Improved lipase biosynthesis by a newly isolated *Penicillium* sp. grown on agricultural wastes

Elisandra Rigo,¹ Jorge L. Ninow,¹ André E. Polloni,² Daniela Remonato,² Francieli Arbter,² Renata Vardanega,² Débora de Oliveira,² Helen Treichel,^{2*} and Marco Di Luccio²

¹Departamento de Engenharia Química e de Alimentos, Universidade Federal de Santa Catarina, UFSC, SC, Brasil

²Programa de Pós-Graduação em Engenharia de Alimentos, Universidade Regional Integrada do Alto Uruguai e das Missões – Campus de Erechim, Av. Sete de Setembro, 1621, 99700-000, Erechim, RS, Brasil

*Corresponding author

Tel: +55 54 35209000

Fax: +55 54 35209090

E-mail: helen@uricer.edu.br

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Abstract

The main objective of this work was the optimization of the lipase production by a newly isolated *Penicillium* sp. in supplemented soybean meal, considering a fixed C/N ratio (6.11). The kinetic behavior of lipase production was also evaluated, quantifying activity at different pH. The production of the enzyme was maximum using a substrate particle size between 1 and 2 mm and inoculum concentration of 2×10^8 spores $\cdot g^{-1}$, at 20°C. Lipase activities of about 200 U $\cdot g^{-1}$ at pH 4.0 were obtained after 120 h of cultivation, but activities at pH 7.0 as high as 317 U $\cdot g^{-1}$ could be obtained after 96 h. The activities measured at pH 9.0 were also promising, ranging from 177 to 191 U $\cdot g^{-1}$. The results obtained are relevant, since high lipase activities, in a wide range of pH (4.0 to 9.0) were obtained from a newly isolated microorganism in a low-cost fermentation medium. The variation in the C/N ratio at the previously optimized conditions showed that the best results were obtained at C/N of 6.11.

Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are a class of hydrolases that catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at an oil–water interface. In addition, lipases can catalyze the hydrolysis of other esters and also reactions of synthesis of esters, transesterification, and interesterification, and may show enantioselective properties. Their ability to perform very specific chemical transformations has made lipases increasingly popular in the food, detergent, cosmetic, organic synthesis, and pharmaceutical industries.¹⁻⁴

Several different lipase-production techniques have been developed in the search for higher yields and more-specific and more-stable lipases, to enable more industrial applications.⁵⁻⁶ Solid state fermentation (SSF) is characterized by the growth of the microorganism in the absence of free water. This kind of technique can be used for production of many bioproducts, to add value to agro-industrial residues, and to increase nutritional quality and antioxidant activities of agro-wastes for use as animal feed.^{6,7} SSF has been presented as an interesting alternative to the production of microbial enzymes. The use of agro-industrial residues can be a feasible means of decreasing the final cost of the enzyme.^{6,8-10}

Studies on lipase production by SSF with different substrates combined to different fungi have been reported.¹⁰⁻¹² *Penicillium restrictum*¹³⁻¹⁵ and *P. simplicissimum*¹⁶⁻¹⁷ have been cultivated in babassu cake. Growth of *P. simplicissimum*¹⁸⁻¹⁹ and *Penicillium verrucosum*²⁰ was studied in soybean meal. Other residues and microorganisms combinations have also been reported, such as nut residues using *Rhizopus oligosporus*²¹, sugarcane bagasse with *R. rhizopodiformis*, *R. pusillus*²² and *R. homothallicus*^{8,23}, wheat bran with *Aspergillus niger*²⁴⁻²⁵ and *Mucor hiemalis*²⁶, a mix of wheat flour and wheat bran with *R. chinensis*²⁷, and sesame (gingelly) oil cake with *A. niger*.²⁸

The effect of medium composition, temperature, pH, moisture content, inoculum concentration, and substrate porosity is of essential importance for bioprocess development. In a general sense, the enhancement on productivity is related to medium optimization.^{8,9,29-32}

The measurement of enzymes activity in different pH values offers a way of screening different lipases, or a pool of them, considering that a variation in the fermentation medium can result in the production of different isoenzymes.^{33–34}

In this context, this work aimed to optimize lipase production by SSF using a newly isolated strain of *Penicillium* sp. and soybean meal as both support and substrate. Different medium supplementations were tested, and the kinetic behavior was followed at different pH. The influence of C/N ratio in the lipase yield was also investigated.

