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Optimization of cultivation conditions for extracellular lipase production by *Yarrowia lipolytica* using response surface method

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A wild strain of *Yarrowia lipolytica*, identified as LMI 91, was isolated from an oleaginous Brazilian fruit called *pequi* (*Caryocar brasiliense* Camb), which is a native species of the Brazilian savannah and which acts as a potent lipase producer. The present study examined the effects of certain factors, such as the combination of casein and meat peptones, as well as the initial pH of a culture medium in lipase produced by *Y. lipolytica* LMI 91. Experiments were conducted within a low concentration of olive oil, at a temperature of 30°C and a fermentation time of 60 h, under orbital shaking. The response surface methodology was used to determine the optimum concentration of the constituents' composition. A 2^2 experimental design with four axial points and three central points was employed in the optimization experiments, and interactive regression analysis was performed to obtain the optimum concentration. The experimental values were found to be in accordance with the predicted values, presenting a correlation coefficient of 0.866. The maximum lipase activity (13.0 U/mL) was obtained by peptone mixture of 70 g/L (1:1) and an initial pH of 5.0.

Key words: *Yarrowia lipolytica*, wild strain, extracellular lipase, fermentation, optimization.

INTRODUCTION

The growing demand for lipases to be incorporated into several biotechnological processes has been stimulating research on novel microbial lipases. As such, the present research focuses on both the screening of microorganisms from different sources and the development of better conditions to obtain higher enzyme production with lower costs (Sharma et al., 2001; Colen et al., 2006; Salihu et al., 2012). Among these the most microorganisms are yeasts, mainly *Candida* sp, which is commonly reported lipase producer in the literature (Salihu et al., 2012), but whose use is limited because it is a human pathogen. On the other hand, *Yarrowia lipolytica,* a non-pathogenic yeast with a Generally Recognized as Safe (GRAS) status, secretes several proteins with high molecular weight, depending on its growth conditions (Glover et al., 1997; Madzak et al.,

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Abbreviations: CCRD, Central composite rotatable design; **RSM,** response surface methodology; **SA,** Sabouraud agar; **X,** coded levels of variables; **Y,** lipase activity (U/mL).

2004). Intracellular, membrane-bound lipases (carboxylesterases) and extracellular lipases (triacylglycerol hydrolases) can all be detected in *Y. lipolytica* (Fickers et al., 2006). These differ from lipases produced by other yeasts through their amino acid compositions, protein chain lengths and enzymatic properties (Pignède et al., 2000; Zhao et al., 2011).

Lipase production depends largely on the microorganisms' source, culture type (liquid or solid fermentations), medium composition and bioreactor design (Salihu et al., 2012).

The knowledge on how wild strains become domesticated represents a common research priority for microbiologists. The diversity of microbial communities and their ecological and metabolic functions have the potential for remarkable scientific, social and economic impacts (Suzzi, 2011). In fact, the evaluation of strain diversity provides a great chance to increase the knowledge of metabolic functions and regulation, as well as to speed up the improvement process of a targeted strain (Wittmann and Heinzle, 2002).

In the development of an industrial fermentation, fermentation medium designing is of critical importance, as the medium's composition can significantly affect the concentration, yield, and volumetric productivity of the product. In addition, the cost of the medium substantially affects the value of overall process (Kennedy and Krouse, 1999). Optimization through factorial design and RSM analysis is a common practice in biotechnology. Various researchers have applied this approach, especially to optimize process parameters, including the pH, temperature, aeration, among others (Gupta et al., 2004). The best conditions for the production of a microbiological enzyme can be gathered by experimental design using response surface methodology (Bornscheuer et al., 2002; Burkert et al., 2004; Rajendran and Viruthagiri, 2007). This methodology consists of a group of techniques used to investigate the relationship among the experimental variables and their responses, in turn allowing for a reduction in the number of experiments without neglecting the interaction among variable parameters (Myers, 1971).

