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Surface response methodology for the optimization of lipase production under submerged fermentation by filamentous fungi

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ABSTRACT

A Plackett–Burman Factorial Design of 16 experiments was conducted to assess the influence of nine factors on the production of lipases by filamentous fungi. The factors investigated were bran type (used as the main carbon source), nitrogen source, nitrogen source concentration, inducer, inducer concentration, fungal strain (*Aspergillus niger* or *Aspergillus flavus* were selected as good lipase producers via submerged fermentation), pH and agitation. The concentration of the yeast extract and soybean oil and the pH had a significant effect ($p < 0.05$) on lipase production and were consecutively studied through a Full Factorial Design 2^3, with the concentration of yeast extract and pH being significant ($p < 0.05$). These variables were optimized using a central composite design, obtaining maximum lipolytic activities with the use of 45 g/L of yeast extract and pH 7.15. The statistical model showed a 94.12% correlation with the experimental data.

Introduction

Enzymes currently have various industrial applications.[1] The industrial market for enzymes continues to grow due to the development of new production technologies, the use of genetic engineering during production and emergence of new fields of application. The global enzyme market in 2007 was 2.3 billion US dollars and is expected to be 2.7 billion US dollars in 2012.[2] Among these enzymes, lipases are widely used. Their applications result from their ability to catalyze reactions, mainly the hydrolysis and inter- and transesterification

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of lipids, making these enzymes useful in the detergent,[3] medical[4] and food industries[5] as well as in the pharmaceutical industry as diagnostic tools, in cosmetics, in tea processing, in medical applications, as biosensors, in degreasing of leather and waste treatment.[6] Other applications include the maturation of cheeses,[7] the synthesis of aromas,[8] the production of lipids with high levels of unsaturated fatty acids[9] and methyl-esters of fatty acids (biodiesel).[10]

Industrial enzymes are produced primarily through submerged fermentation in batch and fed-batch cultures[1] using filamentous fungi. The most-cited genera for lipase production are *Aspergillus*, *Rhizopus*, *Penicillium*, *Mucor*, *Geotrichum* and *Fusarium*.[11,12] Submerged processes have some advantages over solid-state processes, such as higher homogeneity of the culture medium and more facility to control parameters like temperature and pH.[13]

Moreover, Mahadik et al.[14] mentioned that one of the disadvantages of solid state processes is the color of the fermented media, due to the presence of fungal spores, and other components remaining in the extract after the enzyme extraction process. However, submerged fermentation can present difficulty for the transfer of oxygen in liquid media, which is aggravated in the case of fungi due to the filamentous morphology of hyphae in liquid media.[1] Coradi et al.[15] mentioned that lipases have been produced by submerged fermentation because the recovery of extracellular enzymes and the determination of biomass are facilitated by being performed by simple filtration or centrifugation. However, solid-state fermentation can improve the use of agricultural waste and demands less water and energy.

Other factors, such as the types and concentrations of nutrients, pH, agitation and the presence and concentration of inducers can affect the productivity of these bioprocesses. Research that uses isolated microorganisms from new environments and that uses agro-industrial residues in the composition of media is needed to obtain high yields at lower costs.

The statistical optimization of processes has advantages compared to the classical practice of changing one variable at a time,[16,17] such as the lower number of experiments and the possibility of evaluating the interaction effects among variables. Numerous researchers have reported the use of these techniques for the production of lipases by microorganisms.[18–20] An efficient and widely used approach is the application of Plackett–Burman designs that allow efficient screening of key variables for further optimization in a rational way.[21,22] The aim of this work was to screen significant variables for lipase production through submerged fermentation and to optimize these variables through response surface methodology.

Materials and methods

Microorganisms and inoculum preparation

The fungi used in this study were two species of *Aspergillus* that were isolated from dairy effluent (isolate E-19) and soil contaminated with diesel oil (isolated O-8)[23] and that were previously selected as good producers of lipase via submerged fermentation.[24] Both isolates were submitted to genetic identification through Phred/Phrap and Consed, using the methodology cited by Smanioto et al.,[25] at the Center of Nuclear Energy in Agriculture (Cena), University of São Paulo (USP), Brazil.

Sequences were compared to 18S rRNA data obtained from GenBank (http://www.ncbi.nlm.nih.gov). The isolate E-19 was identified as *Aspergillus niger* strain DAOM (100% identity, GenBank accession number: KC545858.1), and the isolate O-8 was identified as *Aspergillus flavus* strain DAOM (99% identity, GenBank accession number: JN938987.1).