Materials & methods

MICROORGANISM & INOCULUM

The microorganism used in this work was previously isolated from samples of soybean meal and identified as *Penicillium* sp.³⁵ The isolation of microorganisms and cultivation was carried out in Petri dishes using potato dextrose agar (PDA; Merck KGaA Darmstadt, Germany) medium at 30°C. The screening was carried out by fermentation in soybean meal, and this strain was identified as a good lipase producer. Spores were maintained at –10°C in cryotubes containing a 20% solution of glycerol in 9 g•L^{–1} aqueous NaCl.

Erlenmeyer flasks (500 mL) with 100 mL of sterile PDA medium were inoculated with 0.5 mL of a spore suspension obtained from stock culture. Flasks were incubated for 7 days at 30°C. Spores were collected by adding 20 mL of a solution of Tween 80 1 mL•L^{–1} in water and sterile glass beads to the flask. The resulting suspension was then used as inoculum, after adjusting the amount of spores to achieve the desired spore concentration, defined in the experimental design.

SOLID STATE FERMENTATION

The substrate and support used in all experiments was soybean meal from a same batch, obtained from a local soybean oil indus-

try (Olfar SA; Erechim, Brazil). The meal is the residue of soybean oil extraction after cold press and extraction with *n*-hexane. The substrate was sieved, and the major fractions collected and stored at –18°C until use. The three major fractions based on the particle diameter were 1–2 mm (Tyler 9–16; WS Tyler, division of Haver & Boecker, Oelde, Germany), 0.5–1 mm (Tyler 16–32), and 0.25–0.50 mm (Tyler 32–60).

Fermentations were carried out with 10 g of dry soybean meal in tray reactors covered with hydrophobic fabric (Polysentric®; Broomfield, Colorado, USA). The effect of composition of the cultivation medium, supplements, moisture content, particle size, inoculum concentration and temperature of fermentation on lipase production was evaluated by a strategy of sequential experimental designs.

After sterilization (121°C for 15 min), each reactor was inoculated with the respective suspension of spores and incubated in a climatic chamber (TE 401, Tecnal; Piracicaba, Brazil) with humid air injection at a temperature defined by the factorial design.

Enzyme extraction was carried out using phosphate buffer (100 mmol•L^{–1}, pH 7.0) at a solid-liquid ratio of 1:4 in an orbital shaker (MA 410; Marconi; Piracicaba, Brazil) at 150 rev•min^{–1}, 35°C for 30 min. After filtration of solids, the supernatant was used for analytical assays.^{18–20}

MAXIMIZATION OF LIPASES PRODUCTION BY *PENICILLIUM* sp.

Variables and studied range for the sequential experimental designs are presented in Table 1. The first was a Plackett-Burman (PB12) type design, used for screening the statistically significant process variables that influence lipase production.

After statistical analysis of the first experimental design (PB12), a two-factor central composite rotational design (CCRD 2²) was carried

Table 1. Ranges of the factors investigated in the three experimental designs

Level	First Experimental Design (Plackett-Burman)				
	–1.41	–1	0	+1	+1.41
Temperature (°C)	–	20	27	34	–
Moisture (g•kg ^{–1})	–	350	550	750	–
Particle size (mm)	–	0.25–0.5	0.5–1	1–2	–
Spore concentration (spores • g ^{–1})	–	1.00x10 ⁷	1.05x10 ⁸	2.00x10 ⁸	–
Supplement* (g•kg ^{–1})	–	10.0	55.0	100.0	–
Second Experimental Design (2 ²)					
Moisture (g•kg ^{–1})	–	550	650	750	–
Supplement* (g•kg ^{–1})	–	6.0	18.0	30.0	–
Third Experimental Design (CCRD 2 ²)					
Moisture (g•kg ^{–1})	610	650	750	850	890
Supplement* (g•kg ^{–1})	1.0	2.4	6.0	9.5	11.0
*Selected supplement concentration with soybean oil and urea (C/N of 6.11)					

out at constant temperature, particle size, and spore concentration. In this design, the effect of moisture content and supplement concentration on lipase production was investigated.

Analysis of this second experimental design showed a need to shift the studied levels for the optimization of enzyme production. A third experimental design (CCRD 2²) was then carried out.