Y. lipolytica is known for its ability to grow in hydrophobic substrates like oils (Pignède et al., 2000). Considering the consumption of fatty acids, it seems that *Y. lipolytica* preferably consumes unsaturated fatty acids (Kar et al., 2012). Unsaturated oleic acid has been pointed out as a potent inducer of *Y. lipolytica* lipase production (Fickers et al., 2004). The strain used in the present work was isolated from a unique biological resource, a typical Brazilian fruit called *pequi* (*Caryocar brasiliensis* Camb.). This fruit is rich in palmitic and oleic acids, containing 442 and 517 g kg^{-1} of each fat acid, respectively (Segall et al., 2006); consequently, a singular lipase production was expected from this wild strain.

Through fractioned factorial planning, the present study investigated the variables that affect the production of extracellular lipase by the wild strain of *Y. lipolytica* LM 91. The influence of different growing conditions (peptones, yeast extract, olive oil concentrations and the initial pH of the culture medium) could be observed at 48, 60 and 72 h of fermentation. The aim of the present work was to determine the best combination of casein and meat peptones, as well as the initial pH of the culture medium's lipase production in the presence of low concentrations of olive oil, during 60 h of fermentation. A response surface methodology was used to compare and optimize the medium's components for lipase production. A 2^2 central composite design matrix was used in the optimization experiments, and interactive regression analysis was performed to obtain the optimum concentration.

MATERIALS AND METHODS

Microorganism, culture media and chemicals

A *Y. lipolytica* LMI 91 strain was isolated from the *pequi* pulp (*C. brasiliense* Camb), an oleaginous fruit that is a native species of the Brazilian savannah (Ferreira, 2007). The yeast was placed in GYMP agar, consisting of 20 g/L dextrose, 5 g/L yeast extract, 10 g/L malt extract and 2 g/L NaH_2PO_4, 20 g/L agar, with a pH of 4.6 at 4°C, and was cultivated in Sabouraud Agar (SA) for production storage. Slants were incubated at 30°C for 72 h.

This experiment used casein peptone, meat peptone, yeast extract, malt extract and agar, all of the BD brand (Becton, Dickinson, and Company, USA). The commercial olive oil (1% acidity) used in this study was from GALLO (Lisboa, Portugal). All reagents were of analytical grade.

Production of lipase by submerged fermentation

A cell suspension containing 10^7 cells/mL, prepared by washing 3 day-old SA slants with sterile distilled water, was used (1 mL) to inoculate each 125 mL Erlenmeyer flask containing 25 mL of the liquid growth medium. Cultivations were carried out in an orbital shaker (Marconi, Rio Piracicaba, SP, Brazil) at 150 rpm and 30°C. The composition of the medium varied according to the design matrix. The basal liquid medium contained $NaNO_3$ (1.0 g/L), KH_2PO_4 (1.0 g/L), $MgSO_4.7H_2O$ (0.5 g/L) and chloramphenicol (0.2 g/L). The initial pH and the concentration of other components (g/L) used in the medium's composition are presented in Table 1. The cultures of each flask were centrifuged (2000 rpm/15 min), and the supernatants were analyzed. Product formation was measured at 48, 60 and 72 h. In the optimization step, the composition of the medium varied according to the matrix design and was made up of: a mixture (1:1) of meat and casein peptones (varying from 55.9 to 84.1 g/L), olive oil (5.0 mL/L) (Table 2) and the basal medium described above. The initial pH was adjusted to 6.0. The lipase activities of cultures after 60 h of incubation were determined in the supernatants after separation of the cells by centrifugation.

Table 1. Experimental values and coded levels for the fractional factorial design and lipase activity during *Y. lipolytica* fermentation.

