DOI:10.1021/jf901816x

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Optimization of Extraction of Lipase from Wheat Seeds (*Triticum aestivum*) by Response Surface Methodology

Morgana K. Pierozan,[†] Roger J. da Costa,[§] Octávio A. C. Antunes,[†] Enrique G. Oestreicher,[†] J. Vladimir Oliveira,[§] Rogério L. Cansian,[§] Helen Treichel,*[,§] and Débora de Oliveira[§]

[†]Centro de Tecnologia, Universidade Federal do Rio de Janeiro (UFRJ), Av. Athos da Silveira Ramos 149, Bloco A, 21941-909 Rio de Janeiro, RJ, Brazil, and [§]Campus de Erechim, Universidade Regional Integrada (URI), Av. Sete de Setembro 1621, 99700-000 Erechim, RS, Brazil

This work aimed to evaluate the effects of particle size, solid/solvent ratio (w/v), and reaction time on hydrolytic and esterification activities of a lipase extract from wheat seeds. The higher hydrolytic activity was 5.9 U/g with a particle size of 425 μm, solid/solvent ratio of 30:60 (w/v), and reaction time of 15 h, with maximum hydrolytic activity of 14.47 U/g after 24 h of precipitation. For esterification activity the best result was 57.88 U/g with a particle size of 425 μm and solid/solvent ratio of 30:120 (w/v) for 5 h, with a maximum value after 10 h of precipitation reaching 208.20 U/g. The partial characterization showed that the optimal pH and temperature were found to be 5.5 and 32−37 °C, respectively. The extract stability at low temperatures was kept after 48 h of storage in terms of esterification activity. The hydrolytic activity was kept constant at −10 °C during 72 h and diminished considerably after 24 h at 4 °C.

KEYWORDS: *Triticum aestivum*; lipases; hydrolytic activity; esterification activity; experimental design; response surface methodology

INTRODUCTION

Lipases (glycerol ester hydrolases, EC 3.1.1.3) are enzymes belonging to the group of hydrolases, the main biological function of which is to work as catalysts in the hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono- and diacylglycerols, and glycerol (*1*−*3*).

Besides their natural function, lipases can catalyze esterification, interesterification, and transesterification reactions in nonaqueous media (*4*, *5*). These biocatalysts have interesting characteristics, such as action under mild conditions, stability in organic solvents, high substrate specificity, and regio- and enantioselectivity (*2*, *3*, *6*, *7*), with optimal activities at 30−40 °C and at pH from 6 to 8 (*5*, *8*, *9*).

These enzymes can be obtained from animals (pancreatic, hepatic, and gastric), microbes (fungi and bacteria), and plants (almond seeds, leaves, and stems), with differences in the catalytic properties as a function of the source. There has been great interest in studies concerning the extraction of plant lipases recently, reducing the production costs and, thus, making intense their use in industrial applications (*10*).

Another important aspect concerns enzyme application to food (natural, kosher, and/or halal restrictions, etc.) or pharmaceutical industries. In this case, regulatory restrictions related to the use of microbial and animal enzymes can be found.

Plant lipases have been isolated from leaves, stems, latex, oils, and seeds of oleaginous plants and, on a lower scale, from cereals seeds, such as rice and wheat (*Triticum* sp.). The wheat seeds belong to the family Poaceae, comprising about 24 species, mainly *Triticum aestivum* and *Triticum durum*. This seed is considered to be the most important cereal in the human diet, presenting an annual cycle of 90−180 days, depending on the genotype, cultivated during winter and spring, because some varieties are dependent on cold temperature and light intensity (*2*).

However, despite the huge importance of such cereals in human life, surprisingly, no work was found in the available literature regarding the use of wheat seeds as lipase sources. Due to the lack of literature, the objective of this work was to establish a protocol for wheat lipase extraction as well as to optimize the hydrolytic and esterification activities of the crude enzyme extracts by response surface methodology, following the extract concentration and the partial characterization of the concentrated enzyme extract.

MATERIALS AND METHODS

Materials. The wheat seeds (*T. aestivum*) Embrapa-Guamirim were kindly donated by Empresa de Sementes Estrela de Erechim-RS (Brazil) and stored at 4 °C until use. All chemicals were purchased from Sigma-Aldrich.

Lipase Extraction. The wheat seeds were triturated in a knife-mill (Marconi MA-340) and sieved following different particle sizes. The samples (30 g) were mixed with chilled acetone at 150 rpm and 4 °C. The contact time and the particle size were defined in the experimental design. Then the samples were filtered under vacuum via a Büchner funnel and washed with acetone. The degreased samples were kept in open flasks

*Corresponding author (telephone +55 54 35209000; fax +55 54 35209090; e-mail helen@uricer.edu.br).

