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Response Surface Analysis for the Production of an Enantioselective Lipase from *Aspergillus niger* by Solid-State Fermentation

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The lipase produced by the *Aspergillus niger* strain AC-54 has been widely studied due to its enantioselectivity for racemic mixtures. This study aimed to optimize the production of this enzyme using statistical methodology. Initially a Plackett-Burman (PB) design was used to evaluate the effects of the culture medium components and the culture conditions. Twelve factors were screened: water content, glucose, yeast extract, peptone, olive oil, temperature, NaH_2PO_4, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2$, NaCl, and $MnSO_4$. The screening showed that the significant factors were water content, glucose, yeast extract, peptone, NaH_2PO_4, and KH_2PO_4, which were optimized using response surface methodology (RSM) and a mathematical model obtained to explain the behavioral process. The best lipase activity was attained using the following conditions: water content (20%), glucose (4.8%), yeast extract (4.0%), and NaH_2PO_4 (4.0%). The predicted lipase activity was 33.03 U/ml and the experimental data confirmed the validity of the model. The enzymatic activity was expressed as μmoles of oleic acid released per minute of reaction (μmol/min).

***Keywords*:** lipase, response surface methodology, *A. niger*

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) catalyze the hydrolysis of triacylglycerol into mono- and di-acyl glycerols, fatty acids, and glycerol at an oil-water interface, however, under certain conditions, they are also able to catalyze synthetic reactions such as esterification in an organic medium (Contesini and Carvalho, 2006). Currently lipases are a popular choice for a biocatalyst since they show unique chemo-, regio-, enantioselectivity which make it possible to produce enantiopure drugs and other refined products (Houng *et al*., 1996).

Fungi are recognized as the best lipase producers and are currently the preferred option, especially *Aspergillus niger*, which is superior to several other microorganisms and produces lipases suitable for many applications (Fu *et al*., 1995). Studies on the production of lipases from this fungi have shown variations amongst different strains, although the requirement for a lipid carbon source remains essential for enzyme production (Folony *et al*., 2006).

The solid state fermentation (SSF) system involves the growth of microorganisms on moist solids without any free flowing water. It has been reported that SSF with fungal strains results in greater productivity than submerged fermentation (Hesseltine, 1972). Of the different solid substrates available for SSF such as gingelly oil cake (Kamini *et al*., 1998) and soybean meal (Vargas *et al.*, 2008), wheat bran, which was used in the present study, is cheap and easy to obtain.

The authors of the present study previously reported that a lipase from *Aspergillus niger* AC-54 had several properties of great industrial importance such as good stability and the ability to preferentially esterify (*R*)-ibuprofen, providing better results for enantioselectivity than other native lipases (Carvalho *et al*., 2005). To improve the enantioselectivity of this enzyme for (*R*)-ibuprofen, the same research group reported the optimization of the reaction parameters (enzyme concentration and molar ratio of propanol: ibuprofen) that affected the enantioselective resolution of (*R*,*S*)-ibuprofen by this lipase (Carvalho *et al*., 2006a) and the use of ionic liquids as the reaction medium (Contesini and Carvalho, 2006).

Considering the immense potential of this *A. niger* lipase, it was worthwhile to optimize the production of this enzyme for maximum yields in an economically viable manner. The usual method used to determine the optimal conditions of these processes is to vary one parameter while keeping the others constant, but such methods are time consuming and cost ineffective and show the additional disadvantage of not including the effects of interactions amongst the variables. To overcome this difficulty, Plackett-Burman (PB) produced designs that require fewer runs than a comparable fractional design, and can be used to identify the more important independent variables from a long list and select them to realize a complete factorial design. Finally, a central composite design (CCD) and response surface methodology (RSM) are used to identify the relationships between the variables and the response, generally resulting in higher production

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Table 1. Experimental range and levels of the independent variables used in the screening design (PB) in terms of actual and coded values

Variables	Coded level		
	-1	0	+1
Medium composition			
Water content (%, m/m)	36	52	68
Glucose (%, m/m)	0.16	1.68	3.2
Yeast extract (%, m/m)	0.16	1.68	3.2
Peptone (%, m/m)	0.16	1.68	3.2
Olive oil (%, m/m)	0.16	1.68	3.2
NaH_2PO_4 (%, m/m)	0.32	1.6	2.88
KH_2PO_4 (%, m/m)	0.08	0.72	1.36
$MgSO_4{\cdot}7H_2O$ (%, m/m)	0.008	0.04	0.072
$CaCl_2$ (%, m/m)	0.008	0.04	0.072
NaCl (%, m/m)	0.4	1.6	2.8
$MnSO_4$ (%, m/m)	0.008	0.04	0.072
Culture conditions			
Temperature (°C)	23	30	37

yields and simultaneously limiting the number of experiments. In addition, the best condition for each variable is obtainable by a differential approximation. RSM has been widely used for the optimization of lipase production (Elibol and Ozer, 2002; Rajendran and Thangavelu, 2007).

