BIOCHEMICAL PROPERTIES OF A LIPASE FROM AN AMAZON FUNGUS IMMOBILIZED ON POLYHYDROXYBUTYRATE

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ABSTRACT

Lipases (EC 3.1.1.3) are enzymes that belong to the class of hydrolases. They can be produced by different microbial sources. Enzyme immobilization, especially in supports such as polyhydroxybutyrate (PHB), provides greater stability, ease of separation and reuse. This study aimed at optimizing the conditions of temperature and pH of the reaction of olive oil hydrolysis. The reaction was catalyzed by a lipase from an amazon filamentous fungus immobilized on PHB functionalized with glutaraldehyde. An experimental design of the type *Central Composite Rotational Design* was employed to optimize the enzymatic hydrolysis reaction. The results demonstrated that the best condition achieved occurred in a reaction medium at pH 7.0 conducted at 46.9 ºC for 5 minutes of reaction, whose hydrolytic activity obtained was of 6602 U g^{-1}, suggesting the hyperactivation of the biocatalyst when immobilized on a material of hydrophobic support.

Keywords: *Oenocarpus bacaba*. Lipase. Immobilization. Hydrolysis. Polyhydroxybutyrate

1 INTRODUCTION

Lipases, or triacylglycerol acyl hydrolases (EC 3.1.1.3), are enzymes that belong to the class of hydrolases, and which can be produced by diverse microbial sources [1]. These enzymes catalyze several types of reactions, especially the hydrolysis of oils and fats[2]. They also catalyze esterification, transesterification, interesterification, alcoholysis and acidolysis reactions, with the water activity of the reaction medium being one of the determining factors for each class of reaction [1]. The application of soluble enzymes in processes involves a high cost for their production and purification, instability of their three-dimensional structure, difficulty in recovery and the loss of activity because of the inhibition caused by the substrate or product or the adverse conditions of the reaction medium [1,3]. Furthermore, soluble enzymes contaminate the desired product and, many times, cannot be recovered from the reaction medium in an active form [4]. Therefore, the application of immobilized lipases can become a promising alternative considering the problems associated to homogeneous biocatalysis. Immobilization consists in confining, physically or chemically, the enzyme on an insoluble solid support in aqueous medium or organic solvent, obtaining a heterogeneous biocatalyst with high activity, specificity, selectivity and stability [5]. Immobilization has been considered the most promising technique to make the application of enzymes competitive in the industrial sector[6]. The application of immobilized enzymes allows the acquisition of heterogeneous biocatalysis that are more robust and resistant to changes in the reaction environment (increase in enzyme stability), their reuse and ease in the separation of the product, besides the possibility of implementing and conducting continuous processes [3,6]. Lipase immobilization has been employed as an essential tool to improve enzymatic activity, selectivity, specificity, stability and purity, as well as to confer resistance to the biocatalyst against inhibitory agents [4].

Among the organic supports, polyhydroxybutyrate (PHB) stands out, a biopolymer produced by microorganisms as a carbon and energy reserve [7]. PHB is prominent for its characteristics, such as biodegradability and good mechanical resistance, in addition to being inert, non-toxic, biocompatible and hydrophobic, characteristics that are desirable for enzyme immobilization [3,6]. The surface of the support can also be functionalized with the insertion of reactive groups that react with nucleophilic groups of the enzyme, providing greater resistance and rigidity to the structure of the heterogeneous biocatalyst against denaturing agents (heat, organic solvents and extreme pH values), besides minimizing the phenomenon of enzyme desorption from the support [6]. Glutaraldehyde is one of the most used reagents for the functionalization of supports, as a spacer arm, given the simplicity of the activation methods used and the acquisition of active and stable enzyme preparations [3]. The glutaraldehyde molecule reacts with the support and the enzyme, which, in turn, is covalently immobilized on the support by a reaction with its amino groups, that bind to the aldehyde groups of the activated support [6]. Considering the above mentioned, the aim of this work was to optimize, by experimental design, the temperature and pH for the hydrolysis of olive oil by the reaction catalyzed by a lipase of an amazon filamentous fungus, immobilized in functionalized PHB.