 Published on Web 10/05/2009

Table 1. Ranges of the Factors Investigated in the Experimental Designs

	hydrolytic activity (U/g)				
	levels used in the first experimental design				
	−1.41	−1	0	+1	+1.41
particle size (μm)	−	425	500	1000	−
solid:solvent ratio (w/v)	−	30:60	30:90	30:120	−
contact time (h)	−	5	10	15	−
	levels used in the second experimental design				
particle size (μm)	355	355	425	500	500
contact time (h)	8	10	15	20	22
	esterification activity (U/g)				
	levels used in the first experimental design				
	−1.68	−1	0	+1	+1.68
particle size (μm)	250	425	500	1000	2000
solid:solvent ratio (w/v)	30:39.6	30:60	30:90	30:120	30:140.4
contact time (h)	1.6	5	10	15	18.4

for about 15 h for solvent evaporation. The product was defined as crude enzyme extract and used for determination of hydrolytic and esterification activities (*11*).

Besides wheat seeds, commercial wheat germ (MaisVitta) and germinated wheat were also tested as lipase sources. The tests using germinated wheat were carried out on the basis of information published in the literature (*4*, *12*, *13*) that stated that only the seeds of castor (*Ricinus communis* L.) and sunflower (*Vernonia galamensis*) do not need germination before enzyme extraction. To carry out germination, 100 g of seed was mixed in distilled water at room temperature (approximately 25 °C) and placed in plates with moist cotton. The mixture was placed in a growth chamber for 48 h at 25 °C with a photoperiod of 12 h. After this procedure, the need to remove the tegument was also evaluated. Afterward, the seeds (20 g) were ground in a pestle and mortar and then added to 100 mL of sodium phosphate buffer (*14*). This procedure was performed in an ice bath, and after 30 min of grinding, the sample was left to rest for 1 h and then filtered via a Büchner funnel and used for hydrolytic activity determination.

It is worth mentioning that the use of organic solvent is necessary to remove the oils present in the seed. The work by Kapranchikov et al. (*15*) demonstrated that lipid removal before enzyme extraction enhanced lipase activity by a factor of 1.8 using wheat germ as enzyme source.

Analytical Methods. *Lipase Hydrolytic Activity*. Lipase activity was assayed by reaction using olive oil as substrate followed by alkali titration. Olive oil (10 wt %/v) was emulsified with arabic gum (5 wt %/v) in 100 mM sodium phosphate buffer, pH 7.0, for approximately 2 min at 3000 rpm using a domestic blender. A 2 mL sample of crude enzyme was added to 18 mL of this emulsion. After incubation in the shaker for 15 min at 37 °C and 150 rpm, the reaction was interrupted by the addition of 20 mL of an acetone/ethanol solution (1:1 v/v). The amount of fatty acids liberated was then titrated with 0.05 M NaOH until pH 11. Reaction blanks were run in the same way, but adding the sample after the addition of acetone/ethanol solution. Lipase activity measurements were carried out in duplicate. One unit of hydrolytic lipase activity (HA) was defined as the amount of enzyme that yields 1 μmol of fatty acids per minute in the assay conditions (*16*).

Lipase Esterification Activity. The enzyme activity was determined as the initial rates in esterification reactions between lauric acid and *n*-propanol at a molar ratio of 3:1, a temperature of 60 °C, and an enzyme concentration of 5 wt % in relation to the substrates. At the beginning of the reaction, samples containing the mixture of lauric acid and *n*-propanol were collected, and the lauric acid content was determined by titration with 0.04 N NaOH. After the addition of the enzyme to the substrates, the mixture was kept at 60 °C for 15 min. Then, the lauric acid consumption was determined. One esterification lipase activity unit (EA) was defined as the amount of enzyme necessary to consume 1 μmol of lauric acid per minute at the established experimental conditions presented previously (*17*). All enzyme activity determinations were replicated at least three times.

Optimization of Lipases Activity by Sequential Experimental Design. On the basis of some preliminary results and some data disclosed in the literature (*10*, *12*, *15*, *18*, *19*), the effects of particle size (mesh), solid/solvent ratio (w/v), and contact time (h) were evaluated using the following experimental designs.

Table 2. Matrix of the 2^3 Full Experimental Design (Coded and Real Values) with the Responses in Terms of Hydrolytic Lipase Activity Using Wheat Germ and Wheat Seed as Lipase Source

				hydrolytic activity (U/g)	
run	particle size (μm)	solid/solvent ratio (w/v)	contact time (h)	wheat germ	wheat seed
1	+1 (1000)	−1 (30:60)	−1 (5)	0.79	0.72
2	−1 (425)	−1 (30:60)	−1 (5)	1.35	1.12
3	+1 (1000)	+1 (30:120)	−1 (5)	0.31	0.85
4	−1 (425)	+1 (30:120)	−1 (5)	1.20	0.38
5	+1 (1000)	−1 (30:60)	+1 (15)	2.45	2.53
6	−1 (425)	−1 (30:60)	+1 (15)	0.97	0.75
7	+1 (1000)	+1 (30:120)	+1 (15)	1.21	1.23
8	−1 (425)	+1 (30:120)	+1 (15)	0.66	1.98
9	0 (500)	0 (30:90)	0 (10)	0.68	1.24
10	0 (500)	0 (30:90)	0 (10)	0.46	1.47
11	0 (500)	0 (30:90)	0 (10)	0.91	1.11

Experimental Design for Lipase Extraction Optimization in Terms of Hydrolytic Activity. A first 2^3 full experimental design was applied in this step. The variables and the related levels are presented in **Table 1**. Statistical analysis of this experimental design permitted us to propose a second central composite rotatable design with two factors, particle size and extraction time. The solid/solvent ratio was fixed at 30:60.