Experiment	Coded variables (concentration)				Y_1	Y_2	Y_3
	X_1	X_2	X_3	X_4	(48 h)	(60 h)	(72 h)
1	-1 (30)	-1 (0)	-1 (3)	-1 (5)	0.00	3.68	0.80
2	+1 (70)	-1 (0)	-1 (3)	+1 (7)	17.90	4.00	0.00
3	-1 (30)	+1 (2)	-1 (3)	+1 (7)	0.00	0.00	0.48
4	+1 (70)	+1 (2)	-1 (3)	-1 (5)	15.03	4.96	1.28
5	-1 (30)	-1 (0)	+1 (7)	+1 (7)	0.00	2.40	1.28
6	+1 (70)	-1 (0)	+1 (7)	-1 (5)	2.40	9.91	11.67
7	-1 (30)	+1 (2)	+1 (7)	-1 (5)	5.75	1.60	0.80
8	+1 (70)	+1 (2)	+1 (7)	+1 (7)	1.76	0.00	0.00
9	0 (50)	0 (1)	0 (5)	0 (6)	8.47	2.44	4.88
10	0 (50)	0 (1)	0 (5)	0 (6)	4.64	1.48	5.62
11	0 (50)	0 (1)	0 (5)	0 (6)	6.71	1.33	4.14

X_1 Peptones (g/L): Mixture of meat and casein peptones (1:1); X_2 yeast extract (g/L); X_3 olive oil (mL/L); X_4 pH; $Y_{1,2,3}$ Lipase activity (U/mL) at 48, 60 and 72 h of fermentation, respectively.

Table 2. Variables and their coded levels and concentrations used in the first factorial at 60 h of fermentation and lipase activity (U/mL).

Run	Peptones (g/L)	pH	Coded levels		Y^c (U/mL)
			$X_1{}^a$	$X_2{}^b$	
1	50	4	-1	-1	0.00
2	90	4	+1	-1	10.69
3	50	6	-1	+1	2.99
4	90	6	+1	+1	1.26
5	41.8	5	-1.41	0	0.00
6	98.2	5	+1.41	0	2.51
7	70	3.59	0	-1.41	5.03
8	70	7.41	0	+1.41	5.66
9	70	5	0	0	2.67
10	70	5	0	0	3.77
11	70	5	0	0	2.83

[a] Concentration of mixture of meat and casein peptones (g/L); [b] pH; [c] lipase activity (U/mL).

Lipase assay

The lipase activity was determined using a titration method (Watanabe et al., 1977). The reaction mixture contained 5.0 mL of 25% (v/v) olive oil emulsion in a 2% (v/v) polyvinyl alcohol solution, prepared at 13.500 rpm for 5 min, using an ultra-turrax dispersor T25 (Ika, Wilmington, NC, USA); 5.0 mL of 0.1 M Tris-HCl buffer, pH 8.0; and 1.0 mL of the enzyme supernatant. The reaction mixture was incubated at 30°C in a reciprocal shaker (Solab, Piracicaba, SP, Brazil) at 45 cycles/min for 10 min. The emulsion was immediately disrupted after incubation by adding 10 mL of an acetone/ethanol (1:1, v/v) solution. The released free fatty acids were determined by titration with 0.05 N NaOH solution, using thymolphthalein as an indicator. A supernatant culture, boiled for 10 min, was used as the control. One unit of lipase was defined as the amount of enzyme required to release one µmol of fatty acid per minute under the specific conditions.

Experimental design

A 2^{4-1} fractional factorial design with three central points was used to calculate the main effects on a desired response. Four independent variables (concentration of peptones, yeast extract, olive oil, and pH) were used, the levels and values of which are presented in Table 1. Eleven experimental runs were carried out, which included eight factorial points and three central points. The lipase activity after 48, 60 and 72 h of fermentation was studied as a response.

Next, the effects of the peptone mixture and the initial pH of the culture medium on the lipase production were determined by modulating the variables according to a two-factor, five-level central composite rotatable design (CCRD). The equivalence of the design and levels are shown in the Tables 2 (first factorial design) and 3 (second factorial design).

A 2^2 experimental design with star (4 axial points) and 3 central

Table 3. Variables and their coded levels and concentrations used in the second factorial at 60 h of fermentation and lipase activity (U/mL).

