CHARACTERIZATION OF A BETA-GLUCOSIDASE FROM A FUNGUS ISOLATED FROM MACAUBA CAKE BIOMASS

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ABSTRACT

Macaúba, *Acrocomia aculeata*, is a palm tree present in a large part of the Brazilian territory. Its large oil production capacity to produce biodiesel and value-added products, such as Macaúba cake, make the use of Macaúba in a biorefinery interesting. Filamentous fungi isolated from the Macaúba cake itself, can produce the enzymes, like β-glucosidase, necessary to degrade the lignocellulosic material. In the present work, five different morphotypes of filamentous fungi were isolated from Macaúba cake biomass from biodiesel industry and were investigated for β-glucosidase production capability. Fungus 5R1D0-SD1 presented an activity of 6.9±1.9 U/mg of β-glucosidase in the extract when cultivated in Macaúba cake as unic carbon source. The β-glucosidase in the extract was characterized. A central composite rotational design (CCRD) was performed to analyze the optimum pH and temperature combination, obtaining a relative activity of at least 60% in the temperature range between 40° and 55° Celsius and at pH between 3.5 and 6. Enzyme kinetics study was also performed and the values of 66.64±5.98 μM/min and 1.12±0.2 mM were obtained for V_{max} and K_m, respectively. These results show the potential of Macaúba in a biorefinery and the capacity of the fungus 5R1D0-SD1 to produce β-glucosidase.

Keywords: Beta-glucosidase. Macaúba. Filamentous Fungi. Biorefinery.

1 INTRODUCTION

The biorefinery is part of a sustainable economic model which value is added to the products that are generated by biomass, such as bioenergy, bioproducts and biofuels.[1] In the context of biorefinery, the use of Macaúba for the production of biofuels, especially biodiesel, stands out for having high oil productivity and a large amount of by-products, such as Macaúba cake.[2] Macaúba cake, a by-product of the biodiesel industry, is a rich biomass that has great potential for biotechnological use, and could be employed for food, cosmetics, and energy purposes.[3]

Filamentous fungi produce all the enzymes necessary to degrade cellulose, hemicellulose, and lignin.[4] Therefore, filamentous fungi play an important role in the production of second-generation biofuel.[5] Among the enzymes with significant industrial value, β-glucosidases stand out as particularly interesting.[6] These enzymes play a crucial role in various processes, including winemaking, biofuel production, and the manufacturing of juices, beverages, and functional foods. As key cellulolytic enzymes, they facilitate the hydrolysis of cellobiose into glucose. β-glucosidase activity is essential for overcoming the inhibitory effect of cellobiose on other cellulolytic enzymes, such as endoglucanases and cellobiohydrolases.[6]

In this study, we characterized a β-glucosidase from a fungus, isolated from Macaúba cake biomass from biodiesel industry, cultivated in Macaúba cake biomass as unic carbon source.

2 MATERIAL & METHODS

Fungi were isolated from Macaúba cake biomass with mineral medium. The enzymatic extract was prepared by cultivation of morphotypes isolated in 15% w/v of Macaúba cake biomass and mineral medium. For this, 125mL Erlenmeyer flasks were used, in which two discs of fungi (replicated in YPD and incubated at 28° Celcius for 7 days) were placed in each one and three repetitions were made for each type of isolated fungus. After five days, acetate buffer (pH 5.0 100mM) was added. The extract was filtered using gases and centrifuged for 10 minutes at 3.500 r.p.m.

The β-Glucosidase activity was carried out through the colorimetric determination of p-nitrophenol, released by the hydrolysis of the synthetic substrate p-nitrophenyl-β-D-glucopyranoside (pNPG) catalyzed by the enzyme β-Glucosidase. The substrate used was pNPG 2mM, diluted 1:1 in acetate buffer 100mM pH5.0. The activity measurement assay was performed in a microplate, where 40μL of acetate buffer, 10μL of enzymatic extract, and 50μL of pNPG were pipetted, and the mixture was incubated in a thermocycler at 50° Celsius for until 60 minutes. After the incubation period, 100μL of 1M sodium carbonate was added to stop the reaction. 150μL of the sample was transferred to the microplate reader and analyzed at λ= 405nm. One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyzes the release of one μmol of product per minute. The enzymatic activity was calculated in U/mL and plotted on the graph based on the morphotype producing the extract. The total protein concentration was estimated using the Bradford method, and the activity was calculated based on this concentration (U/mg).

The influence of pH on enzymatic activity was carried out by assays performed at 50° Celsius in citrate-phosphate buffer (100 mM, pH = 3.0–7.0). The temperature study was performed by measuring the enzymatic activity at pH = 5.0 (100 mM acetate buffer) in the temperature range 20° Celsius – 80° Celsius. Additionally, a complete factorial central composite rotational design (CCRD) for two factors, temperature and pH, was performed to investigate the influence and interaction of these two factors at the same time in β-glucosidase activity.