Experimental Design for Lipase Extraction Maximization in Terms of Esterification Activity. **Table 1** presents the variables and levels studied in the 2^3 full experimental design with axial points applied to maximization of lipase extraction in terms of esterification activity.

The results obtained in terms of hydrolytic and esterification activities were statistically treated by the software Statistica 6.0 (Statsoft Inc., Tulsa, OK).

Concentration of Crude Enzyme Extract by Precipitation with Ammonium Sulfate. The crude enzyme extract (approximately 120 g) was concentrated by adding 500 mL of 0.1 M sodium phosphate buffer, pH 7.0. Ammonium sulfate was added until the desired saturation (60%). This step was carried out in a magnetic stirred ice bath (4 °C) under pH control (7.0, adjusted by the addition of 20% NaOH).

The final solution was filtered and placed in tubes, kept for the preestablished time at −10 °C in a freezer to precipitate the sample. To determine the best condition for concentration of the enzyme extract, the ammonium sulfate concentration of 60% was employed (*20*) and the precipitation time varied from 5 to 24 h.

After the precipitation time, the samples were centrifuged at 16500*g* for 30 min at 4 °C. The supernatant was discarded and the precipitate removed with a minimum amount of 100 mM sodium phosphate buffer, pH 7.0 (*21*). The samples were then lyophilized for 48 h until constant weight and stored at 4 °C for measurement of hydrolytic and esterification activities and protein content according to the methodology described by Bradford (*22*).

Partial Characterization of Concentrated Enzyme Extract. The partial characterization, mainly in terms of optimal temperature and pH and stability of enzyme activity during storage at low temperatures, is of fundamental importance for establishing the application conditions for the enzyme extract.

For determination of optimal values of temperature and pH for the concentrated enzyme extract, a 2^2 full experimental design with triplicate of the central point was carried out. The ranges of studied pH and temperature were 5.5−8.5 and 32−42 °C, respectively.

The concentrated enzyme extracts were stored (samples of 2 g) at 4 and −10 °C during 144 h, the hydrolytic and esterification activities being measured after 0, 6, 12, 24, 48, 72, 96, 120, and 144 h of storage.

RESULTS AND DISCUSSION

Preliminary Tests. *Choice of Organic Solvent*. The parameters solvent polarity and time necessary for complete solvent evaporation

Table 3. Matrix of the Second Experimental Design (Coded and Real Values) with the Responses in Terms of Hydrolytic Lipase Activity

run	particle size (μm)	contact time (h)	hydrolytic activity (U/g)
1	+1 (500)	−1 (10)	1.44
2	−1 (355)	−1 (10)	4.40
3	+1 (500)	+1 (20)	1.37
4	−1 (355)	+1 (20)	0.60
5	0 (425)	0 (15)	4.85
6	0 (425)	0 (15)	5.26
7	0 (425)	0 (15)	5.90
8	+1.41 (1000)	0 (15)	1.52
9	−1.41 (250)	0 (15)	0.63
10	0 (425)	−1.41 (8)	2.71
11	0 (425)	+1.41 (22)	0.63

were taken into account for choosing the organic solvent to be used for lipase extraction. From evaluated solvents (acetone, ethanol, butanol, and hexane), acetone was chosen as having higher polarity and fast evaporation, permitting the extraction process in a short time range. This solvent was also used by Liaquat and Owusu Apenten (*11*) and Sagiroglu and Arabaci (*23*) for lipase extraction of other plant sources.

Choice of Raw Material. **Table 2** presents the matrix of the 2^3 full experimental design (coded and real values) with the responses in terms of hydrolytic lipase activity using wheat germ and wheat seed as lipase source. The results obtained permitted us to observe that the hydrolytic activities for lipases obtained from wheat germ were, in a general way, lower than that obtained when using wheat seed as enzyme source. This can be related to the fact that the wheat germ is a commercialized toasted product and these high temperatures could inactivate the enzymes present in the sample.

The hydrolytic activity was also evaluated in the germinated grain with and without the tegument. Here the results pointed out activities of 1.07 ± 0.08 and 0.66 ± 0.06 U/g, respectively, which can be considered low compared to that obtained using wheat seeds. From this we can infer that the wheat seed does not need the germination step for enzyme activation (*4*).

