratio leads to a decrease in the percentage of hydrolysis (POH%).

On the contrary, Table 6 shows the effects of the variables and their interactions on the percentage of hydrolysis for olive oil, and it can be seen that for olive oil, the pH and concentration of reactants (oil–water ratio) were statistically significant at 95% of confidence. The interactions between the variables were not statistically significant in the range studied for all the three types of oils studied. For free lipase, at 70% oil–water ratio (not shown at Table 2), all oil suspensions have shown a percentage of hydrolysis (POH%) lower than that observed at 50% oil–water ratio, suggesting substrate mass transfer limitations at the highest oil concentrations.

Analysis of variance (Tables 4, 5, and 6) shows that the statistical significance for the responses of the percentage of hydrolysis is appropriate because a high determination coefficient (R^2) of 0.98542, 0.90664, and 0.95227 was obtained for olive, canola, and soybean, respectively. In this part of the study, RSM was used as an approach for determining the region where the percentage of hydrolysis is maximized for the oils tested

Table 4 Effect and ANOVA for the free and encapsulated CRL on the hydrolysis of canola oil obtained by the experimental design employing three levels.

	Effect	Standard error	*p*	SS	*Df*	MS
Free CRL						
Mean/Interc.	3.18	±0.16	0.000007			
Curvature	*6.80*	*±0.53*	*0.000051*	*35.53*	*1*	*35.53*
(1) pH	−0.62	±0.33	0.115881	0.79	1	0.79
(2) Temperature	−0.22	±0.33	0.532231	0.10	1	0.10
(3) Concentration	*−2.37*	*±0.33*	*0.000805*	*11.20*	*1*	*11.20*
1 by 2	−0.20	±0.33	0.570550	0.08	1	0.08
1 by 3	*−1.59*	*±0.33*	*0.004725*	*5.06*	*1*	*5.06*
2 by 3	0.50	±0.33	0.185929	0.51	1	0.51
Error				1.08	5	0.18
Total SS				54.34	12	
Encapsulated CRL						
Mean/Interc.	*4.29**	*±0.02*	*0.000000**			
Curvature	*−3.57**	*±0.07*	*0.000000**	*9.87**	*1*	*9.87**
(1) pH	*−4.82**	*±0.03*	*0.000000**	*46.47**	*1*	*46.47**
(2) Temperature	*0.15**	*±0.03*	*0.016772**	*0.044**	*1*	*0.044**
(3) Loading	0.087	±0.03	0.096542	0.015	1	0.015
1 by 2	*−0.81**	*±0.03*	*0.000007**	*1.349**	*1*	*1.349**
1 by 3	*0.18**	*±0.03*	*0.008934**	*0.062**	*1*	*0.062**
2 by 3	*0.23**	*±0.03*	*0.002696**	*0.109**	*1*	*0.109**
Error				0.018	5	0.003
Total SS				57.939	12	

For free CRL, R^2 =0.95227; for encapsulated CRL, R^2 =0.99926

Table 5 Effect and ANOVA for the free and encapsulated CRL on the hydrolysis of soybean oil obtained by the experimental design employing three levels.

	Effect	Standard error	*p*	SS	*df*	MS
Free CRL						
Mean/Interc.	3.28	±0.22	0.000027			
Curvature	*6.63**	*±0.72*	*0.000254**	*33.78**	*1*	*33.78**
(1) pH	−0.77	±0.45	0.146278	1.18	1	1.18
(2) Temperature	−0.29	±0.45	0.550681	0.16	1	0.16
(3) Concentration	*−2.63**	*±0.45*	*0.002014**	*13.81**	*1*	*13.81**
1 by 2	0.02	±0.45	0.959938	0.001	1	0.001
1 by 3	−0.17	±0.45	0.718635	0.06	1	0.06
2 by 3	−0.38	±0.45	0.433811	0.29	1	0.29
Error				1.99	5	0.39
Total SS				51.27	12	
Encapsulated CRL						
Mean/Interc.	*4.34**	*±0.09*	*0.000000**	*5.80**	*1*	*5.80**
Curvature	*−2.75**	*±0.29*	*0.000232**	*65.78**	*1*	*65.78**
(1) pH	*−5.72**	*±0.18*	*0.000001**	*0.02**	*1*	*0.02**
(2) Temperature	0.09	±0.18	0.653743	0.19	1	0.19
(3) Loading	0.31	±0.18	0.151864	0.01	1	0.01
1 by 2	0.06	±0.18	0.732805	0.11	1	0.11
1 by 3	−0.24	±0.18	0.245813	0.16	1	0.16
2 by 3	0.28	±0.18	0.182921	5.80	1	5.80
Error				0.33	5	0.33
Total SS				72.37	12	

For free CRL, R^2 =0.90664; for encapsulated CRL, R^2 =0.98905

(Fig. 1), confirming that in the ranges studied, temperature (30–44 °C) is at its optimum and pH (4 to 10) is optimum in the lower range of the 4 to 7 interval.

