Characterization of mycelium-bound lipase (*Rhizomucor miehei*) using design of experiments methodology

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ABSTRACT

Unlike extracellular microbial lipases that require extensive purification, these mycelium-associated lipases eliminate the purification step, as the product of interest is in the solid part of the culture. One of the most widely used types of factorial design of experiments (DOE) more used in studies of bioprocess is the Central Composite Rotatable Design (CCRD). This study investigates the characterization of lipases associated with the mycelium of *Rhizomucor miehei* regarding pH and temperature parameters employed a Central Composite Rotational Design (CCRD) to assess its effects about the lipolytic activity. Results showed that the lipases exhibited stable activity over a wide range of pH (4.38 to 8.62) and temperature (25.86°C to 54.14°C), with no significant effects from these variables. This suggests that mycelium-associated lipases could be a cost-effective and versatile alternative to traditional enzymes, suitable for various applications.

Keywords: Mycelium-associated lipases. *Rhizomucor miehei*. Design of Experiments (DOE). Biocatalyst stability.

1 INTRODUCTION

Most of the microbial lipases used are extracellular, thus requiring various separation and purification steps involving solvents and chromatographic processes for their use. Additionally, the post-use recovery of the biocatalyst is complex, often necessitating the immobilization of these enzymes on physical supports to facilitate this step[1,2].

The use of the catalyst associated with this fungal biomass eliminates the purification step, as the product of interest is in the solid part of the culture, unlike enzymes obtained through traditional methods, which require purification processes to obtain only the biocatalyst. Furthermore, the cellular matrix with which the lipase is associated acts as a natural support, stabilizing the immobilized enzyme and minimizing the adverse effects of the reaction medium that could reduce its catalytic capacity[3]. Another favorable aspect of using this biomass containing the biocatalyst is its recovery; being a solid matrix, it can be easily separated from the medium containing the product after the reaction and reused in multiple cycles, which also reduces the overall process costs[4].

In the literature, there are some studies on the production of whole cells associated with lipases as biocatalysts for the hydrolysis of various oils into fatty acids, using filamentous fungi from various genera, such as *Rhizopus.* Additionally, studies have reported the use of these enzymes associated with mycelium in the production of other economically important products, such as biodiesel[3,5,6]. However, fungi of the genus *Rhizopus* are not the only ones used in the synthesis of whole cells with associated enzymes. There are also studies involving whole cells of fungi from other genera, such *as Aspergillus*, with various applications ranging from the treatment of pharmaceutical industry effluents using β-lactamases, to the synthesis of biodiesel and bio-solvent esters with lipases from *Aspergillus oryzae* and *Aspergillus niger*[7,8,9,10].

The use of design of experiments (DOE) strategies the field of bioprocesses has proven advantageous[11]. A Central Composite Rotatable Design (CCRD) is a type of factorial that offers significant advantages for bioprocess studies[12]. CCRD is used to helps identify which variables are most relevant to control in the system, requires a relatively small number of experiments compared to a “one-at-a-time” study design and allows for investigating effects and relationships between variables[11].

Nevertheless, studies on the production of this type of biocatalyst with other filamentous fungi, such as *Rhizomucor miehei*, a commercially widely used producer of lipases, are still scarce. This gap highlights the potential for new research on the production of these lipases associated with mycelium for various applications, ranging from the production of biofuels, such as biodiesel, to lipid modification, such as in the production of dietary triacylglycerols[13]. Thus, this study focuses on the characterization of lipases associated with the mycelium of *Rhizomucor Miehei* in order to later explore possible applications for this catalyst.

2 MATERIAL & METHODS

To evaluate the effect of temperature and pH on the substrate hydrolysis capacity of lipases associated with the mycelium of *Rhizomucor miehei*, design of experiments was employed as a strategic tool. A 2^3 Central Composite Rotational Design (CCRD) was applied, wherein, 9 experiment runs were conducted including 4 factorial points (1,-1), 4 axial points (1.41,-1.41) and 3

replications of central points. The dependent variable were pH (x_1) and temperature (x_2), and response variable was hydrolysis activity (Table 1).

Table 1 Actual and coded values of the independent variables on the central composite rotatable design.