Although many studies reported on the use of SSF and RSM in fungal systems, no reports on lipase production by *A. niger* or the optimized production of an enantioselective lipase using this statistical methodology were found. Thus a response surface methodology approach was used in the present study to optimize the medium constituents for lipase production. A PB design was initially used to screen for significant factors amongst twelve variables in the production of lipase by *A. niger* AC-54 in SSF, such as the carbon source (wheat bran, glucose, and olive oil), nitrogen source (peptone and yeast extract), inorganic compounds (NaH_2PO_4, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2$, NaCl, and $MnSO_4$), water and temperature, followed by a CCD to optimize the process.

Materials and Methods

Microorganism and medium

A. niger AC-54 was obtained from the culture collection of the Biotechnology Laboratory of the Universidade São Francisco (Brazil). It was grown on Sabouraud Agar at 30°C for 4 days and the inoculum was a spore suspension in sterile distilled water ($10^5 \sim 10^6$ spores/ml), adding 10 ml of the suspension to each 250 ml flask containing the production medium. Forty grams of medium were used in each trial and the components and culture conditions varied according to the experimental design (Table 1 and 3). Wheat bran was used as the solid state substrate in all the trials, and the water content was considered as an independent variable, instead of the wheat bran : water content ratio. The lowest value for water content was defined as the minimum percentage possible considering the inoculum (10 ml of distilled water), since the volume of water in the inoculum added to each flask was considered in the final water content.

Chemicals

The yeast extract and Bacto peptone were purchased from Difco Laboratories (USA). The other components in the

Table 2. Plackett-Burman screening design matrix (PB-16) and the enzyme activity after 96 of fermentation

Run	Variables and levels												Response
	WC[a]	GLU[b]	YEX[c]	PEP[d]	OLI[e]	T[f]	NaH_2PO_4	KH_2PO_4	$MgSO_4{\cdot}7H_2O$	$CaCl_2$	NaCl	$MnSO_4$	Lipase activity, 96 h (U/ml)
1	+1	-1	-1	-1	+1	-1	-1	+1	+1	-1	+1	-1	15.03
2	+1	+1	-1	-1	-1	+1	-1	-1	+1	+1	-1	+1	10.81
3	+1	+1	+1	-1	-1	-1	+1	-1	-1	+1	+1	-1	23.46
4	+1	+1	+1	+1	-1	-1	-1	+1	-1	-1	+1	+1	19.77
5	-1	+1	+1	+1	+1	-1	-1	-1	+1	-1	-1	+1	18.46
6	+1	-1	+1	+1	+1	+1	-1	-1	-1	+1	-1	-1	12.13
7	-1	+1	-1	+1	+1	+1	+1	-1	-1	-1	+1	-1	28.47
8	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	-1	+1	30.05
9	+1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	-1	32.68
10	-1	+1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	35.59
11	-1	-1	+1	+1	-1	+1	-1	+1	+1	+1	+1	-1	23.73
12	+1	-1	-1	+1	+1	-1	+1	-1	+1	+1	+1	+1	24.25
13	-1	+1	-1	-1	+1	+1	-1	+1	-1	+1	+1	+1	21.09
14	-1	-1	+1	-1	-1	+1	+1	-1	+1	-1	+1	+1	23.99
15	-1	-1	-1	1	-1	-1	+1	+1	-1	+1	-1	+1	27.94
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	12.40
17	0	0	0	0	0	0	0	0	0	0	0	0	21.09
18	0	0	0	0	0	0	0	0	0	0	0	0	21.09
19	0	0	0	0	0	0	0	0	0	0	0	0	20.82
20	0	0	0	0	0	0	0	0	0	0	0	0	20.82

[a] Water content; [b] glucose; [c] yeast extract; [d] peptone; [e] olive oil; [f] temperature

Table 3. Experimental range and levels for the six significant independent variables used in the response surface methodology in terms of actual and coded values

Variables	Coded variables	Coded level				
		-2.83 (-α)	-1	0	+1	+2.83 (+α)
Medium composition						
Water content (%, m/m)	x_1	20.0	40.7	52.0	63.3	84.0
Glucose (%, m/m)	x_2	0.0	1.52	2.4	3.28	4.8
Yeast extract (%, m/m)	x_3	0.0	1.28	2.0	2.72	4.0
Peptone (%, m/m)	x_4	0.0	1.28	2.0	2.72	4.0
NaH_2PO_4 (%, m/m)	x_5	0.0	1.28	2.0	2.72	4.0
KH_2PO_4 (%, m/m)	x_6	0.0	1.04	1.6	2.16	3.2

Table 4. Central composite design matrix (2^6); lipase activity after 96 h of fermentation