According to Fu et al. [16], a complete hydrolysis can be achieved by either lengthening the reaction time at low free enzyme concentration or increasing the enzyme concentration for a shorter reaction time, to achieve 90–98% hydrolysis with coconut oil and other oils, and lipase from *Aspergillus* sp. The former is preferable for industrial production when using an expensive enzyme.

Encapsulated Lipase

Table 3 shows that for the encapsulated lipase, the region where the percentage of hydrolysis is maximized as a function of pH, temperature, and lipase loading. POH data for canola oil showed significant effects in the statistical analyses for pH and temperature and also for the interactions between pH and temperature and temperature and biocatalyst loading (Table 4). For the olive and soybean oils, it was verified that with encapsulated lipase, the pH was the only variable statistically significant (Tables 5 and 6).

The analysis of variance (Tables 4, 5, and 6) showed that the statistical significance of the responses for the percentage of hydrolysis is appropriate because a high determination coefficient R^2=0.98229, 0.99926, and 0.98905 was obtained for olive, canola, and soybean oil, respectively. Statistical analyses showed significant effects for pH and demonstrate high statistical significance (p<0.05) at 95% confidence level. Tables 3, 4, 5, and 6 and Fig. 2 indicate a region of higher POH where the temperature is around 37 °C, and there is maximum loading of the entrapped lipase for the tested oils. These results were

Table 6 Effect and ANOVA for the free and encapsulated CRL on the hydrolysis of olive oil obtained by the experimental design employing three levels.

	Effect	Standard error	*P*	SS	*df*	MS
Free CRL						
Mean/Interc.	*5.59**	*±0.14*	*0.000000**			
Curvature	*2.91**	*±0.44*	*0.001195**	*6.53**	*1*	*6.53**
(1) pH	*−5.79**	*±0.26*	*0.000004**	*67.18**	*1*	*67.18**
(2) Temperature	0.36	±0.26	0.249549	0.25	1	0.25
(3) Concentration	*−4.78**	*±0.26*	*0.000011**	*45.80**	*1*	*45.80**
1 by 2	−0.36	±0.26	0.249549	0.25	1	0.25
1 by 3	*1.06**	*±0.26*	*0.011774**	*2.24**	*1*	*2.24**
2 by 3	0.15	±0.26	0.578532	0.05	1	0.05
Error				0.74	5	0.15
Total SS				123.04	12	
Encapsulated CRL						
Mean/Interc.	*4.86**	*±0.12*	*0.000000**			
Curvature	*−3.61**	*±0.38*	*0.000220**	*10.00966*	*1*	*10.00966*
(1) pH	*−5.67**	*±0.24*	*0.000002**	*64.30002*	*1*	*64.30002*
(2) Temperature	−0.12	±0.24	0.645975	0.02653	1	0.02653
(3) Loading	0.32	±0.24	0.233711	0.20399	1	0.20399
1 by 2	−0.05	±0.24	0.800594	0.00789	1	0.00789
1 by 3	−0.03	±0.24	0.915946	0.00137	1	0.00137
2 by 3	−0.38	±0.24	0.170894	0.28420	1	0.28420
Error				0.55633	5	0.11127
Total SS				75.39000	12	

For free CRL, R^2 =0.98542; for encapsulated CRL, R^2 =0.98229

experimentally confirmed, and the POH was determined for different vegetable oils using an experimental design. Similar values have been found for fatty acids as previously obtained by our group in other studies [6, 17]. This study demonstrated that the statistical analysis is an efficient tool to unfold the influences of pH, temperature, and lipase loading on the POH.

The results of the experimental design for the encapsulated lipase were also analyzed by the RSM using the software STATISTICA® to find the region where a high POH for canola, soybean, and olive oils can be obtained as a function of pH, temperature, and lipase loading. A high percentage of hydrolysis is important for the biotechnological production processes of biodiesel and fatty acids.