The microorganisms were kept in test tubes with PDA (potato-dextrose-agar) at 4 °C. The inoculum was prepared by inoculation of the fungi in Petri dishes containing 30 mL of solidified PDA medium and incubated at 30 °C for 5 days.

Culture medium and experimental apparatus

The culture medium was prepared with 10% (m/v) bran (wheat or soybean), which was boiled at 100 °C for 30 min. Afterwards, the medium was filtered, and the soluble extract was added to a10% (v/v) saline solution containing also the nitrogen source, inducer and distilled water to complete the final volume. The saline solution contained KH_2PO_4 (2 g/L), $MgSO_4$ (1 g/L) and trace solution (10 mL/L). The composition of the trace solution was $FeSO_4{\cdot}7H_2O$ (0.63 mg), $MnSO_4$ (0.01 mg), $ZnSO_4$ (0.62 mg)and distilled water up to a volume of 1 L.[26] The liquid medium was autoclaved, and the pH was adjusted to values pre-defined by the experimental design using solutions of 1.5 mol/L HCl or 1 mol/L NaOH.

The experiments were carried out in 300-mL Erlenmeyer flasks with 100 mL of medium. The inoculation was accomplished using 10 or 20 mm diameter circular areas containing spores grown in Petri dishes. The Erlenmeyer flasks, containing the inoculated culture medium, were incubated at 30 °C for 10 days in a shaker with a level of agitation pre-defined according to the experimental design. Aliquots (10 mL) were removed at 24 h, 48 h, 72 h and 96 h to measure lipolytic activity.

Experimental design

The optimization of lipase production by submerged fermentation was carried out using three sequential experimental designs. The first step aimed to assess the influence of nine variables on lipase production using a Plackett–Burman Design with 16 trials (1–16). The variables studied were bran type used as a carbon source (wheat bran or soybean bran), nitrogen source (sodium nitrate or yeast extract), nitrogen source concentration (10 or 30 g/L), inducer (soybean or olive oil), inducer concentration (10 or 30 g/L), culture medium pH (5 or 7), fungus strain used (*A. niger* E-19 or *A. flavus* O-8), inoculum diameter (fungal spores growth equal to 1 or 2 cm diameter in Petri dishes containing PDA) and agitation (120 or 160 rpm). Next, a 2^3 Full Factorial Design (FFD) (Trials 17–24) was carried out to study the influence of yeast extract concentration (YEC), soybean oil concentration (SOC) and pH on lipolytic activity. Subsequently, the pH and the concentration of yeast extract were optimized using a Central Composite Rotational Design (CCRD), including 4 factorial points, 4 axial points and 3 central points for evaluating the pure error for a

Table 1 – Real and coded levels of variables used in the Plackett–Burman Design and maximum residual lipolytic activities obtained in experiments carried out in submerged fermentation.