2 MATERIAL & METHODS

The extracellular lipase was produced by an amazon filamentous fungus isolated from bacaba (*Oenocarpus bacaba*) a species of palm tree native to the Amazon Rainforest. Bacaba fruit were harvested in March 2021, at the Community Bosque Menino Jesus, in the municipality of Cametá, Pará, Brazil (2°15'17,0" S, 49°23'47,9" W). The research was registered at the National System for the Management and Access to the Genetic Heritage and Associated Traditional Knowledge – SisGen, under the registration AAC153B.

The submerged cell cultivation occurred in a synthetic medium, pH 5.5, composed of: 5.5 g L^{-1} of K_2HPO_4; 15 g L^{-1} of $KHPO_4$; 0.5 g L^{-1} of $MgSO_4.7H_2O$; 1.0 % (m v^{-1}) of olive oil and 0.2 % of yeast extract (m v^{-1}). The cultivation was conducted in an orbital shaker at 30 °C and 200 rpm for 168 hours, inoculating 500 µL of a suspension of 10^7 spores mL^{-1} of the fungus in *Erlenmeyer* flasks with 50 mL of sterile culture medium. After cultivation, the total content of the flasks was vacuum filtered (cellulose ester membrane filter, 0.45 µm) and the permeate, containing the soluble lipase, was used for the immobilization assays. PHB functionalization was performed following the method adapted [3]. The PHB particles were first washed with ethanol P.A 95 % (v v^{-1}) at the proportion of 1:10 (m v^{-1}) under agitation of 120 rpm and 25 ºC for 6 hours. Subsequently, they were vacuum filtered, dried in an oven for 1 hour and functionalized at 120 rpm and 30 ºC for 18 hours in a solution composed of 25 % glutaraldehyde (v v^{-1}) and sodium phosphate buffer at 5 µmol L^{-1}, pH 7.0.

The immobilization was conducted in a *Dubnoff* Bath at 35 ºC and 175 rpm for 8 hours, adding 1 g of functionalized PHB to 10 mL of enzyme broth, pH 5.5 [7]. The hydrolytic activity of the immobilized enzyme was quantified by titration with NaOH (0.05 mol L^{-1}) using 1 % olive oil (v v^{-1}) as substrate. The enzymatic reaction was performed at 37 ºC and 175 rpm for 5 minutes. One unit of hydrolytic activity (U) was defined as the amount of enzyme that releases 1 µmol of fatty acid per minute of reaction, under the assay conditions. The activities were expressed in U g^{-1} [8].

An experimental design of the type Central Composite Rotational Design (CCRD) was applied for the optimization of the enzymatic reaction of olive oil hydrolysis, whose design matrix (Table 1) was prepared using the software Protimiza Experimental Design®. A total of 11 assays were performed, consisting in 4 factorial points, 4 axial points and 3 repetitions at the central point for the study of the factors: pH (X_1) and temperature (X_2). The levels were defined according to preliminary experimental results. As response variable (Y_1), the hydrolytic activity of the immobilized enzyme (in U g^{-1}) was obtained. The results were analyzed by analysis of variance (ANOVA) [9].

3 RESULTS & DISCUSSION

The experimental design matrix (factors and levels) with their respective responses for the hydrolytic activity of the immobilized lipase is shown in Table 1.

Table 1 Matrix of the *Central Composite Rotational Design* of 11 assays for the response hydrolytic activity of the microbial lipase immobilized on functionalized PHB with the respective real and coded values.

Assay	pH (X_1)	Temperature (X_2) (ºC)	Hydrolytic Activity (U g^{-1})
1	6.0 (-1)	30 (-1)	492
2	8.0 (+1)	30 (-1)	1402
3	6.0 (-1)	44 (+1)	2367
4	8.0 (+1)	44 (+1)	2461
5	5.59 (-1.41)	37 (0)	496
6	8.41(+1.41)	37 (0)	480
7	7.0 (0)	27.1 (-1.41)	978
8	**7.0 (0)**	**46.9 (+1.41)**	**6602**
9	7.0 (0)	37 (0)	994
10	7.0 (0)	37 (0)	954
11	7.0 (0)	37 (0)	975