Run	Peptones (g/L)	pH	Coded levels		Y[c] (U/mL)
			X_1[a]	X_2[b]	
1	60	4	-1	-1	2.22
2	80	4	+1	-1	3.40
3	60	6	-1	+1	0.00
4	80	6	+1	+1	0.89
5	55.9	5	-1.41	0	5.32
6	84.1	5	+1.41	0	2.44
7	70	3.59	0	-1.41	4.29
8	70	7.41	0	+1.41	2.66
9	70	5	0	0	13.53
10	70	5	0	0	12.57
11	70	5	0	0	12.61

[a] Concentration of mixture of meat and casein peptones (g/L); [b] pH; [c] lipase activity (U/mL).

Table 4. Analysis of effects on lipase activity as determined by fractional factorial planning at 48, 60 and 72 h of fermentation.

Factor	Effect			Standard Error			t			P		
	48 h	60 h	72 h	48 h	60 h	72 h	48 h	60 h	72 h	48 h	60 h	72 h
Peptones (g/L)	7.83	2.80	2.40	3.86	1.18	2.29	2.03	2.38	1.04	0.09	0.05	0.34
Yeast extract (g/L)	0.56	-3.36	-2.80	3.86	1.18	2.29	0.15	-2.85	-1.22	0.89	0.03	0.27
Olive oil (mL/L)	-5.75	0.32	2.80	3.86	1.18	2.29	-1.49	0.27	1.22	0.19	0.80	0.27
pH	-0.88	-3.44	-3.20	3.86	1.18	2.29	-0.23	-2.92	-1.39	0.83	0.03	0.21

points, totaling 11 experiments, was carried out to obtain a second order model. The distance of the axial points was 1.414, calculated from Equation 1, where α is the distance of the axial points; k is the number of independent variables.

$$\alpha = (2^k)^{1/4} \quad \text{(Equation 1)}$$

The results of CCRD were used to fit a second-order polynomial equation represented as

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad \text{(Equation 2)}$$

In Equation 2, Y is the predicted response; β_0, the intercept; β_1, β_2, the linear coefficients; β_{12}, β_{11}, β_{22}, the squared coefficients.

Statistical analysis

The Statistica 8.0 software was used to determine the regression and graph analyses of the obtained data. The statistical significance of the aforementioned model equation was determined by Fisher's test value and the proportion of variance explained by the polynomial model was given by the multiple coefficient of determination, R squared (R^2) value. Analysis of variance (ANOVA) was also performed, and the level of significance was set at 0.1.

RESULTS AND DISCUSSION

The present study evaluated several parameters involved in lipase produced by a wild strain of *Y. lipolytica*, including the effect of the culture medium composition, given that sources and concentrations of nutrients directly influence both cell growth and enzyme production. Parameters selected as variables and lipase activity measured after 48, 60 and 72 h of fermentation are shown in Table 1.

Fractioned factorial planning was used as a screening tool to select the variables that affected lipase production. For optimization, other factorial designs with 2 or 3 variables were necessary, depending on the fractioned planning results. Table 4 shows the effect of the variables on lipase activity in *Y. lipolytica* LMI 91.

The parameters were considered significant when respective *p* values were lower than 10% ($p < 0.1$). In this manner, results obtained after 48 h of fermentation indicated that only the dependent variable peptone ($p = 0.09$) was significant in lipase production (Table 4). Therefore, determining the optimal concentration of peptone was required to reach a maximum enzymatic activity. By contrast, the results indicated that any value within the range of the tested pH, as well as all yeast extracts and olive oil concentrations, can be used without interfering in enzyme production.