Run	Variables and levels						Response
	WC[a]	GLU[b]	YEX[c]	PEP[d]	NaH_2PO_4	KH_2PO_4	Lipase activity (U/ml)
1	-1	-1	-1	-1	-1	-1	21.62
2	+1	-1	-1	-1	-1	-1	15.82
3	-1	+1	-1	-1	-1	-1	19.51
4	+1	+1	-1	-1	-1	-1	16.35
5	-1	-1	+1	-1	-1	-1	16.88
6	+1	-1	+1	-1	-1	-1	15.03
7	-1	+1	+1	-1	-1	-1	21.75
8	+1	+1	+1	-1	-1	-1	13.97
9	-1	-1	-1	+1	-1	-1	21.62
10	+1	-1	-1	+1	-1	-1	15.82
11	-1	+1	-1	+1	-1	-1	20.03
12	+1	+1	-1	+1	-1	-1	12.92
13	-1	-1	+1	+1	-1	-1	19.24
14	+1	-1	+1	+1	-1	-1	16.87
15	-1	+1	+1	+1	-1	-1	22.41
16	+1	+1	+1	+1	-1	-1	20.56
17	-1	-1	-1	-1	+1	-1	25.30
18	+1	-1	-1	-1	+1	-1	21.88
19	-1	+1	-1	-1	+1	-1	25.04
20	+1	+1	-1	-1	+1	-1	20.03
21	-1	-1	+1	-1	+1	-1	27.15
22	+1	-1	+1	-1	+1	-1	22.67
23	-1	+1	+1	-1	+1	-1	25.04
24	+1	+1	+1	-1	+1	-1	23.72
25	-1	-1	-1	+1	+1	-1	26.10
26	+1	-1	-1	+1	+1	-1	21.62
27	-1	+1	-1	+1	+1	-1	27.68
28	+1	+1	-1	+1	+1	-1	24.78
29	-1	-1	+1	+1	+1	-1	27.15
30	+1	-1	+1	+1	+1	-1	22.67
31	-1	+1	+1	+1	+1	-1	28.99
32	+1	+1	+1	+1	+1	-1	23.20
33	-1	-1	-1	-1	-1	+1	23.20
34	+1	-1	-1	-1	-1	+1	18.45
35	-1	+1	-1	-1	-1	+1	22.67
36	+1	+1	-1	-1	-1	+1	18.98
37	-1	-1	+1	-1	-1	+1	23.46
38	+1	-1	+1	-1	-1	+1	18.98
39	-1	+1	+1	-1	-1	+1	22.67
40	+1	+1	+1	-1	-1	+1	22.41
41	-1	-1	-1	+1	-1	+1	20.56
42	+1	-1	-1	+1	-1	+1	16.34

Table 4. Continued

Run	Variables and levels						Response
	WC[a]	GLU[b]	YEX[c]	PEP[d]	NaH_2PO_4	KH_2PO_4	Lipase activity (U/ml)
43	-1	+1	-1	+1	-1	+1	21.35
44	+1	+1	-1	+1	-1	+1	18.45
45	-1	-1	+1	+1	-1	+1	18.98
46	+1	-1	+1	+1	-1	+1	18.32
47	-1	+1	+1	+1	-1	+1	23.20
48	+1	+1	+1	+1	-1	+1	18.72
49	-1	-1	-1	-1	+1	+1	23.99
50	+1	-1	-1	-1	+1	+1	21.88
51	-1	+1	-1	-1	+1	+1	26.36
52	+1	+1	-1	-1	+1	+1	23.20
53	-1	-1	+1	-1	+1	+1	29.52
54	+1	-1	+1	-1	+1	+1	20.03
55	-1	+1	+1	-1	+1	+1	27.41
56	+1	+1	+1	-1	+1	+1	22.93
57	-1	-1	-1	+1	+1	+1	26.36
58	+1	-1	-1	+1	+1	+1	20.30
59	-1	+1	-1	+1	+1	+1	27.28
60	+1	+1	-1	+1	+1	+1	21.35
61	-1	-1	+1	+1	+1	+1	26.89
62	+1	-1	+1	+1	+1	+1	22.41
63	-1	+1	+1	+1	+1	+1	27.94
64	+1	+1	+1	+1	+1	+1	23.99
65	-2,83	0	0	0	0	0	21.48
66	+2,83	0	0	0	0	0	14.76
67	0	-2,83	0	0	0	0	19.24
68	0	+2,83	0	0	0	0	23.46
69	0	0	-2,83	0	0	0	18.45
70	0	0	+2,83	0	0	0	21.35
71	0	0	0	-2,83	0	0	18.98
72	0	0	0	+2,83	0	0	22.14
73	0	0	0	0	-2,83	0	13.45
74	0	0	0	0	+2,83	0	27.41
75	0	0	0	0	0	-2,83	16.34
76	0	0	0	0	0	+2,83	25.57
77	0	0	0	0	0	0	21.09
78	0	0	0	0	0	0	21.35
79	0	0	0	0	0	0	20.03
80	0	0	0	0	0	0	22.14

[a] Water content; [b] glucose; [c] yeast extract; [d] peptone

culture media, chemical reagents and the other solvents were obtained from Merck (Germany) and from Sigma-Aldrich Chemical Co (USA), using the highest purity available. Low acidity olive oil (Carbonell, Spain) was purchased at a local market.