Exp.	X_1 (TB)	X_2 (NS)	X_3 (I)	X_4 (NSC)	X_5 (IC)	X_6 (pH)	X_7 (fungus)	X_8 (ID)	X_9 (A)	LA_{MR} (U)[a]
1	−1 (WB)	−1 (SN)	−1 (OO)	−1 (10)	+1 (30)	+1 (7)	+1 (E-19)	+1 (2)	+1 (160)	1.74 ± 0.16
2	+1 (SB)	−1 (SN)	−1 (OO)	−1 (10)	−1 (10)	−1 (5)	−1 (O-8)	+1 (2)	+1 (160)	0.31 ± 0.13
3	−1 (WB)	+1 (YE)	−1 (OO)	−1 (10)	−1 (10)	+1 (7)	+1 (E-19)	−1 (1)	−1 (120)	2.03 ± 0.28
4	+1 (SB)	+1 (YE)	−1 (OO)	−1 (10)	+1 (30)	−1 (5)	−1 (O-8)	−1 (1)	−1 (120)	1.50 ± 0.12
5	−1 (WB)	−1 (SN)	+1 (SO)	−1 (10)	+1 (30)	−1 (5)	+1 (E-19)	−1 (1)	+1 (160)	0.19 ± 0.07
6	+1 (SB)	−1 (SN)	+1 (SO)	−1 (10)	−1 (10)	+1 (7)	−1 (O-8)	−1 (1)	+1 (160)	1.21 ± 0.25
7	−1 (WB)	+1 (YE)	+1 (SO)	−1 (10)	−1 (10)	−1 (5)	+1 (E-19)	+1 (2)	−1 (120)	0.84 ± 0.44
8	+1 (SB)	+1 (YE)	+1 (SO)	−1 (10)	+1 (30)	+1 (7)	−1 (O-8)	+1 (2)	−1 (120)	2.93 ± 0.88
9	−1 (WB)	−1 (SN)	−1 (OO)	+1 (30)	+1 (30)	+1 (7)	−1 (O-8)	+1 (2)	−1 (120)	1.62 ± 0.18
10	+1 (SB)	−1 (SN)	−1 (OO)	+1 (30)	−1 (10)	−1 (5)	+1 (E-19)	+1 (2)	−1 (120)	1.36 ± 0.02
11	−1 (WB)	+1 (YE)	−1 (OO)	+1 (30)	−1 (10)	+1 (7)	−1 (O-8)	−1 (1)	+1 (160)	4.08 ± 0.08
12	+1 (SB)	+1 (YE)	−1 (OO)	+1 (30)	+1 (30)	−1 (5)	+1 (E-19)	−1 (1)	+1 (160)	0.86 ± 0.01
13	−1 (WB)	−1 (SN)	+1 (SO)	+1 (30)	+1 (30)	−1 (5)	−1 (O-8)	−1 (1)	−1 (120)	0.37 ± 0.24
14	+1 (SB)	−1 (SN)	+1 (SO)	+1 (30)	−1 (10)	+1 (7)	+1 (E-19)	−1 (1)	−1 (120)	1.39 ± 0.06
15	−1 (WB)	+1 (YE)	+1 (SO)	+1 (30)	−1 (10)	−1 (5)	−1 (O-8)	+1 (2)	+1 (160)	3.51 ± 0.54
16	+1 (SB)	+1 (YE)	+1 (SO)	+1 (30)	+1 (30)	+1 (7)	+1 (E-19)	+1 (2)	+1 (160)	1.68 ± 1.00

Exp., experiment; TB, type of bran; NS, nitrogen source; I, inducer; NSC, nitrogen source concentration (g/L); IC, inducer concentration (g/L); ID, inoculum diameter (cm); A, agitation (rpm); WB, wheat bran; SB, soybean bran; SN, sodium nitrate, YE, yeast extract; OO, olive oil; SO, soybean oil; LA_{MR}, maximum residual lipolytic activity.

[a] Mean ± standard deviation.

Table 2 – Coded and real levels of the 2^3 Full Factorial Design and results of maximum residual lipolytic activity obtained from submerged fermentation.

Experiment	X_1 (YEC, g/L)	X_2 (SOC, g/L)	X_3 (pH)	LA_{MR} (U)[a]
17	−1 (10)	−1 (10)	−1 (5.0)	0.23 ± 0.13
18	+1 (30)	−1 (10)	−1 (5.0)	0.89 ± 0.01
19	−1 (10)	+1 (30)	−1 (5.0)	0.00 ± 0.00
20	+1 (30)	+1 (30)	−1 (5.0)	0.95 ± 0.14
21	−1 (10)	−1 (10)	+1 (7.0)	0.84 ± 0.01
22	+1 (30)	−1 (10)	+1 (7.0)	2.00 ± 0.48
23	−1 (10)	+1 (30)	+1 (7.0)	0.85 ± 0.08
24	+1 (30)	+1 (30)	+1 (7.0)	0.95 ± 0.06

YEC, yeast extract concentration (g/L); SOC, soybean oil concentration (g/L); LA_{MR}, maximum residual lipolytic activity; fixed variables, type of bran (wheat bran), fungus (O-8), inoculum diameter (2 cm) and agitation (120 rpm).

[a] Mean ± standard deviation.

Table 3 – Coded and actual values of pH and yeast extract concentration (YEC) used in the Central Composite Rotational Design (CCRD) for the optimization of lipase production by *Aspergillus flavus* (O-8) via submerged fermentation.