All analyses were performed in duplicate and triplicate at the central point of the experimental design. Statistical analysis was carried out using Statistica 6.1 software (Statsoft Inc., Tulsa, Oklahoma, USA).

EFFECT OF C/N RATIO ON LIPASE PRODUCTION

The effect of different carbon-to-nitrogen ratios (C/N of 2.00, 6.11, and 10.22) on lipase production was evaluated. Urea and soybean oil were added to achieve the desired C/N ratios as presented in Table 2.

Table 2. Soybean oil and urea concentrations used to achieve the desired C/N ratio

C/N ratio	Soybean oil (g•kg ⁻¹)	Urea (g•kg ⁻¹)
2.00	2.0	41.2
6.11	2.0	0.6
10.22	39.5	0.6

DETERMINATION OF HYDROLYTIC ACTIVITY

Lipase hydrolysis was assayed using an emulsion of olive oil (10 wt%) in arabic gum (5 wt%) in different buffers to evaluate lipase production, in terms of enzyme activity, at different pH. Buffers used were sodium acetate 0.1 mol•L⁻¹ pH 4.0, sodium phosphate 0.1 mol•L⁻¹, pH 7.0, and tris-HCl 0.1 mol•L⁻¹, pH 9.0. After incubation for 15 min at 37 °C and 160 rev•min⁻¹, the reaction was stopped by addition of a solution of acetone-ethanol (1:1). The fatty acids produced by the hydrolysis were titrated with NaOH 0.05 mol•L⁻¹ in a pH-stat (Mettler-Toledo DL50; Columbus, Ohio, USA). Control assays (blanks) were carried out adding the acetone/ethanol solution right after enzyme addition. One unit of lipase activity was defined as the amount of enzyme preparation necessary to produce 1 μmol of free acid per minute in the assay conditions. The results are expressed in terms of units per gram of dry substrate (U•g⁻¹).¹⁸

Results & discussion

FIRST EXPERIMENTAL DESIGN

The matrix of the Plackett-Burman experimental design (PB12) is presented in Table 3. The coded values of the variables temperature (°C), moisture content (g•kg⁻¹), soybean meal particle size (mm), spore concentration (spores•g⁻¹), and supplementation (a mixture of urea and soybean oil, g•kg⁻¹) concentration are also presented, as well as the response in terms of hydrolytic activity after 96 h of fermentation.

Table 3. Matrix of the first experimental design (PB) with coded values of the variables temperature (°C), moisture content (g•kg⁻¹), particle size (mm), spores (spores•g⁻¹), and supplement (urea plus soybean oil, g•kg⁻¹) concentrations on lipase production after 96 h of fermentation

Run	Experimental Conditions					Hydrolytic activity (U•g ⁻¹ dry soybean bran) 96 h of fermentation		
	T	MC	Particle Size	Spores	Supplement	pH 4.0	pH 7.0	pH 9.0
1	+1	-1	+1	-1	-1	16.20	0.65	2.70
2	+1	+1	-1	+1	-1	16.84	6.22	5.80
3	-1	+1	+1	-1	+1	43.49	97.26	70.01
4	+1	-1	+1	+1	-1	6.79	4.31	2.67
5	+1	+1	-1	+1	+1	13.39	37.62	10.54
6	+1	+1	+1	-1	+1	11.96	6.73	2.25
7	-1	+1	+1	+1	-1	204.27	266.18	177.68
8	-1	-1	+1	+1	+1	8.02	7.35	3.11
9	-1	-1	-1	+1	+1	16.02	22.14	4.53
10	+1	-1	-1	-1	+1	29.33	14.54	3.54
11	-1	+1	-1	-1	-1	147.12	175.85	200.96
12	-1	-1	-1	-1	-1	50.16	61.03	30.66
13	0	0	0	0	0	129.85	123.85	97.13
14	0	0	0	0	0	106.42	124.08	88.69
15	0	0	0	0	0	106.28	121.54	102.34

Run 7 (20°C, moisture content 750 g•kg⁻¹, particle size 1–2 mm, 2×10⁸ spores•mL⁻¹, and 10 g•supplement kg⁻¹) and Run 11 (20°C, moisture content of 750 g•kg⁻¹, particle size 0.25–0.50 mm, 1×10⁷ spores•mL⁻¹, and supplement 10 g•kg⁻¹) demonstrated higher lipase activities, at all tested pH. It is worth noting that the lipase activities are high compared to others reported, which vary from 30 U•g⁻¹ (*P. simplicissimum* cultivated on soybean bran) to 40 U•g⁻¹ (*P. verrucosum* cultivated on soybean bran)^{18,20}, further demonstrating the potential of this microorganism for industrial applications.