On the basis of the results presented above, the following steps of the present work were carried out using the wheat seed as lipase source.

Optimization of Lipase Hydrolytic Activity by Sequential Experimental Design. Table 2 presents the matrix of the 2^3 full experimental design with the responses in terms of hydrolytic lipase activity using wheat seed as lipase source. From these results, we can observe that experiment 5 led to higher hydrolytic activities, followed by experiment 8. One can also verify the good reproducibility obtained in the experiments, demonstrated by the triplicate of the central point. The data presented in **Table 2** were statistically treated, and at a significance of 90% the contact time and the interaction between the particle size and the solid/solvent ratio presented a significant effect on lipase activity. The solid/solvent ratio did not present a significant effect, this variable being kept constant at the lower value (30:60), reducing the solvent costs. From studies presented in the literature (*12*) and the previous results obtained here, the effect of particle size was also evaluated in the second experimental design. As the contact time presented a positive significant effect on lipase extraction and the literature points out an extraction time of 16 h for extraction of lipases from castor seed (*Ricinus communis* L.) (*12*), we have decided also to verify the effect of this variable in the next experimental design.

Table 3 presents the responses obtained in the second experimental design, in terms of hydrolytic activity, as a function of contact time and particle size, where one can observe that higher lipase activities were obtained in the central point.

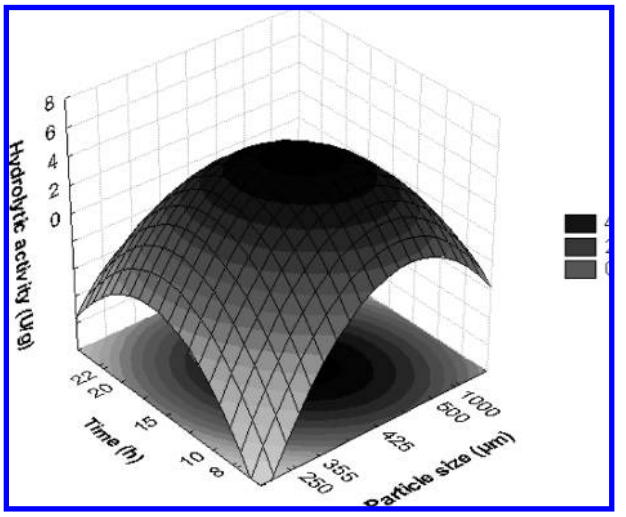

Figure 1. Response surface for extraction of lipases from wheat seeds as a function of hydrolytic activity. Experimental data and conditions are shown in **Table 3**.

The analysis of variance (ANOVA) was used for determining the significant parameters that influence hydrolytic lipase activity. ANOVA consists of classifying and cross-classifying statistical results and testing whether the means of a specified classification differ significantly. This was carried out by Fisher's statistical test for the analysis of variance. The F value is the ratio of the mean squares due to regression and the mean squares due to the error. This test indicates the significance of each controlled factor on the tested model. The model F value of 19.66, higher than the listed F (4.53), implied that the model was significant, making it possible to validate an empirical coded model (eq 1) and build the response surface and contour curve presented in **Figure 1**.

$$\mathrm{HA} = 5.34 - 1.99 \times \mathrm{PS}^2 - 0.85 \times \mathrm{T} - 1.69 \times \mathrm{T}^2 - 0.93 \times PS \times \mathrm{T} \quad (1)$$

where HA is hydrolytic activity, PS is particle size, and T is contact time.

The hydrolytic activities in the optimized experimental conditions (5.9 U/g) are higher than that obtained (0.195 U/g) by Kapranchikov et al. (*15*) using olive oil and polyvinyl alcohol to prepare the emulsion for hydrolytic activity determination.

Comparing the results obtained here with some others presented in the literature using seeds with high fatty acids content as lipase sources, we can consider the hydrolytic activities from wheat seeds low. Sagiroglu and Arabaci (*23*), for example, obtained hydrolytic activities of 920 U/g using sunflower seeds (*Helianthus annuus* L.) as lipase source. Activities of 28 U/g were achieved in the work of Cavalcanti et al. (*12*) using castor seed (*R. communis* L.). On the other hand, Prabhu et al. (*24*), extracting lipases from rice (*Oryza sativa*), which has low lipid concentration, obtained hydrolytic activities of 0.85 U/g.