Experiment	X_1 (pH)	X_2 (YEC, g/L)	LA_{MR} (U)[a]
25	−1 (6.0)	−1 (20)	1.87 ± 0.12
26	+1 (8.0)	−1 (20)	2.20 ± 0.10
27	−1 (6.0)	+1 (40)	2.98 ± 0.05
28	+1 (8.0)	+1 (40)	2.92 ± 0.12
29	−1414 (5.5)	0 (30)	1.67 ± 0.10
30	+1414 (8.5)	0 (30)	2.11 ± 0.07
31	0 (7.0)	−1414 (15.9)	1.96 ± 0.02
32	0 (7.0)	+1414 (44.1)	2.98 ± 0.05
33	0 (7.0)	0 (30)	2.75 ± 0.02
34	0 (7.0)	0 (30)	2.93 ± 0.10
35	0 (7.0)	0 (30)	3.04 ± 0.02

Fixed variables, type of bran (wheat), fungus (O-8), soybean oil concentration (1%), inoculum diameter (2 cm), agitation (120 rpm); LA_{MR}, maximum residual lipolytic activity.

[a] Mean ± standard deviation.

total of 11 experiments (Trials 25–35). All experiments were carried out in replicates. Lipolytic activity was used as the response in all factorial designs. Tables 1–3 present coded and original values of the Plackett–Burman Design, the 2^3 Factorial Design and the 2^2 CCRD, respectively.

Lipolytic activity determinations

The samples were filtered with cotton wool to remove the hyphae, and the filtrates were used to measure lipolytic activity. Enzymatic activity was determined following the methodology of Burkert et al.,[18] which is based on the NaOH titration of fatty acids released by the action of the lipase enzyme in the enzymatic extract on triacylglycerols of olive oil emulsified in arabic gum.

One unit of lipolytic activity was defined as the amount of enzyme that releases 1 μmol of fatty acid per minute per mL of enzyme extract (1 U = 1 μmol/min/mL) under the test conditions.

Treatment of data

The results of the lipolytic activity over time were subtracted from the values of lipolytic activity obtained at the beginning of fermentation. These activities can be attributed to the presence of free fatty acids in the culture medium before fungal growth and lipase production. The results of maximum residual lipolytic activities obtained were submitted to analysis of variance (Anova). The estimated effects of variables and the regression coefficients of the generated models were calculated.

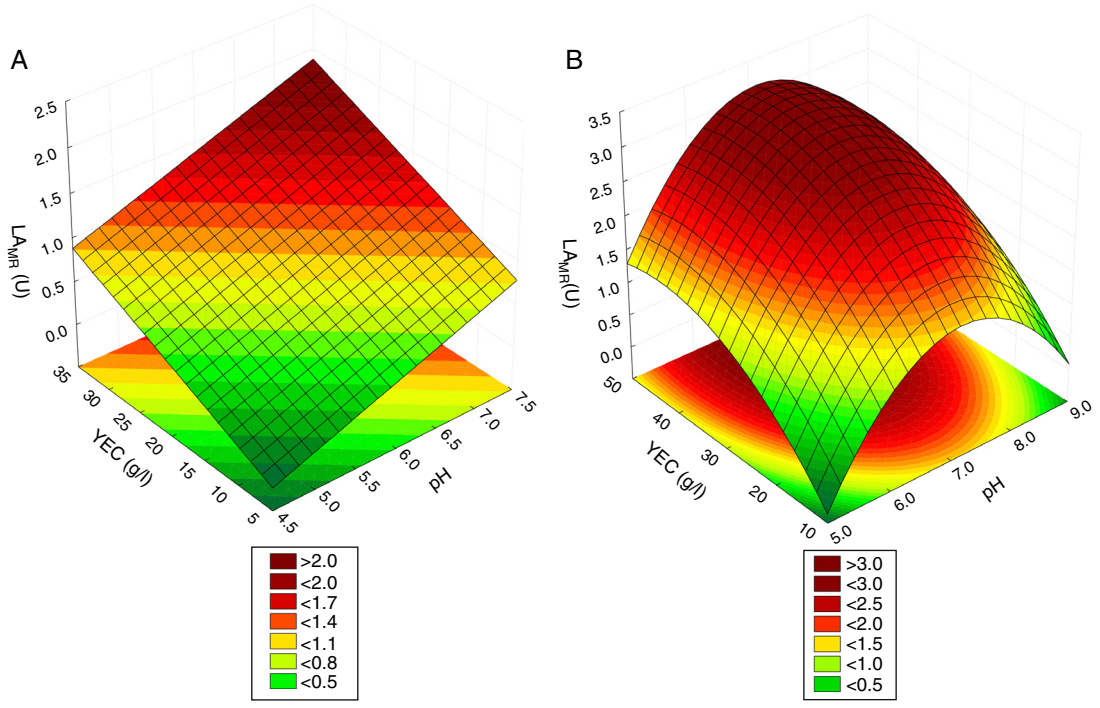

Fig. 1 – Response surface of the maximum residual lipolytic activity (LA_{MR}) as a function of pH and yeast extract concentration (YEC) for lipase production by submerged fermentation according to (A) 2^3 FFD and (B) CCRD.