Analyzing the curvatures in Tables 4, 5, and 6, one concludes that the POH profile approximates to the optima region for the tested oils utilizing entrapped enzyme. RSM for entrapped lipase (Fig. 2) shows that the typical POH profiles is different from that of free lipase (Fig. 1), while the pH effect is very significant for the entrapped lipase (Fig. 2) with all tested oils; for the free enzyme, the POH is more affected by pH only for olive oil. Maximum hydrolysis was observed at lower pH for the entrapped lipase, whereas for the free enzyme, the maximum hydrolysis occurred at pH 7, for canola and soybean oils. POH was generally smaller for lower loadings of entrapped enzyme, as shown in Tables 3, 4, 5, and 6. This could be due to the limitation of substrate diffusion toward the biocatalyst surface and into the pores of the support because of its microporous structure.

Recently, the RSM has been used to determine the kinetic constants of enzymatic reactions as well as for the optimization of reactions. Boyaci [18] used RSM and the

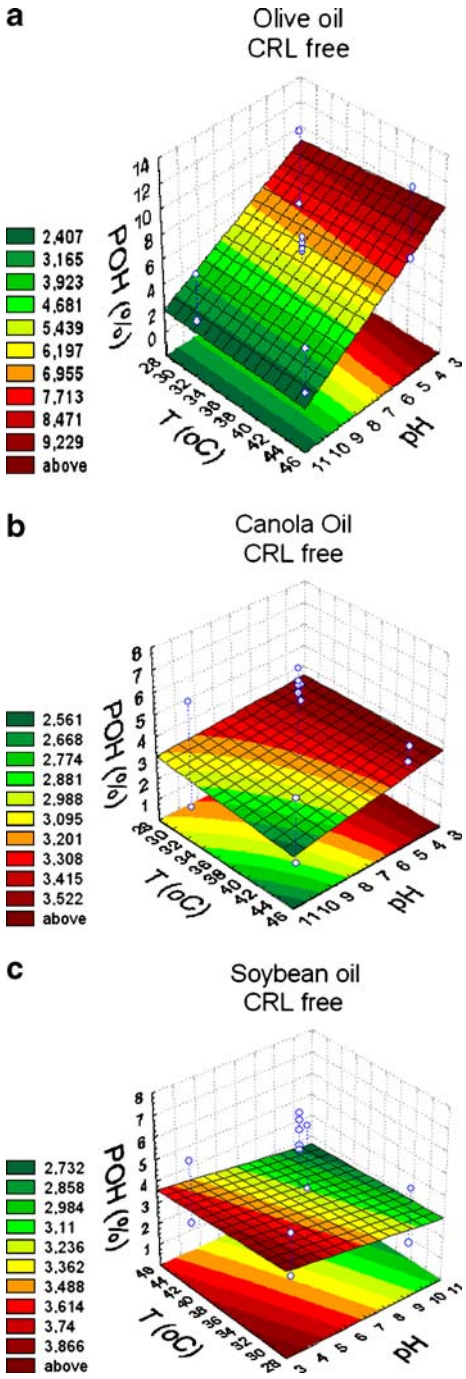

Fig. 1 Surface response plot of free CRL hydrolysis of olive (**a**), canola (**b**), and soybean oil (**c**) showing the POH dependence on pH, temperature, and substrate concentration