Experimental Design for Maximization of Lipase Esterification Activity. Table 4 shows the matrix of the experimental design (coded and real values) with the responses in terms of esterification lipase activity. Analyzing the data presented in this table, we can observe that higher activity (57.88 U/g) was obtained at a particle size of 425 μm (mesh 35), solid/solvent ratio of 30:120, and contact time of 5 h. This result was already expected because higher solvent content leads to high contact surface between the seeds and the solvents, facilitating the enzyme extraction. As the lipase is intracellular, from a general sense, the lower the

Table 4. Matrix of the Experimental Design (Coded and Real Values) with Responses in Terms of Esterification Lipase Activity

run	particle size (μm)	solid:solvent ratio (w/v)	contact time (h)	esterification activity (U/g)
1	+1 (1000)	−1 (30:60)	−1 (5)	7.06
2	−1 (425)	−1 (30:60)	−1 (5)	31.43
3	+1 (1000)	+1 (30:120)	−1 (5)	19.53
4	−1 (425)	+1 (30:120)	−1 (5)	57.88
5	+1 (1000)	−1 (30:60)	+1 (15)	29.16
6	−1 (425)	−1 (30:60)	+1 (15)	55.32
7	+1 (1000)	+1 (30:120)	+1 (15)	23.41
8	−1 (425)	+1 (30:120)	+1 (15)	35.93
9	0 (500)	0 (30:90)	0 (10)	22.58
10	0 (500)	0 (30:90)	0 (10)	21.20
11	0 (500)	0 (30:90)	0 (10)	21.89
12	0 (500)	0 (30:90)	−1.68 (1.6)	31.39
13	0 (500)	0 (30:90)	+1.68 (18.4)	14.72
14	0 (500)	−1.68 (30:39.6)	0 (10)	10.98
15	0 (500)	+1.68 (30:140.4)	0 (10)	19.41
16	+1.68 (2000)	0 (30:90)	0 (10)	7.60
17	−1.68 (250)	0 (30:90)	0 (10)	44.03

particle size, the higher the lipase activity. This statement can be considered true until a limit when a predominance of the starch present in the wheat seed is observed. Results from experiment 13 permit us to prove this statement.

Another important observation, comparing the hydrolytic and esterification activities obtained from wheat seeds, is that the last ones were considerably higher. These values can be considered to be relevant because no promising results were found in the literature concerning the esterification activity of lipases from plant sources. Villeneuve et al. (*25*) evaluated the feasibility of lipase-catalyzed esterification of canola phytosterols with oleic acid. The influence of various reaction parameters was evaluated. Among tested lipases, namely, plant lipases from *Carica papaya* (commercial product from Sigma and Recco Ltd.) and *R. communis* (castor bean lipase, a noncommercial enzyme preparation obtained by extraction with acetone) and microbial lipases (*Rhizomucor miehei*, *Candida antarctica* B, or *Candida rugosa*), the best results were obtained with the last ones. In a general way, lower esterification degrees were obtained compared to microbial lipases. The esterification degree obtained by plant lipases was compared to the absence of biocatalyst.

The results obtained in **Table 4** were statistically treated at a significance level of 95%, where one can verify that the particle size, the solid/solvent ratio, the contact time, and the interaction between particle size and contact time presented a significant effect on esterification activity.

The ANOVA analysis permitted us to validate an empirical coded model (eq 2) for esterification activity, because the calculated F value (6.33) was higher than the listed one (3.21) with a correlation coefficient of 0.88 ($p < 0.05$), and to build the response surface presented in **Figure 2**.

$$EA = 21.15 + 11.92 \times PS + 3.88 \times PS^2 + 2.05 \times SSR + 2.90 \times T^2 - 3.01 \times PS \times T - 8.01 \times SSR \times T \quad (2)$$

where EA is esterification activity, PS is particle size, SSR is solid/solvent ratio, and T is contact time.

From **Figure 2a** one can verify that with lower the particle sizes higher esterification activities were achieved, independent of solid/solvent ratio. **Figure 2b** permits us to observe that when lower particle sizes and contact time were applied, higher esterification activities were achieved, demonstrating the significance