Results and discussion

Table 1 shows the maximum lipolytic activities obtained in the Plackett–Burman (PB) Design experiments. The maximum lipolytic activities for all experiments were found between the third and fourth days of cultivation. In this way, the values of lipolytic activity obtained at these times of cultivation were used to evaluate the influence of the variables studied in the PB design on lipase production. The highest lipolytic activities were obtained in trials 3, 8, 11 and 15, all of which used yeast extract as the nitrogen source. In relation to the type of bran used as a carbon source, trials 3, 11 and 15 were carried out with wheat bran, and trial 8 was carried out with soybean meal. In trials 3 and 11, olive oil was used as the inducer, and in trials 8 and 15, the inducer was soybean oil. In trials 3, 8 and 11, a pH of 7.0 was used, and pH 5.0 was used in experiment 15. The isolate E-19 (*A. niger*) was used in trial 3; the isolate O-8 (*A. flavus*) was used in trials 4, 11 and 15. Trials 3 and 8 were carried out with an agitation rate of 120 rpm, and trials 11 and 15 were carried out with an agitation rate of 160 rpm.

The results of lipolytic activity obtained in the Plackett–Burman Design trials were evaluated through analysis of variance and showed that the type of bran (TB), inducer (I), inoculum diameter (ID) and agitation had no significant influence ($p > 0.10$) on lipase production through submerged fermentation. Thus, these variables were fixed in the next stages of optimization.

Wheat bran was used in this sequence of statistical optimization of lipase production in submerged fermentation, because it provided the most homogeneous culture medium after boiling. According to Pinto et al.,[27] agro-industrial residues can be recovered and used in fermentative processes. The advantage of there being no difference between the type of bran used is that the bran type can be chosen in accordance with market conditions, such as price, transportation and product availability.

Soybean oil was chosen as the inducer in the optimization sequence because it is cheaper than olive oil. The result was similar to that obtained in the study by Burkert et al.,[18] who assessed the influence of oil type (olive oil and soybean oil) on lipase production by *Geotrichum* sp. and found no significant difference between the results obtained using 1% of the inducers.

Teng and Xu[19] studied the influence of variables on lipase production. Agitation and initial inoculum concentration were significant variables and influenced the morphology of mycelia. The formation of groups of clumps was observed at an agitation rate of 200 rpm, and this type of morphology was determined to be important for lipase synthesis. At agitations lower than 150 rpm, dispersed mycelia were obtained. High initial concentrations of inoculum led to the formation of dispersed mycelia; this formation was a factor that decreased lipase synthesis by *Candida cylindracea*. These results were contradictory to those obtained by Haack et al.,[1] who reported that the presence of dispersed hyphae, rather than *clumps* or *pellets*, facilitates the synthesis of lipase by strains of filamentous fungi because the other morphological forms cause diffusion problems for the substrate and product. However, the presence of dispersed hyphae causes increased viscosity of the medium, which reduces oxygen transfer. In our study, the presence of pellets was not observed, but there was an increase in the visual viscosity of the media during the fermentation process, which characterizes the presence of dispersed hyphae.

Table 4 – Analysis of variance of the maximum residual lipolytic activity results of the CCRD experiments.

Effect	SS	DF	MS	$F_{calculated}$	$F_{critical}$
Regression	5.137	4	1.284	33.077	2.965
Error	0.660	17	0.039		
Total	5.797	21			

SS, sum of squares; DF, degrees of freedom; MS, mean square. The effect of interaction, which was not significant, was ignored in the implementation of analysis of variance.

The production of lipases was not affected by agitation or by the initial inoculum concentration, as indicated by the analysis of variance of the PB design experiments ($p > 0.10$). Thus, in subsequent experiments, the inoculation was carried out using a 20 mm diameter of fungal growth after preparing the inoculum and using an agitation rate of 120 rpm.