Enzymatic assays

The hydrolytic activity of the lipase was determined at 35°C and pH 7.0, by titration of the free fatty acids with 0.05 N KOH released from olive oil hydrolysis (Thomson *et al*., 1999). The enzymatic activity was expressed as μmoles of oleic acid released per minute of reaction (μmol/min).

Optimization experiments

Two successive designs were carried out. The first was a screening design to evaluate the medium composition and the culture conditions on the response (lipase activity). The most important variables were then selected for further optimization using RSM.

Plackett-Burman (PB) design

The influence of twelve variables on the production of *A. niger* lipase was investigated using PB methodology (Table 1 and 2). Each independent variable was tested at two levels, a high (+1) and a low (-1) one. This screening is of particular importance when large numbers of factors are considered for optimization (Plackett and Burman, 1946). Sixteen experiments were carried out with four center points at the mid-level of each variable, to estimate the experimental error (Table 2). The statistical analyses were performed using

Table 5. Main effects of the analysis for lipase activity from the PB design after 96 h of fermentation

Factor	Effect	Std. error	t-value	p-value
Mean interaction[a]	22.19	0.420	52.774	0.00000[a]
Water[a]	-2.93	0.940	-3.119	0.016875[a]
Glucose[a]	2.60	0.940	2.768	0.027763[a]
Yeast extract[a]	1.81	0.940	1.927	0.095304[a]
Peptone[a]	1.88	0.940	1.927	0.085954[a]
Olive Oil	1.28	0.940	1.367	0.214019
Temperature	0.76	0.940	0.806	0.446781
Monobasic sodium phosphate[a]	11.63	0.940	12.370	0.000005[a]
Monobasic potassium phosphate[a]	6.49	0.940	6.903	0.000231[a]
Magnesium sulfate	1.15	0.940	1.226	0.259680
Calcium chloride	-0.23	0.940	-0.245	0.813260
Sodium chloride	-0.03	0.940	-0.035	0.973025
Manganese sulfate	-0.89	0.940	-0.946	0.375595

[a] Statistically significant effects

Statistica® software (version 5.1) to determine the effect, *p* values and confidence levels. Variables with a confidence level greater than 90% were considered to have a significant influence on lipase production. The exclusion process of the not significant variables was based on a confidence level of 90% to avoid the elimination of factors with some importance that would be eliminated if used a higher confidence level. The PB experimental design was based on a first-order model with no interaction amongst the factors.

Central composite design (CCD) and response surface methodology (RSM)

The parameters selected in the PB design were then further investigated to define the optimal process conditions (Table 3 and 4). The central points and amplitude parameters (Table 3) were chosen based on the previous results to cover the possible optimum region. A 2^6 full factorial design with 2 axial points (+\–) at a distance of α=2.83 was used, with four replicates at the center point. A total of 80 runs were performed to study the selected variables (Table 4). The olive oil concentration and temperature were maintained constant at 0.16% and 25°C, respectively.

The variables were coded according to Equation 1:

$$x_i = \frac{X_i - X_c}{\Delta X_i} \quad i = 1, 2, 3, ..., k \quad \text{(Equation 1)}$$

Where x_i is the dimensionless coded value of an independent variable, X_i is the real value of an independent variable, X_c is the real value of an independent variable (X_i) at the center point and ΔX_i is the step change value. The system behavior was determined by a second-order polynomial equation, based on the equation below (Equation 2):