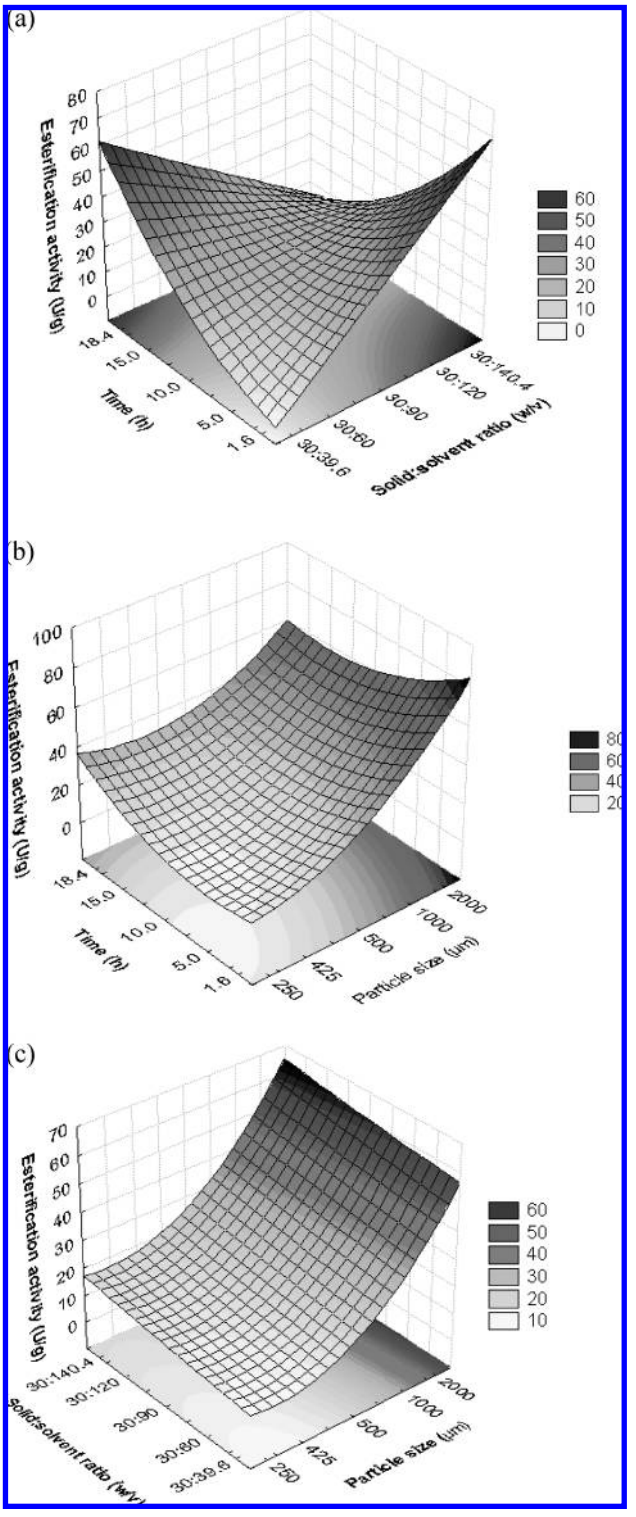

Figure 2. Response surface for (**a**) the effect of time and solid/solvent ratio (w/v), (**b**) time (h) and particle size (μm), and (**c**) solid/solvent ratio (w/v) and particle size on lipase esterification activity. Experimental data and conditions are cshown in **Table 4**.

of these parameters. Higher esterification lipase activities were obtained using higher extraction times and lower solid/solvent ratio, as can be seen in **Figure 2c**. This response is coherent because when a high solvent amount is used, high extraction times are generally not necessary. On the other hand, the use of a low amount of solvent makes necessary a higher contact time for effective enzyme extraction.

Table 5. Hydrolytic and Esterification Activities of the Enzyme Extracts before and after the Concentration with Ammonium Sulphate and the Respective Specific Activities (U/mg of protein)

	variable	hydrolytic activity[a] (U/g)			esterification activity[a] (U/g)		
Run	Time (h)	before precipitation	after precipitation	specific activity (U/mg of protein)	before precipitation	after precipitation	specific activity (U/mg of protein)
1	5	3.00	5.80	0.18	38.80	197.81	6.85
2	10	4.80	1.73	0.05	33.56	208.20	7.79
3	15	3.38	9.58	0.35	34.17	94.67	3.29
4	24	3.52	14.47	0.64	43.67	26.86	1.19

[a] Media of triplicate runs. Standard deviations lower than 5%.

Concentration of Crude Enzyme Extract by Precipitation Using Ammonium Sulfate. The precipitation using ammonium sulfate was carried out to obtain a concentrated lipase, enhancing the activity of the crude extract. The enzyme extraction was performed at the maximized experimental conditions obtained previously. A saturation of 60% was used in all experiments, on the basis of results presented in the literature (*20*). In this step, different precipitation times were evaluated, as presented in **Table 5**.

From this table, we can observe that, in general, the enzyme activity had an enhancement after precipitation.

From an analysis of the hydrolysis activity, the best result was obtained in experiment 4, when the lipase activity was enhanced from 3.52 to 14.47 U/g. Comparing this result with that presented by Kapranchikov et al. (*15*), we have obtained a higher hydrolytic activity, because the referenced authors achieved a hydrolytic activity from the concentrated extract of 5 U/g.

With regard to esterification activity, the best results were observed at lower precipitation times, the higher one being of 208.20 U/g after 10 h of precipitation. It is worth mentioning that, besides these results having not been already presented in the literature, high enzyme activities were achieved, making possible future commercial use of this extract.

Table 5 also presents the specific activities of the concentrated enzyme extracts. The protein content was determined by using the Bradford method (*22*). Related to the hydrolysis activity, higher lipase activity was achieved after 24 h of precipitation with ammonium sulfate (0.64 U/mg of protein). Kapranchikov et al. (*15*) obtained a specific activity of 0.014 U/mg of protein for lipases from wheat seed germ. The higher esterification specific activities were obtained in experiments 2 (7.79 U/mg of protein) and 1 (6.85 U/mg of protein).

Partial Characterization of Concentrated Enzyme Extract. From the best conditions for concentration of the crude enzyme extract, an experimental design was carried out to evaluate the effect of temperature and pH on hydrolytic activities of the extract. The hydrolytic activities varied from 3 to 10 U/g.