The nitrogen source (NS) and fungus strain were significant ($p < 0.001$ and $p = 0.012$, respectively), having the estimated effects of 1.15 and −0.68, respectively. This means that lipase activity increased approximately 1.15 U by varying the nitrogen source from the lowest (sodium nitrate) to the highest level (yeast extract) of the PB design. However, after varying the level of the variable fungus from the lowest (*A. flavus*) to the highest level (*A. niger*), a decrease of 0.68 U was observed in the lipolytic activity. Thus, yeast extract (nitrogen source used at the top level) and the fungus O-8 (*A. flavus*, corresponding to the lowest level) were used throughout the experiments.

Quantitative variables (nitrogen source concentration: NSC, soybean oil concentration: SOC, and pH) that showed a significant influence on lipase production at a 10%level of significance were studied using a 2^3 Full Factorial Design; the results are shown in Table 2.

The maximum lipolytic activity in the 2^3 FFD (2.00 U) was obtained in trial 22, carried out with 30 g/L of yeast extract and 10 g/L of soybean oil at pH 7. Comparing the maximum lipolytic activities obtained in the upper and lower concentrations of yeast extract, it seems that increasing the concentration from 10 g/L to 30 g/L caused an increase in lipolytic activities. Increasing the soybean oil concentration from 10 g/L to 30 g/L caused a decrease in lipolytic activity when comparing trial 17 with 19 and 22 with 24 and caused an increase when comparing trials 18 and 20 and trials 21 and 23. This seemingly contradictory behavior could be explained by alias effects, which are common in PB designs. However, the statistical analysis of the results will better explain if the effect of this variable on lipase production is significant. When assessing the effect of pH, the best results were obtained at pH 7.0.

The YEC and the pH were significant for lipolytic activity according to the analysis of the variance of the data, with levels of significance (p) less than 0.001 for both variables. The effects of these variables were positive, 0.71 for yeast extract and 0.64 for pH. This result indicates that the greatest increases in lipolytic activities were obtained using higher levels of these variables, i.e., 30 g/L of yeast extract and pH 7.0. The concentration of soybean oil and the interactions between the variables had no significant effect on lipolytic activity ($p < 0.05$).

The mathematical model of maximum lipolytic activity presented a determination coefficient (R^2) of 0.714 and an $R^2_{adjusted}$ of 0.669, as shown in Eq. (1) (X_1: yeast extract concentration, X_3: pH). The F value for regression was 16.22, and the $F_{critical}$ was 3.80, indicating that most of the variability of the model was caused by regression, which statistically validates the mathematical model. Fig. 1(A) shows the response surface of the maximum residual lipolytic activity as a function of pH and yeast extract concentration;

Table 5 – Estimated effects of variables used in the CCRD and significance levels.

Effect	Estimated effect	t-Test	p
Mean	2.922	38.204	<0.001
X_1 (L)	0.225	2.404	0.035
X_1 (Q)	−0.867	−7.775	<0.001
X_2 (L)	0.838	8.947	<0.001
X_2 (Q)	−0.278	−3.002	0.029

X_1, pH; X_2, yeast extract concentration; L, linear effect; Q, quadratic effect; p, significance level.

$$LA_{MR} = 0.839 + 0.357X_1 + 0.320X_3 \quad (1)$$

The significant variables in the 2^3 FFD (pH and yeast extract concentration) were optimized using a CCRD, being the matrix design and the results of maximum lipolytic activity presented in Table 3. As the effects of these variables were positive in the 2^3 FFD, their levels were increased in the CCRD. The highest lipolytic activities in the CCRD experiments were obtained in 4 days of fermentation, and these results were used for the analysis of variance. The lipolytic activity results at 4 days of fermentation were subtracted from the lipolytic activity values at the initial time.

The analysis of variance of maximum residual lipolytic activity obtained in the CCRD trials, presented in Table 4, showed that the $F_{calculated}$ value for the regression was 33.08 while the $F_{critical}$ value ($p = 0.05$, degrees of freedom for regression: 4; degrees of freedom for residual: 17) was 2.96, validating the surface response obtained shown in Fig. 1(B). The mathematical model generated is shown in Eq. (2), and a coefficient of determination (R^2) of 0.89 and an adjusted coefficient of determination ($R^2_{adjusted}$) 0.86 are presented.