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \quad \text{(Equation 2)}$$

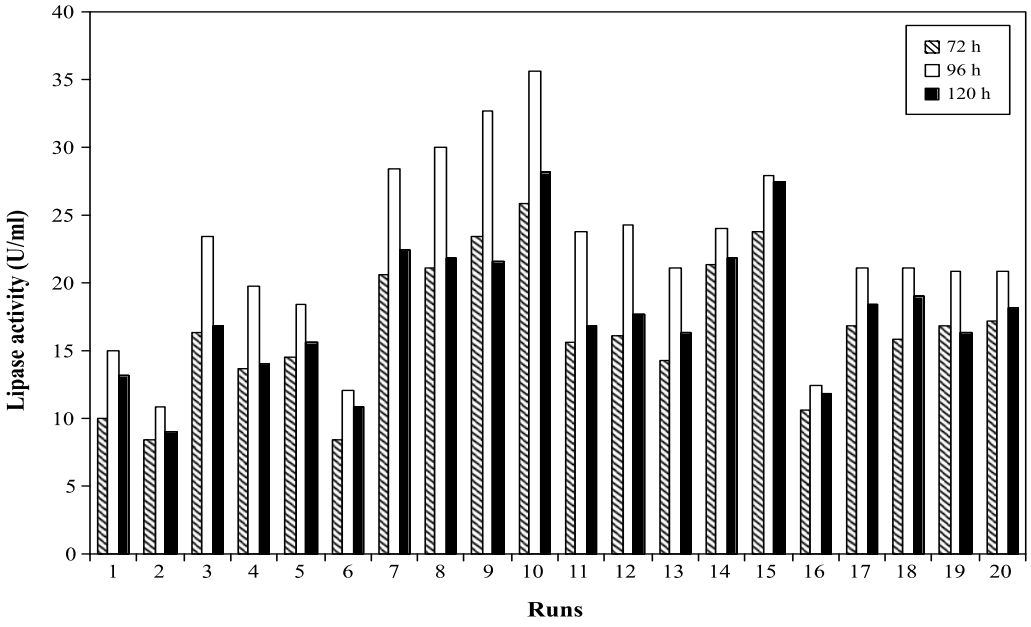

Fig. 1. Lipase activity as a function of time for the PB design.

Where Y is the predicted value for the response, β_0 is the offset term, β_i is the linear effect coefficient, β_{ii} is the squared effect coefficient and β_{ij} is the interaction effect. The x_ix_j represents the interaction between different coded values, where i is one parameter and j is other. The regression coefficient, *p* values and confidence level were determined using Statistica® (version 5.1). Variables with a confidence level greater than 95% were considered to have a significant influence on lipase production.

Validation of the experimental model

The experimental model was validated by carrying out the fermentation process with the statistically significant variables at their optimal concentrations, in the best time, with average of three repetitions.

Results and Discussion

Placket-Burman design

The experimental results for lipase production using the PB desigh are shown in Table 5. The effects of the six factors considered to be statistically significant ($P<0.1$) at the 90% confidence level: water content, glucose, yeast extract, peptone, monobasic sodium phosphate, and monobasic potassium phosphate were calculated to be -2.93, 2.60, 1.81, 1.88, 11.63, and 6.49, respectively. Based on this, it can be observed that the two salts showed the most positive effects, indicating that they were really important for high lipase activity while it was important to maintain the water content low because it had a negative effect. The activity of the *A. niger* lipase was observed to be strongly influenced by the studied parameters, varying from 10.81 to 35.59 U/ml in the 20 experiments (Table 2). There were great variations in lipase activity depending on the medium composition, but not on the culture conditions (temperature). The variables temperature, olive oil, $MgSO_4 \cdot 7H_2O$, CaCl, NaCl, and $MnSO_4$ had no significant effect on the production of *A. niger* lipase up to 96 h, and therefore were of no significant interest (at $p<0.1$) to the process.

All the experiments were carried out for 72 h, 96 h, and 120 h (Fig. 1). Maximum activity was reached after 96 h for all assays, and after this, a decrease in activity occurred as previously described in the literature (Pokorny *et al*., 1997). Other published studies reported 72 h as the optimal fermentation time for *A. niger* lipase grown in solid- state fermentation, with a subsequent decrease in activity seen at 96 h and 120 h (Kamini *et al*., 1998; Mala *et al*., 2007). On the other hand, Mahadik *et al.* (2002) reported that the optimal time for *A. niger* lipase production was 120 h, with the activity decreasing only after this time.

Table 6. Coefficient regressions for lipase activity from the central composite design after 96 h of fermentation