Independent variable	-1.41	-1	0	1	1.41
Temperature (°C)	25.86	30	40	50	54.14
pH	4.38	5	6.5	8	8.62

The lipolytic activity of the lipases associated with the fungal biomass was analyzed following the olive oil hydrolysis method[14]. Finally, the statistical analysis of the effects of the variables (pH and temperature) on these responses from the CCRD was analyzed using Protimiza Experimental Design (http://experimentaldesign.protimiza.com.br/).

3 RESULTS & DISCUSSION

Compared to other types of factorial designs such as CCD, CCRD presents advantages, such as the inclusion of axial points, which allows factorial's rotatability providing a more complete analysis of the bioprocess, as it has more comprehensive study ranges[11] (Nascimento 2024). Based on the employed design (CCRD), lipolytic activities were evaluated in the pH range of 4.38 to 8.62 and the temperature range of 25.86°C to 54.14°C, with the results presented in Table 2. The highest relative hydrolytic activity values were obtained at points 3, 100%, and 4, 73.49%, both performed at 50C.

Table 2 Matrix of the 2^3 factorial composite central rotatable design showing coded (in parenthesis) and real values of each independent variable and relative activity (%).

Batch	pH	Temperature (°C)	relative activity (%)
1	5 (-1)	30 (-1)	54.66
2	8 (1)	30 (-1)	57.01
3	5 (-1)	50 (1)	100
4	8 (1)	50 (1)	73.49
5	4.38 (-1.41)	40 (0)	26.95
6	8.62 (1.41)	40 (0)	55.35
7	6.5 (0)	25.86 (-1.41)	61.46
8	6.5 (0)	54.14 (1.41)	50.83
9	6.5 (0)	40 (0)	70.15

With the statistical treatment of the results obtained regarding hydrolytic activity (Table 2), a Pareto chart (Figure 1) was generated, where the effects of the variables pH and temperature on the process can be observed. Observing only the relative activity values presented in Table 1, a variation of about 50% can be noted within the studied range. The statistical treatment revealed that the effects of both linear and quadratic variables pH (x_1) and Temperature (x_2), as well as the interaction between them ($X_1.X_2$), did not present significant effects ($p<0.05$) on the relative activity of the lipase bound to the mycelium within the range studied in this work.

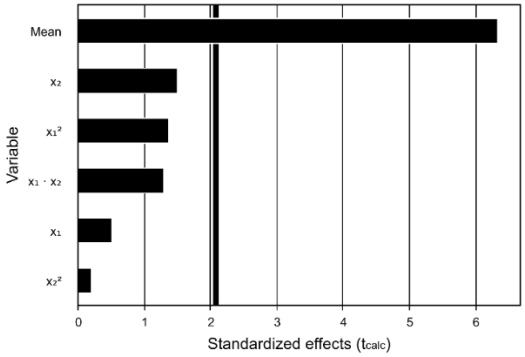

Figure 1 Pareto plot obtained by Protimiza®.

In Figure 1 (Pareto chart) generated with the aid of the software, it is observed that neither the individual variables nor their interaction causes a considerable effect on the lipolytic activity of these lipases. This result is confirmed by analyzing the R^2 value obtained when attempting to fit a model to describe the influence of these parameters on the studied biocatalysts. Therefore, with a low R^2 (24.42%), the model does not fit into the range studied, and it is likely that there is little influence of pH and temperature on the activity of this catalyst. This is reinforced when we look at table 2 and see that there is minor variation in the response, enzymatic activity, in the different conditions described.

However, this result is interesting considering the evaluated range of the parameters (pH from 4.38 to 8.62 and temperature from 25.86°C to 54.14°C). Given the broad intervals, the indication that the lipases present in the mycelium have the capacity to function in both more acidic and alkaline reaction environments, as well as across a wide temperature range (considering it is a biocatalyst), is advantageous for the application of this biocatalyst different bioprocess. This behavior is expected for enzymes associated with mycelia, as the biomass itself acts as a protection against possible effects that temperature and compounds in the reaction medium might have on these catalysts, similar to the effect observed with enzymes immobilized on physical supports[2,4].

4 CONCLUSION

Therefore, it can be concluded that lipases associated with the mycelium of *Rhizomucor miehei* exhibit good lipolytic activity over a wide range of pH and temperature, without significant effects from these variables This low variation in activity over this wide range suggests that these biocatalysts may be suitable for diverse reactions, offering a more economical and versatile alternative compared to enzymes obtained through traditional methods.

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