Data in Table 3 was statistically treated and, from the five studied variables, three presented statistically significant effects (p<0.05) on lipase production. Temperature and supplement concentration showed a negative effect, and moisture content of the solid matrix a positive one, in lipase production. The observation of considerable activities (as per activity measurement) from the same experimental condition but different pH suggests that a pool of lipases was produced.

SECOND EXPERIMENTAL DESIGN

A second experimental design was carried out, based on the results obtained in the Plackett-Burman design, varying the ranges of moisture content and supplement concentration. The matrix of the second experimental design is presented in Table 4, where the respective responses in terms of hydrolytic activity after 96 h of fermentation could be found. Temperature, particle size, and spore concentration were kept constant at 20°C, 1–2 mm, and 2×10⁸ spores•g⁻¹, respectively. The highest lipase activities (200–300 U•g⁻¹) were achieved in the experimental condition of Run 2, corresponding to a moisture content of 750 g•kg⁻¹ and 6 g•kg⁻¹ of supplement mix, which in turn correspond to the superior limits of these variables in the experimental design.

The statistical analysis of the experimental data resulted in an empirical coded model for lipase activities at pH 4.0 and 7.0, which are presented in Equations 1 and 2, respectively. The models were validated by analysis of variance, which yielded correlation coefficients of 0.98 (at pH 4.0) and 0.96 (at pH 7.0) and a good performance in F-test for regression (calculated values approximately 6.3 and 2.8 higher than those tabulated for pH 4.0 and 7.0, respectively). In Equations 1 and 2, LP4 and LP7 correspond to lipase

production at pH 4 and 7, respectively, MC is moisture content, and URSO ("urea soy"), the supplement concentration.

$$LP4 = 123.85 + 41.48 \cdot MC - 38.51 \cdot URSO - 28.01 \cdot MC \cdot URSO \quad (1)$$

$$LP7 = 157.06 + 40.93 \cdot MC - 37.80 \cdot URSO - 46.93 \cdot MC \cdot URSO \quad (2)$$

The hydrolytic activities measured at pH 9.0 after 96 h of fermentation at the experimental conditions presented in Table 4 indicated that the moisture content and the URSO concentration did not have a significant (p<0.05) effect on lipase production.

Table 4. Matrix of the second (CCRD 2²) experimental design with real and coded values of the variables moisture content (MC; g•kg⁻¹) and supplement concentration (urea plus soybean oil, g•kg⁻¹) on lipase production after 96 h of fermentation

Run	Experimental condition		Hydrolytic activity (U•g ⁻¹ dry soybean bran) 96 h of fermentation		
	MC (g•kg ⁻¹)	Supplement (g•kg ⁻¹)	pH 4.0	pH 7.0	pH 9.0
1	-1 (550)	-1 (6)	93.27	107.23	74.25
2	+1 (750)	-1 (6)	232.25	282.95	325.96
3	-1 (550)	+1 (30)	72.26	125.48	65.84
4	+1 (750)	+1 (30)	99.22	113.48	44.60
5	0 (650)	0 (18)	111.42	151.87	48.52
6	0 (650)	0 (18)	123.76	178.81	33.76
7	0 (650)	0 (18)	134.77	139.64	34.22

Table 5. Matrix of the third (CCRD 2²) experimental design with real and coded values of the variables moisture content (MC; g•kg⁻¹) and supplement concentration (urea plus soybean oil, g•kg⁻¹) on lipase production after 96 h of fermentation