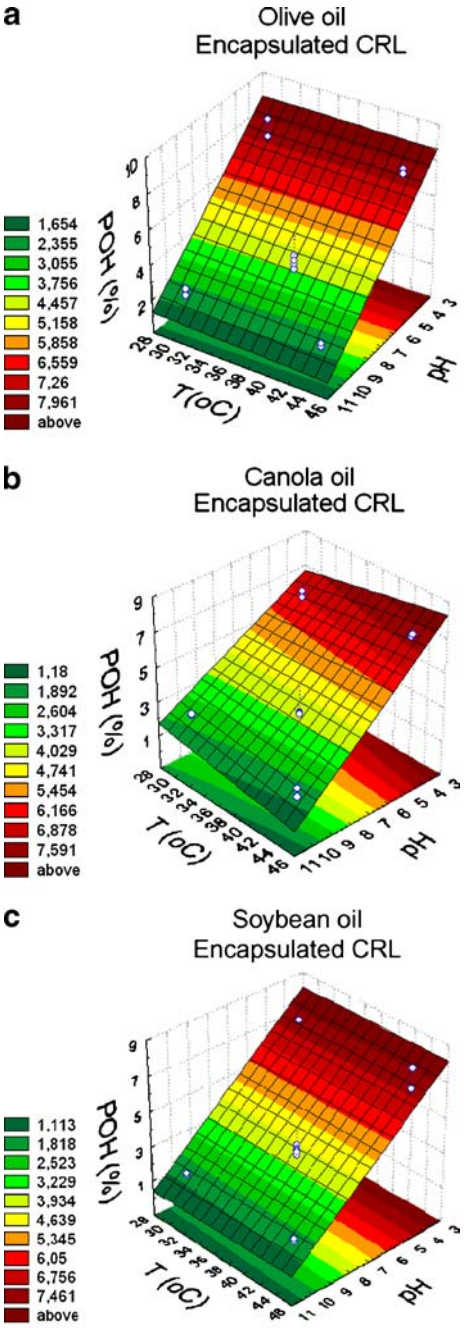

Fig. 2 Surface response plot of encapsulated CRL hydrolysis of olive (**a**), canola (**b**), and soybean oil (**b**) showing the POH dependence on pH, temperature, and lipase loading

conventional method to determine the kinetic constants of glucose oxidase as a function of reaction temperature and pH. The accuracy of the RSM for determining K_m and V_{max} was tested by comparing the results of these two methods. According to the author, reasonable results in the range of tested parameters were reached. A quite good correlation between the

kinetic constants of horse liver alcohol dehydrogenase obtained from conventional methods and those obtained from RSM was also reported by Andersson and Adlercreutz [19].

Conclusions

For free lipase, the POH varied from 0.62 to 11.69%, for temperatures from 30 to 44 °C, pH from 4 to 10, and oil/water ratio from 40 to 70%. The results show that the initial concentration of reactants (oil-to-water ratio) is the most important variable, with a negative effect, which means that an increase in the oil/water ratio leads to a decrease in the POH. In the case of canola and soybean oils, better results were observed using 50% oil–water ratio at 37 °C, pH 7, whereas for olive oil, the pH and concentration of reactants (oil–water ratio) were statistically significant at 95% of confidence, giving higher POH when lower pH (4) and lower substrate concentration (30%) were used for olive oil. The interactions between the variables were not statistically significant in the range studied for all the three types of oils studied. For free lipase, at 70% oil–water ratio, all oil suspensions have shown a POH lower than that observed at 50% oil–water ratio, suggesting substrate mass transfer limitations at the highest oil concentrations. Analysis of variance showed that the statistical significance for the POH responses were appropriate because a high determination coefficient (R^2) was obtained for the three oils. The RSM was used for determining the region where the percentage of hydrolysis is maximized for the oils tested and showed that in the range of the variables studied, temperature is approximately at its optimum (30–44 °C) and the lower pHs between 4 and 7 are optimum for the oils tested.

For the encapsulated lipase, the analysis of variance of the POH data showed that the statistical significance obtained is appropriate too because a high determination coefficient (R^2) resulted for the three oils. Statistical analyses showed significant effects for pH only and demonstrate high statistical significance at 95% confidence level. The results of the experimental design for the encapsulated lipase were also analyzed by the RSM to find the region where a high POH for the tested oils can be obtained as a function of pH, temperature, and lipase loading, giving temperatures approximately 37 °C and maximum entrapped lipase loading (9 mg mL^{-1}). The POH profiles obtained by the RSM show the optima region for the tested oils hydrolyzed by the entrapped lipase and the typical profiles for encapsulated lipase differs from that of free lipase, while the pH effect is very significant for the entrapped lipase with all tested oils; for the free enzyme, the POH is more affected by pH only for olive oil. Maximum hydrolysis was observed at lower pH for the entrapped lipase, whereas for the free enzyme, the maximum hydrolysis occurred at pH 7, for canola and soybean oils. Decreasing immobilized enzyme loading resulted in a reduction in the POH, and this effect was attributed to microporous diffusion limitations. A high percentage of hydrolysis is important for the processes of production of biodiesel and fatty acids.

This study demonstrated that the RSM is appropriate for the maximization of the hydrolysis of olive, canola, and soybean oils by free and sol–gel-entrapped lipase as a function of pH, temperature, and enzyme loading. Examining the curvature of the RSM graphics allowed concluding that the profiles studied approximate to the optima for the experiments carried out using different vegetable oils. This methodology also makes it possible to locate a desirable working region where a better performance for the hydrolysis reaction can be achieved.

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