It was observed that the highest value of hydrolytic activity (6602 U g^{-1}) was obtained for the enzymatic reaction conducted at pH 7.0 and the temperature of 46.9 ºC (Table 1) suggesting the occurrence of a hyperactivation of the biocatalyst when immobilized on the hydrophobic support material. In addition, preliminary results indicated that the hydrolytic activity values of the immobilized biocatalyst decreased at reaction temperatures above 50 ºC. Regarding the assays performed at the central point, it was verified that the values achieved for hydrolytic activity presented low variation, thus indicating good process reproducibility. For this experimental condition, the mean enzymatic activity was of 974.33 ± 20.01 U g^{-1}. Central points that present high variations are not properly controlled, which directly influences the quality of the results obtained with the experimental design [10].

The preliminary screening of olive oil hydrolysis indicated that only temperature (X_2), for the levels of the factors evaluated, showed statistically significant effects on the enzymatic reaction for a significance level of 5 % ($p < 0.05$), as demonstrated in the Pareto Chart (Figure 1). Temperature is a parameter which directly influences enzymatic activity in terms of reaction speed, since the agitation of the molecules increases with the rise in energy and, for this reason, the probability of intermolecular collisions also increases. Nevertheless, the rise in temperature may cause enzyme denaturation, by breaking bonds that stabilize the spatial structure of the protein due to the intense agitation of the molecules [11]. Consequently, studies that demonstrate temperature ranges with the optimal activity for the biocatalysts become fundamental [12].

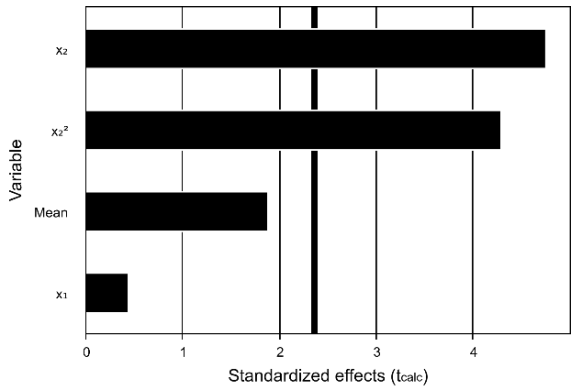

Figure 1 Pareto Chart for the hydrolytic activity. Factors of the experimental design: X_1 – pH of the reaction medium; X_2 – temperature of the reaction medium.

From the results of the experimental design, the statistical model of second order was obtained (Equation 1), in terms of temperature, for the hydrolytic activity of the immobilized microbial lipase, within the ranges evaluated. The regression model presented a correlation coefficient of 85.10 % with the experimental data, suggesting a good adjustment of the model with the values observed. By the *F test*, it was verified that the value of $F_{calculated}$ (22.8) was higher than the value of $F_{tabulated}$ (3.35), which validated the proposed model, at the level of 5 % of significance.

$$U\,g^{-1} = 637.59 + 1360.94\,x_2 + 1398.44\,{x_2}^2 \quad (1)$$

The results of hydrolytic activity indicated that the PHB functionalized with glutaraldehyde was a promising hydrophobic support material for the immobilization of the microbial enzyme. Lipases present a rise in activity when immobilized on hydrophobic supports because of conformational changes that make their active site accessible to the substrate [13]. In the presence of a hydrophobic surface, lipase undergoes interfacial activation, in which its hydrophobic polypeptide chain, called lid, is displaced and the active site is completely exposed to the reaction medium. Thus, the lipase is immobilized in its active conformation[14].

4 CONCLUSION

By studies with experimental design of the CCRD type, it was possible to evaluate the influence of the temperature and pH of the reaction medium on the hydrolysis of olive oil catalyzed by a lipase from an amazon fungus immobilized on PHB functionalized with glutaraldehyde. The best condition achieved in this study occurred in a reaction medium at pH 7.0 conducted at 46.9 ºC for 5 minutes, whose hydrolytic activity was of 6602 U g^{-1}, which suggests the hyperactivation of the biocatalyst when immobilized on the hydrophobic support material.

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