Run	MC (g•kg ⁻¹)	Supplement (g•kg ⁻¹)	Hydrolytic activity (U•g ⁻¹ dry soybean bran) 96 h of fermentation		
			pH 4.0	pH 7.0	pH 9.0
1	-1 (650)	-1 (2.4)	129.30	144.92	172.48
2	+1 (850)	-1 (2.4)	168.05	300.79	209.78
3	-1 (650)	+1 (9.5)	106.38	175.83	176.49
4	+1 (850)	+1 (9.5)	209.49	222.64	240.70
5	0 (750)	0 (6.0)	173.68	330.02	317.68
6	0 (750)	0 (6.0)	158.52	304.68	340.08
7	0 (750)	0 (6.0)	161.55	317.34	324.36
8	0 (750)	-1.41 (1.0)	167.74	197.02	310.90
9	0 (750)	+1.41 (11.0)	136.48	200.17	276.53
10	-1.41 (610)	0 (6.0)	95.90	112.58	173.16
11	+1.41 (890)	0 (6.0)	139.42	195.67	143.68

THIRD EXPERIMENTAL DESIGN

The results of the second experimental design suggested that for optimizing the lipase production, a third plan should be performed. The matrix of this experimental design with the respective responses in terms of hydrolytic activity is presented in Table 5. The kinetic behavior of the enzymatic activity measured at pH 4.0 (A), 7.0 (B) and 9.0

(C), related to each experimental condition of Table 5, is presented in Figure 1.

The statistical analysis was carried out with the data from 96 h of fermentation, since more-pronounced differences were observed at this fermentation duration. When monitoring activity at pH 4.0, neither moisture content nor supplement concentration showed significant ($p < 0.05$) effects on lipase production. Equation 3 gives the coded empirical model for lipase production measured at pH 7.0, which was validated by analysis of variance, yielding a correlation coefficient of 0.79, and calculated F-test regression of 1.15. The empirical model generated a response surface shown in Figure 2.

Figure 1. Kinetic behavior of lipase production in SSF measured at: pH 4.0 (top), pH 7.0 (middle), and pH 9.0 (bottom), at experimental conditions presented in Table 5

Figure 2. Response surface for lipase production measured at pH 7.0 after 96 hours of fermentation, as a function of moisture content and supplement concentration

Figure 3. Response surface for lipase production measured at pH 9.0 after 96 hours of fermentation, as a function of moisture content and supplement concentration

$$LP7 = 304.54 + 25.97 \bullet MC - 44.37 \bullet MC^2 + 8.76 \bullet URSO - 66.74 \bullet URSO^2 - 27.26 \bullet MC \bullet URSO \quad (3)$$

Equation 4 presents the coded empirical model for lipase production measured at pH 9.0, which was validated by analysis of variance with a correlation coefficient of 0.90 and F-test for regression of 2.70. The empirical coded model generated a response surface presented in Figure 3.

$$LP9 = 327.47 + 6.65 \bullet MC - 23.41 \bullet MC^2 - 0.83 \bullet URSO - 91.46 \bullet URSO^2 - 6.72 \bullet MC \bullet URSO \quad (4)$$

The results presented herein suggest that the newly isolated micro-organism is able to produce a pool of lipases with potential for industrial applications, presenting high activities of 201.11 and 177.64 ($\text{U} \cdot \text{g}^{-1}$ dry soybean bran) at 96 h at pH 4.0 and 9.0, respectively. These results accord with those of other reported studies that indicate that the use of a supplementary carbon source in SSF can favor the synthesis of different enzymes.¹⁵ The low amount of urea added to the fermentation medium should not be considered economically inconvenient, as it is a relatively low-cost compound.

The use of inducers in the production of lipases has been reported elsewhere. The most-studied inducers include olive^{27,36} and soybean oil.³⁷ Lipase productivity, however, is affected by the medium supplementation added. The addition of vegetable oils to the fermentation medium can also cause inhibition, decreasing lipase production, possibly due to the action of proteases.³⁸

The use of urea has also been reported by Rodriguez et al⁸ and Diaz et al;²³ those studies of supplementation of sugarcane bagasse with urea and soybean oil demonstrated that supplementation can increase lipase production by *R. homothallicus* up to sixtyfold when compared to basal medium. Alkan et al³⁹ also showed that the addition of urea and olive oil ($10 \text{ g} \cdot \text{kg}^{-1}$) improved lipase production by SSF using sugarcane bagasse and *Bacillus coagulans*.