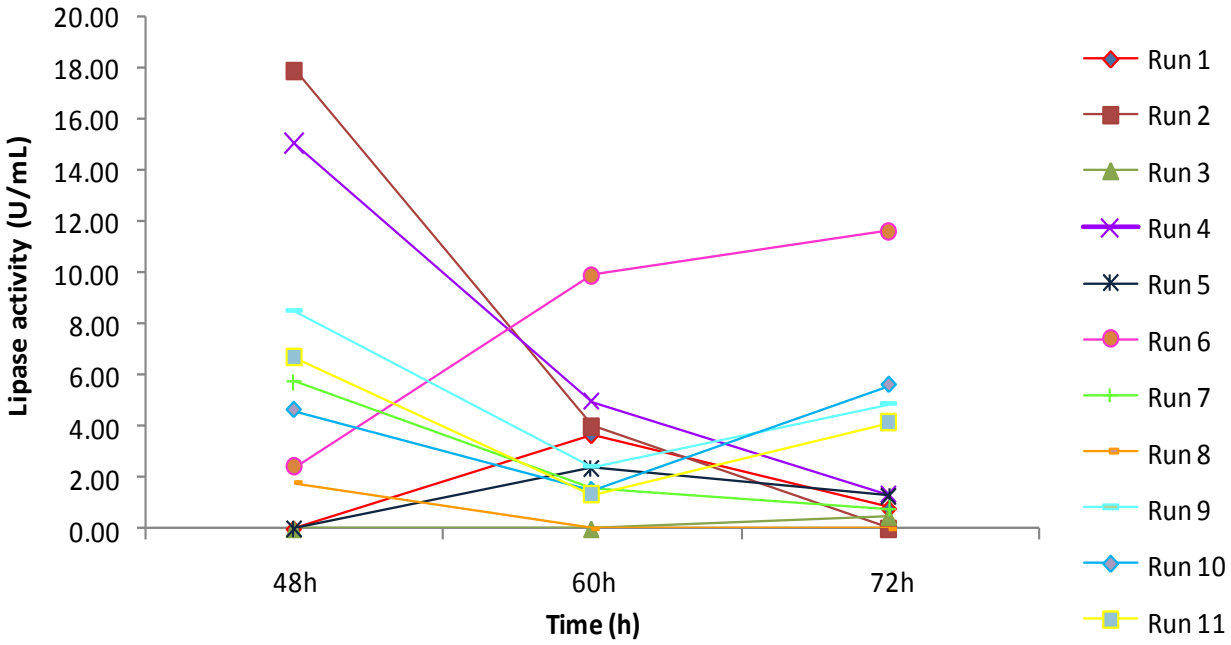


Figure 1. Lipase activity in function of fermentation time in the fractionated factorial planning.

A change in the influence of factors affecting lipase production could be observed after 60 h of fermentation (Table 4). At this time, in addition to the increase in peptone concentration, two other parameters presented statistical significance ($p < 0.1$): a decrease in the concentration of the yeast extract and a lower pH. By contrast, variations in the amount of olive oil present in the medium did not affect lipase production at 60 h of incubation. Since the yeast extract did not show statistically significant effects, this ingredient can be removed from the medium's composition without decreasing lipase production, given that level -1 represents an absence of yeast extract in the culture.

According to the statistical analyses, lipase activity was not affected by any of the studied variables at 72 h of fermentation (Table 4). According to Figure 1, lipase activity was higher at 48 h of fermentation (assays 2 and 4), decreasing abruptly at 60 h, and reaching nearly zero at 72 h. Assay 6 shows a high lipase activity from 48 to 60 h, at which time it reached 9.9 U/mL, remaining almost constant from then on.

Rathi and collaborators (2002) showed that the decrease in lipase activity may well be due to increased protease activity in the post-exponential phase of microorganism growth due to the decrease of oil availability or to the accumulation of fatty acids. Corzo and Revah (1999) also agree that lipase activity is strongly inhibited by the presence of oleic acid. Dalmau et al. (2000) reported that lipase activity decreases quickly after reaching the maximum production due to lipase proteolysis catalyzed by proteases produced during cell growth. *Y. lipolytica* is a dimorphic yeast that secrets several large proteins, such as alkaline proteases and at least one acid protease.

The first step in the process of seeking optimum conditions is to identify the input variables that have the greatest influence on the experimental response. According to Kennedy and Krouse (1999), there are many challenges associated with medium design. Designing the medium is a laborious, expensive, open-ended, often time-consuming process involving many experiments. Many constraints occur during the design process, and an industrial scale must be kept in mind when designing the medium. The present study verified that the most important variables for lipase production by the wild strain after 60 h of fermentation in orbital shake flasks were peptone concentrations and the initial pH of the culture medium and that variations in the amount of olive oil did not affect lipase production, despite prior reports (Fickers et al., 2004) describing that oleic acid enhanced lipase production in *Y. lipolytica*.

