OPTIMIZATION OF *Aspergillus niger* LB-02-SF LIPASE ACTION USING SOYBEAN OIL AS SUBSTRATE

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ABSTRACT

Lipases are enzymes widely used in the industry. Methods have been tested to increase its operational stability and activity in order to employ them more efficiently. However, it is crucial to comprehend the different parameters that show influence on the catalytic performance of lipase. In this study, the influence of pH, temperature, agitation, time, and substrate concentration on the catalytic action of lipase from *Aspergillus niger* LB-02- was investigated SF in submerged cultivation. The experimental planning was carried out to evaluate the parameters mentioned before. Based on the response surface methodology, the highest lipolytic activity of 16.60 $U.mL^{-1}$ was obtained at 28°C, pH 6.0, 300 rpm, 180 minutes, and soybean oil concentration of 5.0% (m/v). This condition has been experimentally validated. All parameters evaluated demonstrated a significant influence on the catalytic activity of lipase, highlighting pH, time, agitation as the main determinants.

Keywords: Lipase. Optimization. Catalytic activity. pH effect. Reaction time.

1 INTRODUCTION

Enzymes are an alternative to meet the global demand for process sustainability. Among the most industrially used groups of enzymes, lipases stand out. Lipases are a multipurpose biological catalyst, being applicable in several industries such as biodiesel, energy, waste treatment, foods and drinks, leather, textile, detergents, pharmaceuticals and medicals. The main reaction catalyzed by lipases is the conversion of substrates, such as oils and fats, mainly triacylglycerols, into shorter-chain fatty acids and glycerol [1].

As it is a very versatile enzyme, it acts on different substrates. The conditions of the enzymatic reaction catalyzed by lipase can be different depending on the substrate and reaction conditions such as pH, temperature, agitation, time, and substrate concentration. When these conditions are not specified for each substrate, this becomes a bottleneck in the industrial process, since the yield of the reaction catalyzed by the enzyme decreases [1,2]. Therefore, it is necessary to use statistical tools to predict the behavior of the production process to identify the condition in which there is greater enzymatic activity [3]. In this context, the present work had the general objective of optimizing the parameters of pH, temperature, agitation, time, and substrate concentration on the catalytic capacity of *Aspergillus niger* LB-02-SF lipase in submerged cultivation.

2 MATERIAL & METHODS

The microorganism used was *Aspergillus niger* LB-02-SF, belonging to the collection of cultures of the Laboratory of Bioprocess of the University of Caxias do Sul (Caxias do Sul, Brazil). This strain was deposited in the Collection of Reference Microorganisms on Health Surveillance at Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) under the code INCQS 40371.

The microorganism was kept at 4°C in a potato-dextrose-agar (PDA) medium and incubated at 30ºC for 96 hours before the inoculum preparation. A total of 10^6 conidia/mL *A. niger* LB-02-SF were grown in 500 mL Erlenmeyer flasks, containing the following composition 36.6 $g.L^{-1}$ of glucose; 6 $g.L^{-1}$ of soybean oil; 10 $g.L^{-1}$ of yeast extract and 30.6 $g.L^{-1}$ mineral salt solution, The saline solution contained ($g.L^{-1}$): $(NH_4)_2SO_4$, 4.0; $MgSO_4$, 1.0; KH_2PO_4, 2.0; $FeSO_4.H_2O$, $6.3x10^{-5}$; $ZnSO_4$, $6.2x10^{-5}$; $MnSO_4$, $1.0x10^{-6}$. The flasks were covered with gauze and hydrophobic cotton wool and maintained at 30ºC for 96 hours, under reciprocal agitation of 250 rpm [4].

Extracellular enzyme extraction was performed after the end of cultivation. The culture was centrifuged at 2000 x g for 15 minutes to separate the fungal biomass from the extracellular lipase. The supernatant was used aiming to optimize the conditions of enzymatic catalysis [5]. In order to evaluate the effects of parameters such as temperature, pH, agitation, substrate concentration, and time on enzymatic activity, a factorial design of the central composite design (CDD) type was performed. In order to verify the lipases activity, the method of titrating the fatty acids released by the reaction catalyzed by the enzyme was used. In this method, one unit (U) was defined as the amount of enzyme that catalyzes the conversion of one µmol of substrate per minute, under the test conditions [5].

The experiment was performed according to a matrix generated with six variables and three repetitions of the central point, with each condition being evaluated in triplicate, totaling 57 tests. The orthogonality alpha used was 1, the independent variables considered were temperature 28°C and 36°C (x_1); agitation 60 to 180 rpm (x_2); pH 6 to 8 (x_3); substrate concentration (soybean oil) from 1 to 5% (m/v) (x_4) and time from 30 to 180 minutes (x_5). The response condition of the experiment is lipolytic activity.