EFFECT OF C/N RATIO ON LIPASE PRODUCTION AT OPTIMIZED EXPERIMENTAL CONDITIONS

The effect of different carbon and nitrogen (C/N) ratios on lipase production was evaluated at the experimental conditions of the central point (Table 5). The temperature, particle size, spore concentration, moisture content, and supplement concentration were kept constant at 20°C, 1–2 mm, 2×10^8 spores $\cdot \text{g}^{-1}$, $750 \text{ g} \cdot \text{kg}^{-1}$, and $6 \text{ g} \cdot \text{kg}^{-1}$, respectively. The C/N ratio at these conditions was 6.11, which was the ratio of soybean meal and all tested media in these experiments. The results from this step are presented in Table 6. Significant differences ($p < 0.05$) among the lipase activities were obtained at different C/N ratios for each pH tested, but C/N ratios other than 6.11 did not lead to enhanced lipase production, although good activities ($>100 \text{ U} \cdot \text{g}^{-1}$) could be obtained at C/N ratio of 10.2.

Mahadik et al²⁵ observed that lipase production was highly influenced by carbon content on fermentation media. It seems that this behavior is directly related to the microorganism's regulation

Table 6. Effect of different carbon and nitrogen concentrations on lipase production measured at pH 4.0, 7.0, and 9.0, at 96 hours of fermentation, using soybean meal as substrate and *Penicillium* sp.

C/N ratio	Hydrolytic activity ($\text{U} \cdot \text{g}^{-1}$ of soybean bran)		
	pH 4.0	pH 7.0	pH 9.0
2.00	$14.86^a \pm 2.09$	$18.62^a \pm 6.24$	$14.97^a \pm 7.88$
6.11	$197.18^b \pm 3.45$	$301.26^b \pm 5.07$	$309.05^b \pm 2.34$
10.2	$101.97^c \pm 0.78$	$165.45^c \pm 2.68$	$117.25^c \pm 2.88$

*Means $\pm$ standard error followed by different superscripts in the same column, in same pH in 96 h of fermentation are significantly different ($p < 0.05$) by Tukey's test.

mechanism; accordingly, some studies have related that medium supplementation with lipids is not efficient.²⁸ Supplementation with $10 \text{ g} \cdot \text{kg}^{-1}$ of tributyrin led to the maximum alkaline lipase production using *Pseudomonas* sp. The use of other complementary carbon sources does not necessarily increase lipase production, as shown by Kiran et al.⁴⁰ The presence of other carbon sources, like sugars, may strongly inhibit lipase production, via catabolic repression. In some cases, carbon limitation may increase lipase production.⁴⁰ In the present work, the increase in the carbon source to detriment of nitrogen caused first an increase, then a decrease, in lipase production, which is in agreement with the observations of Kiran et al⁴⁰ and Mahadik et al.²⁵ Azeredo et al¹³ showed that the ideal C/N ratio for lipase production by *P. restrictum* using babassu cake as substrate was 13.13; that data indicated peak production after 24 h of fermentation ($17.4 \text{ U} \cdot \text{g}^{-1}$) when olive oil was used. Gombert et al¹⁵ showed that the increase in C/N ratio from 11.7 to 14.0 could lead to higher lipase production by *P. restrictum* in babassu cake. Our studies indicate that the excess of carbon source seems to be inhibitory to lipase production, and higher yields are obtained at lower C/N ratios (6.11).

The presence of a nitrogen source reportedly is not an absolute requirement for the production of lipase. Kiran et al⁴⁰ showed that supplementation of liquid medium with peptone, casein, and meat extract inhibits lipase production; however, higher yields are obtainable when the medium is supplemented with urea and corn steep liquor.^{41,42}

Conclusions

The kinetic evaluation of the fermentative process using *Penicillium* sp. showed that medium supplementation improved the production of the enzyme. Optimal lipase production was found in soybean bran supplemented with $6 \text{ g} \cdot \text{kg}^{-1}$ of urea and soybean oil (C/N 6.11), at different pH, moisture content $750 \text{ g} \cdot \text{kg}^{-1}$, particle size between 1 and 2 mm, a temperature of 20°C, and with a spore concentration of 2×10^8 spores $\cdot \text{g}^{-1}$. Lipase production as high as $317 \text{ U} \cdot \text{g}^{-1}$ of dry soybean bran could be obtained at 96 h and pH 4.0. The effect of the C/N ratio on lipase production indicated that the optimal ratio was that of the crude soybean meal (6.11). These results demonstrate that a pool of lipases with high activities at pH ranging from 4.0 to 9.0 are obtainable.

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