According to Gupta et al. (2004), the emphasis has shifted towards medium optimization using response surface methodology (RSM). The factorial design of a limited set of variables is advantageous when compared with the conventional method of manipulating a single parameter per trial, as the latter approach frequently fails to locate the optimal conditions for the process due to a

Table 5. ANOVA for the first factorial design.

Source of variation	Sum of square	Degrees of freedom	Mean square	F ratio	P
Regression	58.09	2	29.05	7.18	< 0.02
Residual	32.39	8	4.05		
Total	90.48	10	9.05		

R^2 = 64.2; F tab. 2; 8; 0.1 = 3.11.

Table 6. Regression coefficient for lipase activity (Y) for the second factorial design.

Factors	Regression coefficient	Standard error	t(5)	P	Estimate interval (90%)	
					Lower limit	Upper limit
X_1 (L)	-0.3	0.75	-0.3	0.7500	-1.75	1.25
X_1 (Q)	-4.5	0.89	-5.1	0.0038	-6.31	-2.74
X_2 (L)	-0.9	0.75	-1.2	0.2913	-2.38	0.62
X_2 (Q)	-4.7	0.89	-5.3	0.0031	-6.51	-2.94
X_1 X_2	-0.1	1.05	-0.1	0.9468	-2.20	2.05

L, Q: Linear and quadratic terms.

failure to consider the effect of possible interactions between factors. Moreover, the factorial design makes it possible to take advantage of practical knowledge about the process during the final RSM analysis.

Lipid carbon sources appear to be essential to obtaining a high lipase yield. However, according to Tamilarasan and Dharmendira (2011), higher oil concentration can affect the oxygen transfer in the medium. Although the lipid carbon source is essential, Treichel et al. (2010) claim that nitrogen sources and essential micronutrients should also be carefully considered for growth and production optimization. These nutritional requirements for microbial growth are fulfilled by several alternative media, including those based on defined compounds (synthetic medium), like sugars, oils and complex components, such as peptone, yeast extract, malt extract media, as well as agroindustrial residues containing all the components necessary for microorganism development.

According to Table 5, which shows the values of the variance analysis for the first factorial planning (Table 2), it could be observed that, although the F_{calc} for regression (7.18) is significantly greater than the F_{tab} (3.11), the percentage of variance (R^2) explained by the template proved to be unsatisfactory, approximately 64.2%. Moreover, there was a lack of adjustment for the model, since the F_{calc} in relation to the pure error (14.92) was greater than the F_{tab} (9.33). In this manner, since a response surface was impossible to obtain, a second factorial planning was required to determine new levels for the variable peptones (Table 3). The results obtained by means of CCRD (Table 3) were used to calculate the regression coefficients shown in Table 6, which gave rise to Equation 3, in which the variables are coded values, represented as: lipase activity (Y) as a function of the peptone mixture (variable X_1) and the initial pH (variable X_2); a second order regression equation (Equation 3), which shows the dependency of the medium's components for lipase activity. Only the quadratic terms proved to be significant model terms for the response ($p < 0.1$).

$$Y = 11.90 - 4.52\ X_1^2 - 4.73\ X_2^2 \quad \text{(Equation 3)}$$

The analysis of variance (Table 7) indicated that the second-order polynomial model (Equation 3) was highly significant and adequate enough to represent the relation between the response (lipase production) and the variables studied, with a very small p-value and a satisfactory coefficient of determination (R^2 = 0.866). This provides proof that the model accurately describes the studied region.