The activity evaluation was carried out by adding the amount of substrate established in the test, 2 mL of sodium phosphate buffer solution (0.1 mol.L^{-1}) at the pH value also established in the test, and 10 mL of crude extract. The reaction temperature and time were by the tests. In all experiments, reactions were stopped by adding 5 mL of an inhibition solution composed of acetone, ethanol, and water (1:1:1)[5].

The data were processed in the statistical software Minitab 18 with a confidence level of 5% ($p < 0.05$). In order to validate the model predictions, the fermentation tests were carried out under conditions predicted by the models.[6]

3 RESULTS & DISCUSSION

Table 1 shows the results of lipolytic activity considering the effects of temperature, agitation, pH, substrate concentration, and time. Lipolytic activity results ranged from 0,20 U. mL^{-1} to 16,60 U.mL^{-1}. The values to be considered as maximum are the temperature of 28°C, pH equal to 6.0, agitation at 300 rpm, substrate concentration of 5% (m/v), and time of 180 minutes.

Table 1 Enzymatic activity of lipase from *Aspergillus niger* LB-02-SF as a function of temperature, agitation, pH, substrate concentration and time.

Test	Temperature (°C)	Agitation (rpm)	pH	Substrate (% m/v)	Time (minutes)	Lipolytic activity (U.mL^{-1})
1	28 (-1)	60 (-1)	6 (-1)	1 (-1)	180 (1)	0,75
2	36 (1)	60 (-1)	6 (-1)	1 (-1)	30 (-1)	3,77
3	28 (-1)	60 (-1)	8 (1)	1 (-1)	30 (-1)	1,43
4	36 (1)	60 (-1)	8 (1)	1 (-1)	180 (1)	0,29
5	28 (-1)	300 (1)	6 (-1)	1 (-1)	180 (1)	3,71
6	36 (1)	300 (1)	6 (-1)	1 (-1)	180 (1)	3,66
7	28 (-1)	300 (1)	8 (1)	1 (-1)	30 (-1)	1,27
8	36 (1)	300 (1)	8 (1)	1(-1)	30 (-1)	1,43
9	28 (-1)	60 (-1)	6 (-1)	5 (1)	30 (-1)	5,77
10	36 (1)	60 (-1)	6 (-1)	5 (1)	180 (1)	1,02
11	28 (-1)	60 (-1)	8 (1)	5 (1)	180 (1)	0,27
12	36 (1)	60 (-1)	8 (1)	5 (1)	30 (-1)	0,93
13	28 (-1)	300 (1)	6 (-1)	5 (1)	180 (1)	16,60
14	36 (1)	300 (1)	6 (-1)	5 (1)	180 (1)	6,18
15	28 (-1)	300 (1)	8 (1)	5 (1)	30 (-1)	0,43
16	36 (1)	300 (1)	8 (1)	5 (1)	30 (-1)	1,43
17	32 (0)	180 (0)	7 (0)	3 (0)	105 (0)	2,22
18	32 (0)	180 (0)	7 (0)	3 (0)	105 (0)	2,38
19	32 (0)	180 (0)	7 (0)	3 (0)	105 (0)	2,63

The values obtained for the coefficient of determination (R2) of 0.9995 showed that the model is reliable and statistically significant ($p \leq 0.05$). Equation 1, presented below, is predictive and only the terms that showed effects were represented.

$$\text{Lipolytic activity} = 44{,}82 - 0{,}54\ \text{Temperature} + 0{,}02\ \text{Agitation} - 5{,}012\ \text{pH} + 1{,}420\ \text{Substrate concentration} + 0{,}06\ \text{Time} - 0{,}00170\ \text{Temperature}^2 + 0{,}08\ \text{pH}^2 + - 0{,}002907\ \text{pH*Time} \quad (1)$$

Figure 1 presents the Pareto diagram that allows the effects evaluation of each factor on the catalysis reaction of lipase from A. niger LB-02-SF. The parameters that showed significant effects were pH followed by time. In general, the pH range of greatest activity for lipases synthesized by fungi is 4.0 to 6.0[7]. According to the authors, the quantification of lipolytic activity in a crude extract can be influenced by the presence of other enzymes, such as proteases, which can act also on lipase denaturation. Controlling pH can improve lipase activity and inhibit the protease performance. The more acid the pH the greater the lipase activity, possibly due to the more favorable environment for this enzyme and less adequate environment for protease action[6].