The enzyme kinetic parameters were obtained by measuring the initial rate of pNPG hydrolysis (V_0) at various substrate concentrations ranging from 0.05 to 3.0 mM in the standard reaction mixture (at 50° Celsius in 100 mM sodium acetate buffer, pH = 5.0). The apparent Michaelis constant (K_m) and the maximal reaction velocity (V_{max}) were assessed from hyperbolic Michaelis-Menten plot.

3 RESULTS & DISCUSSION

From the isolation process, five distinct morphotypes of filamentous fungi were obtained from a Macaúba cake biomass obtained in biodiesel industry. The morphotypes were named according to the biomass-to-medium weight/volume ratio and the repetition from which the aliquot was taken, in addition to the day it was collected (30R3D7-esporos, 15R1D7-1000, 5R1D1-SD, 5R1D0-SD1, 5R2D2-10). The five morphotypes of isolated fungi were cultivated by seven days in a medium containing Macaúba cake as the primary substrate, in a solid-to-liquid ratio of 15% (w/v), added by salt medium. The mixture was used for obtaining enzymatic extracts.

β-glucosidase activity assays were conducted to investigate the presence of this enzyme in the enzymatic extracts obtained from the cultivation of the morphotypes on Macaúba cake. In the initial 60-minute activity measurement attempt, two out of the five fungal morphotypes showed insignificant activity (15R1D7-1000 and 5R1D1-SD), while one morphotype exhibited a relatively low activity value (30R3D7-esporos), and other two morphotypes (5R2D2-10 and 5R1D0-SD1) demonstrated moderate and high activity levels, respectively (Figure 1).

Another β-glucosidase activity assay was conducted based on incubation time, using the extracts related to the two fungal morphotypes that showed the best results in the initial test (5R2D2-10 and 5R1D0-SD1). The activity was measured at 8, 12, 16, and 20 minutes. The fungus 5R1D0-SD1 presented an activity of 6.9±1.9U/mg and 0.38±0.02U/mL and fungus 5R2D2-10 1.2±0.5 U/mg at 12 minutes. According to literature, *Penicillium pinophilum*, grown on wheat bran as biomass, produces 0.15±0.0U/mL.[7] This shows the potential of Macaúba cake biomass as a substrate to stimulate the production of β-glucosidase.

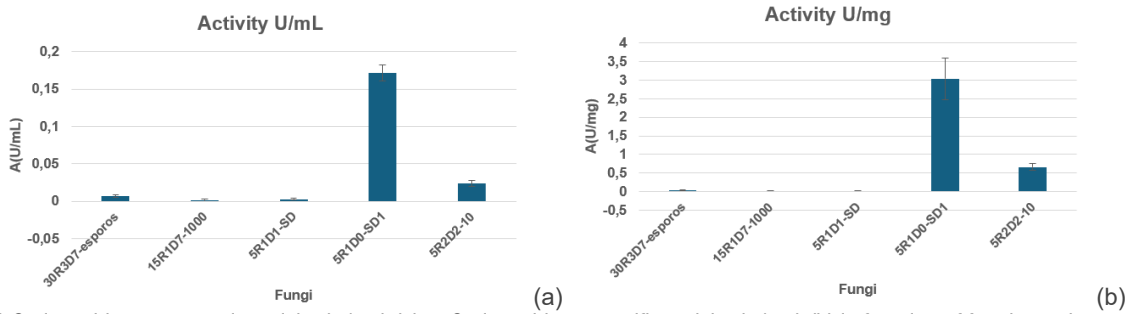


Figure 1. (a) β-glucosidase enzymatic activity (U/mL) (a) or β-glucosidase specific activity (U/mg) (b) in function of fungi morphotype detected in enzymatic extracts produced by cultivation of fungi in Macauba cake as sole carbon source. The values are means and deviations from two repetitions.

The fungus 5R1D0-SD1 was chosen for a more in-depth study in which pH and temperature influences in extract enzymatic activity were investigated.

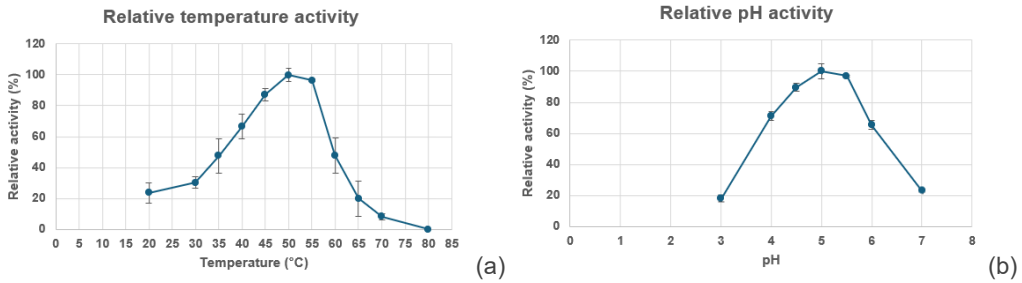
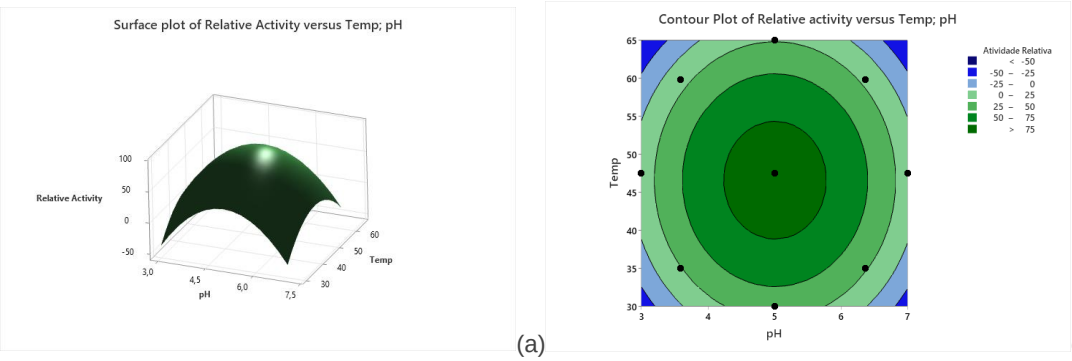