Variable	Coded variables	Regression coefficient	Standard error	t(3)	p
Mean/Interc.[a]		22.3235	0.3891	57.3759	0.0000[a]
1-WC (L) (Linear)[a,b]	x_1	-1.8959	0.0973	-19.4774	0.0003[a]
1-WC (Q) (Quadratic)[a,b]	$(x_1)^2$	-0.3791	0.0942	-4.0259	0.0275[a]
2-GLU (L)[a,c]	x_2	0.4966	0.0973	5.1016	0.0146[a]
2-GLU (Q)[c]	$(x_2)^2$	0.0240	0.0942	0.2553	0.8150
3-YEX (L)[a,d]	x_3	0.4566	0.0973	4.6905	0.0183[a]
3-YEX (Q)[d]	$(x_3)^2$	-0.1569	0.0942	-1.6669	0.1941
4-PEP (L)[e]	x_4	0.1892	0.0973	1.9441	0.1471
4-PEP (Q)[e]	$(x_4)^2$	-0.0747	0.0942	-0.7932	0.4856
5-NaH_2PO_4 (L)[a]	x_5	2.5903	0.0973	26.6106	0.0001[a]
5-NaH_2PO_4 (Q)	$(x_5)^2$	-0.0911	0.0942	-0.9679	0.4045
6-KH_2PO_4 (L)[a]	x_6	0.7659	0.0973	7.8682	0.0043[a]
6-KH_2PO_4 (Q)	$(x_6)^2$	-0.0253	0.0942	-0.2690	0.8054
1L by 2L	x_1x_2	0.0803	0.1088	0.7377	0.5141
1L by 3L	x_1x_3	0.1297	0.1088	1.1917	0.3191
1L by 4L	x_1x_4	-0.0350	0.1088	-0.3216	0.7689
1L by 5L	x_1x_5	-0.1626	0.1088	-1.4944	0.2319
1L by 6L	x_1x_6	0.0391	0.1088	0.3594	0.7431
2L by 3L	x_2x_3	0.2738	0.1088	2.5159	0.0865
2L by 4L	x_2x_4	0.2409	0.1088	2.2132	0.1138
2L by 5L	x_2x_5	-0.0268	0.1088	-0.2459	0.8216
2L by 6L	x_2x_6	0.1668	0.1088	1.5322	0.2230
3L by 4L	x_3x_4	0.1503	0.1088	1.3809	0.2612
3L by 5L	x_3x_5	0.1379	0.1088	1.2674	0.2945
3L by 6L	x_3x_6	0.0926	0.1088	0.8512	0.4572
4L by 5L	x_4x_5	0.2944	0.1088	2.7050	0.0735
4L by 6L [a]	x_4x_6	-0.5250	0.1088	-4.8236	0.0170[a]
5L by 6L [a]	x_5x_6	-0.5868	0.1088	-5.3911	0.0125[a]

[a] Statistically significant effects
[b] Water content; [c] glucose; [d] yeast extract; [e] peptone

Table 7. ANOVA for the central composite design after 96 h of fermentation

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-value
Regression	959.95	8	119.99	46.02
Residual	185.14	71	2.61	
Lack of fit	182.86	68	2.69	
Pure error	2.27	3	0.76	
Total	1145.09	79		

Regression coefficient: $R^2=0.8383$/ $F_{0.05;8;71}=2.1$

The water content was also important, with increasing amounts leading to an appreciable decrease in lipase activity (Table 2). In addition, in SSF, the water content had a great impact on the physical properties of the solid substrate (Pokorny *et al*., 1997). Higher than optimum water levels decreased porosity, lowered oxygen transfer, and altered the wheat bran particle structure (Pandey, 1992). Likewise, a lower than optimum water content decreased the solubility of the solid substrate, lowered the degree of swelling and produced a high water tension (Mahadik *et al*., 2002). Optimal activity of the lipase from *A. niger* was reported to be obtained when 60% water was used (Kamini *et al*., 1998; Mala *et al*., 2007).

Glucose was an important factor in improving lipase production, possibly due the rapid assimilation of this carbohydrate by the cells compared to lipids, leading to a faster growth rate and higher lipase activity (Macris *et al*., 1996), which is in agreement with Mahadik *et al.* (2002). Macris *et al.* (1996) studied the production of *A. niger* lipase and found greater activity when the carbon source was a mixture of olive oil and sucrose at 1.5 and 0.5% (w/w), respectively.

Yeast extract and peptone are important nitrogen sources in this process and showed a great influence. This results is in agreement with the literature datas. Hatzinikolaou *et al.* (1996) reported that peptone as nutrient source combined with corn oil yielded the highest lipase activity (40.5 U/ml).

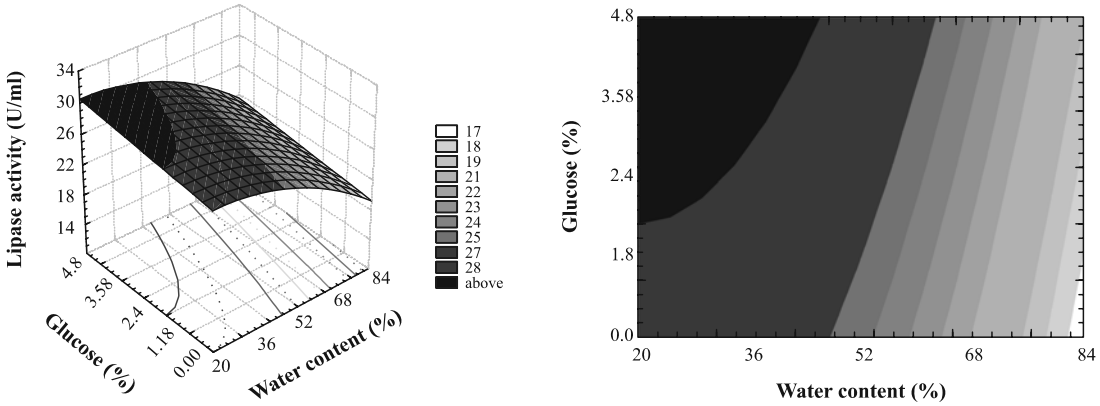

Fig. 2. Response surface and contour plot for lipase activity as a function of moisture content and glucose. All other factors were fixed at the central points.