The statistical analysis of the data obtained permitted us to validate ($p < 0.05$) an empirical coded model ($R = 0.97$), presented in eq 3, for hydrolytic activity (HA) in terms of optimal temperature and pH.

$$\mathrm{HA} = 7.35 - 2.45 \times \mathrm{pH} + 0.89 \times \mathrm{pH} \times \mathrm{temperature} \quad (3)$$

Model validation by variance analysis permitted us to build the response surface, shown in **Figure 3**, of hydrolytic activity in terms of the interaction between the pH and temperature. From this figure we can observe that higher hydrolytic activities were obtained at lower pH values for temperatures from 32 to 37 °C.

The literature points out optimal temperatures in the range from 45 to 55 °C and optimal pH from 7.5 to 8.0 for lipases

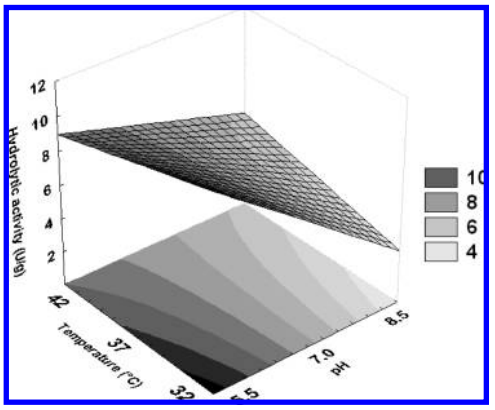

Figure 3. Response surface for the interaction between pH and temperature for hydrolytic activity of the concentrated enzyme extract.

extracted from plant sources (*23*, *26*, *27*). Cavalcanti et al. (*12*), for example, obtained higher hydrolytic activities using castor seeds in acid pH (approximately 4.0) and temperature of 30 °C. Analysis of the data presented in the literature permits us to observe that most microbial and plant lipases present optimal pH ranging from 7.0 to 9.0. This behavior was not observed for lipases from wheat seeds, making it possible to propose new applications for the enzyme obtained in this work, mainly when acid conditions are required.

The stability of concentrated extract to low temperatures was evaluated by submitting the samples to cooling (4 °C) and freezing (−10 °C) temperatures. Hydrolytic activities kept their values until 24 and 72 h of storage, at 4 and −10 °C, respectively. After this period of time, the enzyme lost its activity. The esterification activity showed a similar behavior, keeping constant its value during 48 h of storage in both tested temperatures.

The development of the present work permitted us to conclude that acetone was efficient for lipase extraction and, among all raw materials evaluated, wheat seed showed to be a more promising lipase source than wheat germ and germinated seed. Using different experimental designs it was possible to measure important enzyme parameters.

ACKNOWLEDGMENT

We dedicate this work in memory of Professor Octavio Augusto Ceva Antunes (his wife and little boy), whose lives were far prematurely taken from our existence on the 447 Air France flight tragedy. Professor Antunes will remain as a reference scientist, an unforgettable friend, and an example of courage, always enlightening us with his experience and creativeness.

LITERATURE CITED

(1) Paques, F. W.; Macedo, G. A. Lipases de látex vegetais: propriedades e aplicações industriais. *Quim. Nova* **2006**, *29*, 93–99.
(2) Hasan, F.; Shah, A. A.; Hameed, A. Industrial applications of microbial lipases. *Enzyme Microb. Technol.* **2006**, *39*, 235–251.
(3) Treichel, H.; Oliveira, D.; Mazutti, M. A.; Di Luccio, M.; Oliveira, J. V. A review on microbial lipases production. *Food Bioprocess Technol.* **2009**, DOI: 10.1007/s11947-009-0202-2.
(4) Villeneuve, P. Plant lipases and their applications in oils and fats modification. *Eur. J. Lipid Sci. Technol.* **2003**, *105*, 308–317.
(5) Castro, H. F.; Mendes, A. A.; Santos, J. C. Modificação de óleos e gorduras por biotransformação. *Quim. Nova* **2004**, *27* (1), 146–156.
(6) Snellman, E. A.; Sullivan, E. R.; Colwell, R. R. Purification and properties of the extracellular lipase, LipA, of *Acinetobacter* sp. RAG-1. *Eur. J. Biochem.* **2004**, *269* (23), 5771–5779.