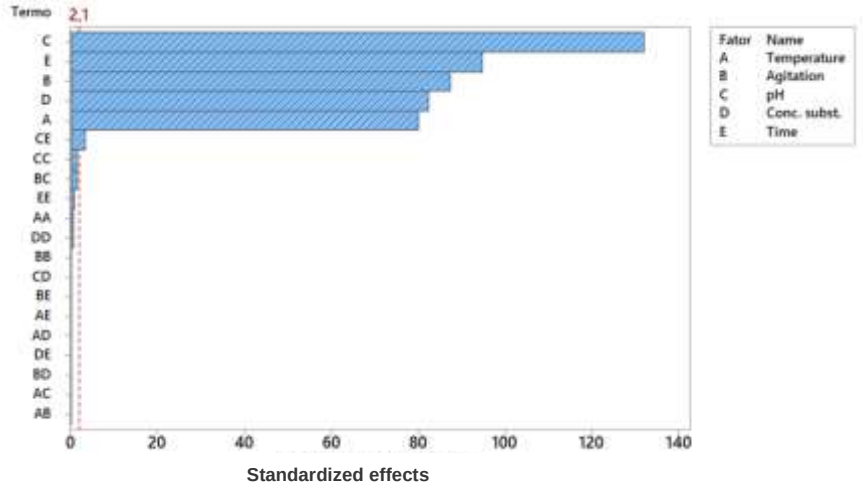

Figure 1 Pareto chart of standardized effects for the lipolytic activity of *Aspergillus niger* LB-02-SF

In addition to pH, other variables that had effects on the lipase catalysis reaction were time and agitation, as represented in Figure 2. In Figure 2A it can be seen that a lower pH value and longer process time favor the lipase activity. Regarding agitation, it can be observed that at lower pH, the enzymatic activity is favored when agitation remains above 200 rpm (Figure 2B). Superior agitation and prolonged time increased the interaction of the enzyme with the substrate, resulting in a positive effect on the catalytic action.

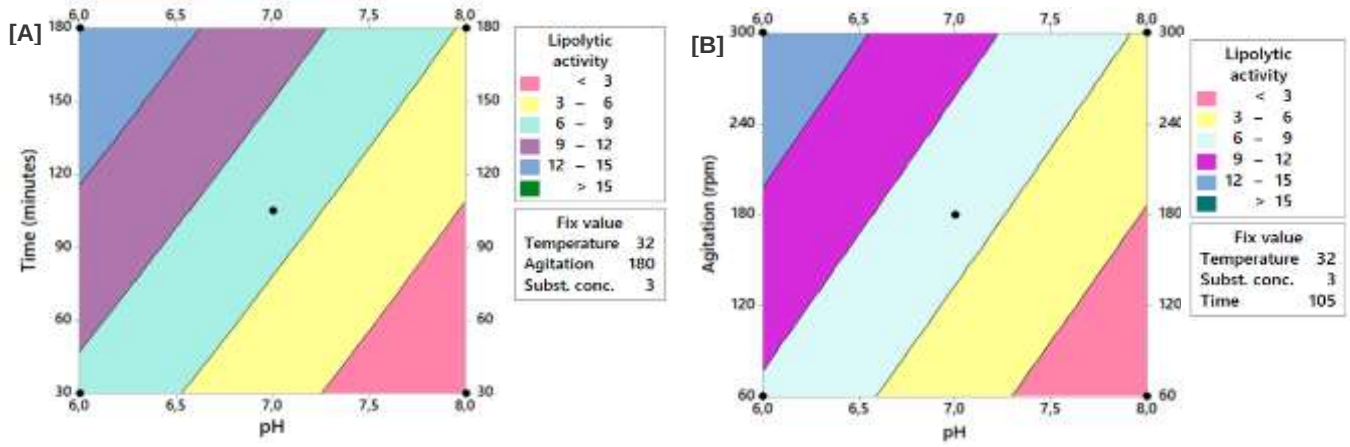

Figure 2 Contour curve of lipolytic activity of *Aspergillus niger* LB- 02-SF and the interaction of independent variables. [A] Effect of lipolytic activity on pH and time at 32°C, 180 rpm, and substrate concentration of 3% (m/v). [B] Effect of lipolytic activity on pH and agitation at 32°C, time of 105 minutes, and substrate concentration of 3% (m/v).

In a system with a more alkaline pH and agitation below 180 rpm, a decrease in enzyme activity was observed. It can be due to the stability of the enzyme against protonation and deprotonation, and when exposed to a more basic pH the enzyme is destabilized. In systems with agitation superior to 200 rpm the rate of catalysis could be increased due to a better enzyme-substrate contact and more acidic pH indicates that the enzyme was in the protonated form[8]. Due to the strong correlation between the predicted values, the empirical results obtained and the analyzes of variance of the generated models, the model was able to satisfactorily predict the behavior of lipase reaction[6].

4 CONCLUSION

In this study, the influence of parameters such as pH, temperature, agitation, time, and substrate concentration on the catalysis of soybean oil by lipase from *Aspergillus niger* LB-02-SF was investigated. The optimization of these parameters was based on the response surface methodology. The conditions that promote the highest lipase activity were 28°C, pH 6.0, 300 rpm, 30 minutes, and 5.0% (m/v) substrate. Among these factors, pH, time, and agitation stood out as the most important on the catalytic capacity of lipase. The results showed the importance of understanding and controlling these parameters for the industrial application of lipases.

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