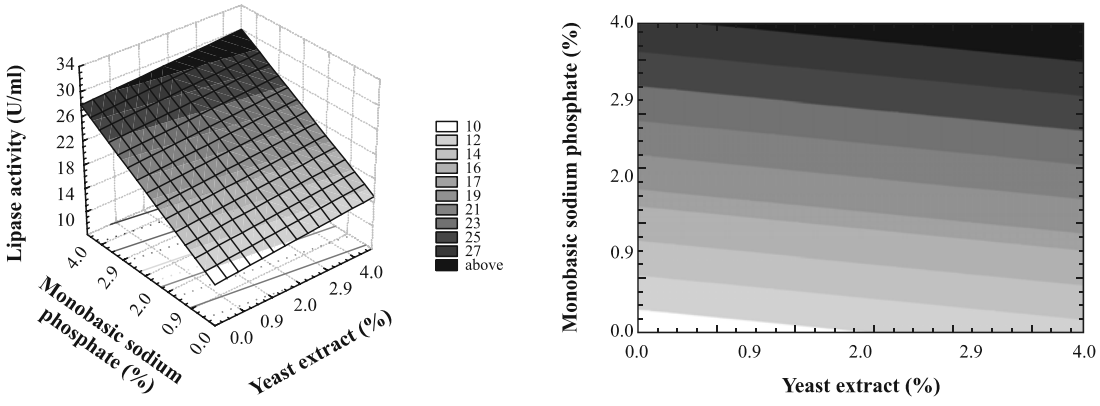

Fig. 3. Response surface and contour plot for the lipase activity as a function of yeast extract and NaH_2PO_4. All other factors were fixed at the central points.

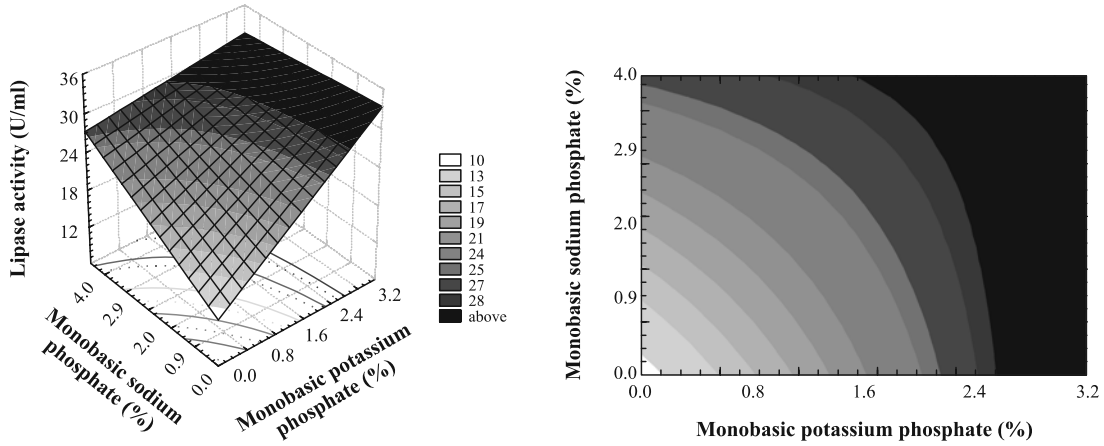

Fig. 4. Response surface and contour plots for lipase activity as a function of KH_2PO_4 and NaH_2PO_4. All other factors were fixed at the central points.

However, Kamini *et al.* (1998) showed that severeal nitrogen sources such as peptone, urea, and ammonium nitrate did not improve the production of *A. niger.*

The types of lipid materials used are particularly important in lipase synthesis. When different lipids in the cultivation medium were compared, the amount of lipase secreted by *A. niger* E.I. 202 were altered and olive oil was reported to be the best inducer (Pal *et al*., 1978).

Central composite design and response surface methodology

The experimental design and the values predicted using RSM, are presented in Table 3 and 4. The levels of the variables for the CCD experiments were selected according to the results of the PB experiments. The results obtained from the CCD were analyzed by a standard analysis of variance (ANOVA), which gave the following regression equation (in terms of the coded factors) (Equation 3).