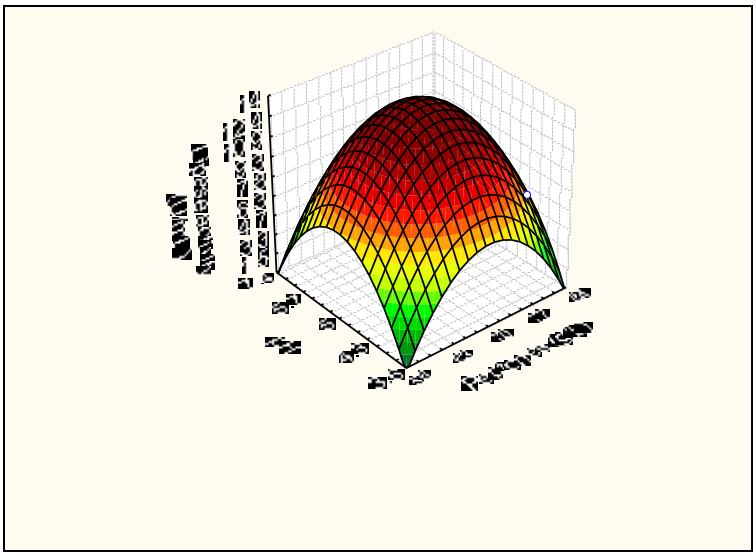
The response surface described by the model equation (Y) is represented in Figure 2a and by the contour diagrams in Figure 2b. The maximum lipase activity (13.0 U/mL) was obtained using 70 g/L of peptones and an initial pH of 5.0.

The results obtained in prior literature are diverse. Under optimum conditions, Muralidhar et al. (2001) reported the use of response surface methodology to optimize lipase production by *Candida cylindraceae*. Although, this study examined a different microorganism, the carbon source used was the same, olive oil, but in a much larger amount (33.7 g/L), resulting in a higher

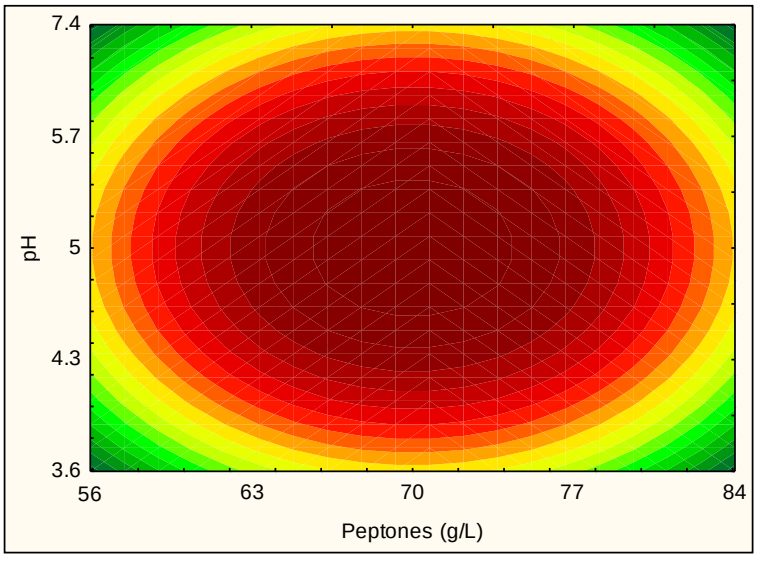
Table 7. ANOVA for the second factorial design.

Source of variation	Sum of square	Degrees of freedom	Mean square	F ratio	P
Regression	186.88	2	93.44	25.839	<0.0001
Residual	28.93	8	3.62		
Total	215.81	10	21.58		

R^2 = 0.866 ; F tab. 2; 8; 0.1 = 3.11.



(a)



(b)

Figure 2. (a) Response surface plot showing the effect of concentration of peptones mixture and initial pH on lipase production from *Y. lipolytica* wild strain under conditions predicted by the model; (b) Contour diagrams for the lipase activity as a function of concentration of peptones mixture and initial pH of the culture medium.

activity (47.25 U/mL). Rajendran and Viruthagiri (2007) also optimized the conditions of culture mediums for lipase production by another yeast, *Candida rugosa* NCIM 3462, using response surface methodology, and reached maximum activity of 5.95 U/mL in 50 h of fermentation. Burkert et al. (2004), optimizing the culture medium conditions using olive oil as a carbon source, reached the maximum activity of 17 U/mL of lipase produced by the fungus *Geotrichum* sp. In another study, Papagora et al. (2013) optimized lipase production, using a *Debaryomyces hansenii* strain isolated from dry-salted olives, and reached a productivity of 7.44 U/mL.