The pH and yeast extract concentration had significant linear and quadratic effects (Table 5). The linear effects of both variables were positive, with a greater effect caused by the concentration of the yeast extract (0.838 U). The quadratic effects of both variables were negative, indicating the presence of points of maximum activity for both variables, as shown in

the surface response presented in Fig. 1(B):

$$LA_{MR} = 2.922 + 0.113pH - 0.433pH^2 + 0.419YEC - 0.139YEC^2 \quad (2)$$

The optimum pH levels and yeast extract concentration to obtain maximum lipolytic activity were also carried out by equaling the first derivative of lipolytic activity as a function of pH and YEC to zero. The maximum lipolytic activities were obtained at a pH level of +0.154 and a YEC level of +1.507, which corresponds to a pH of 7.15 and a yeast extract concentration of 45 g/L.

Several studies have shown the importance of a nitrogen source in lipase production. Sharma et al.[5] reviewed the conditions of cultivation to obtain maximum lipolytic fungal activities and reported the use of many nitrogen sources, such as yeast extract, peptone, soybean extract, ammonium chloride, and amino acids, among others.

The sources of organic nitrogen have been used for the production of lipases by fungi, as reported by several authors. Peptone was used by Kaushik et al.[17] to produce lipases in submerged fermentation using *Aspergillus carneus* and by Teng and Xu[19] and Wang et al.[20] using *Rhizopus chinensis*. Yeast extract and peptone were used in combination by Mahadik et al.[14] for the production of lipase by *A. niger*. Miranda et al.[28] evaluated the production of lipases using oil refinery waste in the presence of nitrogen sources such as ammonium sulfate, urea and ammonium chloride, with the best results being obtained using ammonium chloride. For lipase production by *Antrodia cinnamomia*, Lin et al.[29] used organic and inorganic sources of nitrogen, including ammonium and nitrate salts, proteins, peptides and amino acids, and higher activities were obtained with sodium and potassium nitrates, ammonium chloride and asparagine. In our study, yeast extract generated higher lipolytic activities compared to sodium nitrate, a result that could be explained because yeast extract contains other components besides a nitrogen source that can act as co-enzymes of the aerobic metabolic pathway, for example, carbon skeletons and complex B vitamins.

Several authors consider pH to be an important variable in the production of lipase by submerged fermentation. Muralidhar et al.[30] used an initial pH of 6.5 in the culture of *C. cylindracea*. Teng and Xu[19] studied the effect of pH on the production of lipases by *Rhyzopus chinensis* between pH 5 and 7, and the best results were achieved at pH 5.5. In this study, pH proved to be an important variable for the production of lipases, with the optimal value being approximately 7.0. This result is in agreement with Gopinath et al.,[31] who studied pH 4, 7 and 10 in the production of lipase by *Geotrichum candidum*. Among the factors studied by Wang et al.[20] for the production of lipases by *R. chinensis*, pH was the variable that had a lower effect on production. Each microorganism has a pH for optimal growth. In this case, pH could have affected lipase synthesis beyond microorganism growth.

By evaluating the values of lipolytic activity reached during the experimental designs, *A. flavus* showed maximum lipolytic activities of 4 U in the Plackett–Burman Design and 2 U in the 2^3 Full Factorial Design, which can be attributed to the loss of the fungi'sability to produce lipases. According to Makhsumkhanov et al.,[32] this result may be related to the depletion of the maintenance resources during storage. Furthermore, there may be an accumulation of certain metabolites, decreasing the culture's productivity. The loss of the fungi's ability can be solved during periods of maintenance by using reactivation media containing vegetable oil as the sole assimilable carbon source.[32] However, the maximum lipolytic activity obtained in the CCRD was 3.04 U, while the value predicted by the statistical model for the highest lipolytic activity of the fungus O-8 was 3.24 U. In experiments carried out previously for the selection of lipase-producing fungi by submerged fermentation, the fungus *A. flavus* had a lipolytic activity of approximately 2 U for 4 days of cultivation, and the lipolytic activity at baseline was 1 U, meaning 1 U of enzyme was produced during this time. Compared with the amount of lipolytic activity obtained after optimization, there was an increase, which means that the optimization of variables was successfully performed.

Conclusion

The optimization of lipase production in submerged fermentation was possible through the use of sequential experimental design. The optimized conditions were obtained using the fungus *A. flavus*, 100 g/L wheat bran as the main component of the culture medium, 45 g/L yeast extract as the nitrogen source, 20 g/L soybean oil as the inducer and pH 7.15. The optimization of pH and yeast extract concentration using a central composite design led to a mathematical model with a correlation coefficient of 94.12% with the experimental data.

Conflicts of interest

The authors declare no conflicts of interest.

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