$$Y = 21.95 - 1.90x_1 - 0.33x_1^2 + 0.50x_2 + 0.45x_3 + 2.59x_5 + 0.76x_6 - 0.52x_4x_6 - 0.58\ x_5x_6 \quad \text{(Equation 3)}$$

where Y was the response variable (lipase activity) and x_1, x_2, x_3, x_4, x_5, and x_6 were the coded values of the independent variables: water content, glucose, yeast extract, peptone, NaH_2PO_4, and KH_2PO_4, respectively. The significance of each of the coefficients were checked using the p values. Coefficients with $p<0.05$ at a 95% confidence level were considered statistically significant. The coefficients selected for the linear effects were water content, glucose, yeast extract, NaH_2PO_4 and KH_2PO_4, and the quadratic effect of the water content. The interactive effects between peptone and NaH_2PO_4 and between KH_2PO_4 and NaH_2PO_4 were also significant (Table 6). The ANOVA for the responses (Table 7) indicated that the model was significant. The R^2 value (0.8383) for lipase production indicates the accurancy of the model and provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The statistical significance of the second-order model equation was evaluated by the *F*-test analysis of variance, which revealed that this regression was statistically significant ($p<0.05$) at the 95%

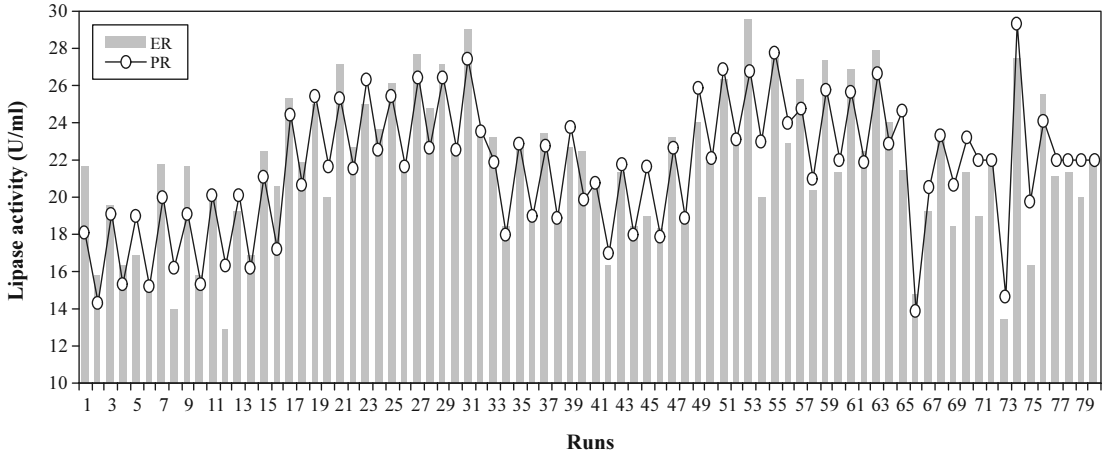

Fig. 5. Predicted and experimental responses for *A. niger* AC-54 lipase production. ER corresponds to the experimental response and PR corresponds to the predicted response.

confidence level.

Three-dimensional response surfaces were plotted for a statistically significant model (Equation 3) to understand the interaction between the medium components and determine the optimum concentration of each component required for optimum production. A maximum predicted lipase activity of 33.03 U/ml was determined using mathematical methods (equation derivation). In this case the values obtained for the concentrations of water, glucose, yeast extract, and NaH_2PO_4 were 20%, 4.8%, 4.0%, and 4.0%, respectively. Decreases in the concentration of any of these factors would lead to a decrease in enzyme production (Fig. 2, 3, and 4). However, as can be seen in Fig. 4, monobasic sodium phosphate and monobasic potassium phosphate showed a negative interaction effect with each other (-0.5868). Considering the greater effect of NaH_2PO_4 on the process, KH_2PO_4 was not included in the validation experiments.

Upon comparison (Fig. 5), a strong correlation was observed between the predicted and experimental data. The behaviors of both were synchronized, though there were some variations according to the R^2 value (0.8383).

Verification of the optimal conditions

To confirm the results, the fermentation process was conducted using the statistically significant variables at their optimal concentrations, i.e. water content (20%), glucose (4.8%), yeast extract (4.0%), and NaH_2PO_4 (4.0%), plus olive oil and temperature at 0.16% and 25°C, respectively. The observed lipase activity was 28.99 U/ml (average of three repetitions), and this is not statistically different than predicted value (33.03 U/ml). The result was higher compared to the lipase production prior to optimization. It was observed a ten-fold improvement of the activity of this lipase when compared to the production by SSF in a medium composed of just wheat bran and water (60:40) (Silva, 2004) and more than six-fold by submerged fermentation in a medium with a initial pH of 6.0 consisted of 2% peptone, 0.5% yeast extract, 0.1% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 2% of olive oil (Carvalho *et al*., 2006b). These results indicated a great improvement in the yield of the process, which is important in the commercial point of view. In addition, a cost reduction of lipase production could be performed by using raw material (wheat bran) as a substrate for fungal growth, considering that culture medium usually ranges 25~50% of the total production costs (Burkert *et al*., 2004). Thus, this might result in a feasible industrial process that could be regarded as possible and economically attractive, considering the enantioselective properties of this enzyme.

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