To optimize the culture medium, peptones were used as a nitrogen source, as can be seen in the works of Muralidhar et al. (2001) and Rajendran and Viruthagiri (2007). Rajendran and Viruthagiri (2007) used only peptones and observed increased activity upon increasing the peptone concentrations, while Muralidhar et al. (2001) used peptone in a much smaller concentration, together with yeast and malt extracts. Another study on the production of lipase by a species of *Bacillus* corroborated with the finding that peptone is the preferred source of organic nitrogen (Gupta et al., 2004). Almeida et al. (2012) evaluated the effects of inorganic (ammonium chloride, sulphate, and nitrate) and organic (peptone and tryptophan yeast extract) nitrogen sources in the growth and production of lipase by the yeast *Candida viswanathii*. These authors reported that ammonium nitrate was the source that most influenced both enzyme productivity and cell growth. However, yeast extract was considered the better nitrogen source for cell growth and lipase production.

Organic nitrogen sources can provide many cell growth factors and amino acids, which are required for cell metabolism and enzyme synthesis (Darvishi et al., 2009; Tamilarasan and Dharmendira, 2011; Almeida et al., 2012).

Lipase production is substantially increased when working with two situations: the use of mutant strains obtained by molecular biology methods (Tan et al., 2003; Fickers et al., 2006; Bussamara et al., 2010) and the increase in the oxygen transfer rate, usually as a result of shaking the cultures, which also contributes to a better access of microorganisms to culture medium components, thus accelerating and increasing enzyme production.

Improvements in microbial strains for the overproduction of industrial products have been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the processes through increased productivity and may also possess some specialized and desirable characteristics (Karanam and Medicherla, 2008).

Despite the smaller productivity of wild strains, the screening of microorganisms from new sources, such as that used in the present work, is of utmost importance due to the industrial demand for new sources of lipases with different catalytic characteristics that need to be isolated from new strains (Sharma et al., 2001; Colen et al., 2006). In the present study, the target strain was isolated from the *pequi* fruit, an oil-rich fruit typically found in the Brazilian savannah (Segall et al., 2006).

Analyzing the results, it was possible to observe the behavior of *Y. lipolytica* LMI 91 towards different compositions of culture media and the influence of fermentation time on the parameters of cultivation conditions for lipase production. The composition of the medium and cultivation time interfere directly with cell growth, as well as in lipase production, since this production increases and reaches maximum levels in the growth phase and begins to decrease in the mid-stationary phase (Fickers et al., 2006; Almeida et al., 2012). It is also interesting to note the influence that peptone concentrations had on enzyme production, regardless of the time of fermentation.

By contrast, yeast extract, another nitrogen source used in fractional factorial planning, did not influence lipase activity. Therefore, it was possible to remove this component from the growth medium, making it less costly and easier to be controlled, since a variable had been removed. In addition to the yeast extract, olive oil also showed little interference in the production of the enzyme, even if it is the main and only source of cell energy; therefore, this study opted for a medium formulation with a low concentration of olive oil (5.0 mL/L, which corresponds to approximately 4.5 g of oil). The results obtained in this work made it possible to consider that this wild yeast strain of *Y. lipolytica* LMI 91 is a good and promising lipase-producing strain, indicating its importance for possible future work aimed at biotechnological applications, considering that it was able to reach a relatively high lipase activity.

Conclusion

In the present study, using orbital shake flasks, the optimum conditions to obtain a high-level production of extracellular lipase by the wild strain of *Y. lipolytica* LMI 91 included 70 g/L of a casein and meat peptone mixture and a culture medium with an initial pH of 5.0, with the basal experimental conditions presenting a low concentration of olive oil (5.0 mL/L). Under these conditions, an enzyme activity of 13 U/mL was achieved. According to the results found in this study, *Y. lipolytica* LMI 91 is a promising lipase producer. Since this is a wild strain, yield improvement can be achieved by increasing the scope of further developments and applications using *Y. lipolytica* LMI 91 for lipase production to supply the growing biotechnological needs for the supply of lipase

in the world market.

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