(7) Jaeger, K. E.; Reetz, M. T. Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol.* **1998**, *16*, 396–403.
(8) Macrae, A. R.; Hammond, R. C. Present and future applications of lipase. In *Biotechnology and Genetic Reviews*; Intercept: Newcastle upon Tyne, U.K., 1986.
(9) Abdel-Fattah, Y. R.; Gaballa, A. A. Identification and over-expression of a thermostable lipase from *Geobacillus thermoleovorans* Toshki in *Escherichia coli*. *Microbiol. Res.* **2008**, *163*, 13–20.
(10) Mukherjee, K. D.; Kiewitt, I. Specificity of *Carica papaya* latex as biocatalyst in the esterification of fatty acids with 1-butanol. *J. Agric. Food Chem.* **1996**, *44*, 1948–1952.
(11) Liaquat, M.; Owusu Apenten, R. K. Synthesis of low molecular weight flavor esters using plant seedling lipases in organic media. *J. Food Sci.* **2000**, *65*, 295–299.
(12) Cavalcanti, E. D. C.; Maciel, F. M.; Villeneuve, P.; Lago, R. C. A.; Machado, O. L. T.; Freire, D. M. G. Acetone powder from dormant seeds of *Ricinus communis* L. *Appl. Biochem. Biotechnol.* **2007**, *136*−*140*, 57–66.
(13) Ncube, I.; Gitlesen, T.; Adlercreutz, P.; Read, J. S. Fatty acid selectivity of a lipase purified from *Vernonia galamensis* seed. *Bioch. Biophys. Acta* **1995**, *1257*, 149–156.
(14) Alfenas, A. C. *Eletroforese de Isoenzimas e Proteínas Afins* − *Fundamentos e Aplicações em Plantas e Microrganismos*, 1st ed.; Viçosa Editora UFV: Viçosa, Brazil, 1998 (in Portuguese).
(15) Kapranchikov, V. S.; Zherebtsov, N. A.; Popova, T. N. Purification and characterization of lipase from wheat (*Triticum aestivum* L.) germ. *Appl. Biochem. Microbiol.* **2004**, *40*, 84–88.
(16) Freire, D. M. G.; Gomes, P. M.; Bom, E. P. S.; Sant'Anna, G. L. Jr. Lipase production by a new promising strain *Penicillium restrictum*. *Rev. Microbiol.* **1997**, *28*, 6–12.
(17) Oliveira, D.; Feihrmann, A. C.; Dariva, C.; Cunha, A. G.; Bevilaqua, J. V.; Destain, J.; Oliveira, V.; Freire, D. M. G. Influence of compressed fluids treatment on the activity of *Yarrowia lipolytica* lipase. *J. Mol. Catal. B: Enzym.* **2006**, *39*, 117–123.
(18) Gandhi, N. N.; Mukherjee, K. D. Synthesis of designer lipids using papaya (*Carica papaya*) latex lipase. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 271–277.
(19) Enujiugha, V. N.; Thani, F. A.; Sanni, T. M.; Abigor, R. D. Lipase activity in dormant seeds of the African oil bean (*Pentaclethra macrophylla* Benth). *Food Chem.* **2004**, *88*, 405–410.
(20) Menoncin, S.; Domingues, N. M.; Freire, D. M. G.; Toniazzo, G.; Cansian, R. L.; Oliveira, J. V.; Di Luccio, M.; Oliveira, D.; Treichel, H. Study of the extraction, concentration and partial characterization of lipases obtained from *Penicillium verrucosum* using solid state fermentation of soybean bran. *Food Bioprocess Technol.* **2008**, DOI 10.1007/s11947-008-0104-8.
(21) Shu, C. H.; Xu, C. J.; Lin, G. C. Purification and partial characterization of a lipase from *Antrodia cinnamomea*. *Process Biochem.* **2006**, *41*, 734–738.
(22) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(23) Sagiroglu, A.; Arabaci, N. Sunflower seed lipase: extraction, purification and characterization. *Prep. Biochem. Biotechnol.* **2005**, *35*, 37–51.
(24) Prabhu, A. V.; Tambe, S. P.; Gandhi, N. N.; Sawant, S. B.; Joshi, J. B. Rice bran lipase: extraction, activity and stability. *Biotechnol. Prog.* **1999**, *15*, 1083–1089.
(25) Villeneuve, P.; Turon, F.; Caro, Y.; Escoffier, R.; Baréa, B.; Barouh, B.; Lago, R.; Piombo, G.; Pina, M. Lipase-catalyzed synthesis of canola phytosterols oleate esters as cholesterol lowering agents. *Enzyme Microb. Technol.* **2005**, *37*, 150–155.
(26) Giordani, R.; Moulina, A.; Verger, R. Tributyroylglycerol hydrolase activity in *Carica papaya* and other latices. *Phytochemistry* **1991**, *30* (4), 1069–1072.
(27) Caro, Y.; Dhuique-Mayer, C.; Turon, F.; Pina, M.; Reynes, M.; Graille, J. The biocatalytic activity of bromelain in ester synthesis reaction: difference between the intrinsic lipase activity and thermal catalysis. *Biotechnol. Lett.* **2001**, *23*, 2035–2039.

Received May 28, 2009. Revised manuscript received September 14, 2009. Accepted September 15, 2009. We thank FAPERJ, CAPES, CNPq, Intecnial SA, and URI-Campus de Erechim for financial support and scholarships.