Figure 2. (a) β-glucosidase relative activity in function of temperature (°C) in acetate buffer 50mM pH5.0. (b) β-glucosidase relative activity in function of pH in citrate-phosfate buffer 50mM at 50°Celsius. The values are means and deviations from three repetitions.

The enzimatic extract charatherized presented optimal β-glucosidase activity at 50°Celsius and pH 5.0 (Figure 2). But the fungus enzimatic extract still maintains a relative activity of at least 50% in the temperature range between 40° and 60°Celsius and at pH between 3.5 and 6.5 (Figures 2 and 3) and a good activity maintenance is observed in various pH and temperature combinations in that interval (Figure 3).

Fungal β-glucosidase usually have an pH optimum between 4 to 6.5,[6] presenting an important characteristic for the industry, mainly in biomass hydrolysis processes. One β-glucosidase that present relative high activity in temperatures above 40°C is important to SSF (simultaneous sacarification and fermentation) process in ethanol production.[8] The extracts containing β-glucosidase activity charactherized in this work could be applied in this propose.



(a) (b)

Figure 3. Surface (a) and Contour (b) plots for relative activity (adjusted values) of β-glucosidase in function of temperature (°C) and pH obtained by response surface analysis and adjust.

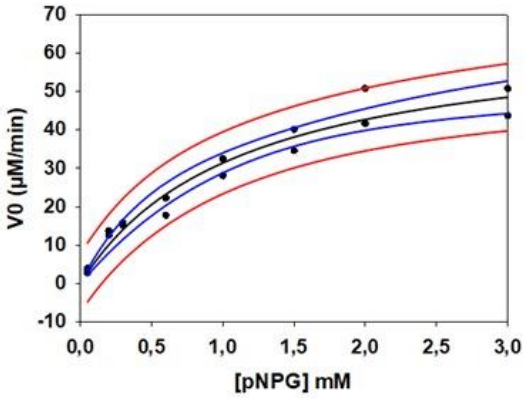


Figure 4. Michaelis-Menten plot.

For study the kinetics potential of the β-glucosidase present in the extracts, the change of initial velocity with pNPG concentration was analyzed using the non-linear Michaelis-Menten equation and the apparent values for Michaelis-Menten parameters were estimated. The K_m and V_{max} values estimated were 1.12±0.2mM and 66.64±5.98µM/min, respectively. Km values between 0.1 and 44 mM of pNPG have been reported for fungal β-glucosidases, and these variations could be related to differences in enzyme assay conditions and substrate preferences.[8] *Aspergillus sp. DHE7* presented a K_m of 0.4mM, showing a high affinity of the enzyme with pNPG.[8] The K_m and V_{max} values presented by the 5R1D0-SD1 fungus enzymatic extract revel great potential of these extracts in be applied for biomass scarification and other biotechnological applications, specially taken in count that the enzyme was not purified for the characterization.

4 CONCLUSION

In the present work we demonstrate the potential use of Macaúba cake as unic carbon source for production of enzymatic cocktail with beta-glucosidase activity to be applied in ethanol production. The development of process sustainable and based in biodiversity like this is important to integrate the two major biofuels matrixes in Brazil: biodiesel and bioethanol.

REFERENCES

[1] SALVADOR, G. 2021. Biorrefinaria de Macaúba em Pequena Escala: Uma Análise Técnica, Econômica, Ambiental e Geoespacial. Dissertação (Mestrado) – Programa de Pósgraduação em Engenharia Química, Universidade Federal do Rio de Janeiro.

[2] BIESDORF, E. 2021. Caracterização e aproveitamento de biomassa foliar senescida de Macaúba (Acrocomia aculeata (Jacq.) Lodd. Ex Martius).

[3] ANISHA, G. S. 2022. Microbial α-galactosidases: Efficient biocatalysts for bioprocess technology. Bioresource Technology. Volume 344, Part B. DOI: https://doi.org/10.1016/j.biortech.2021.126293

[4] TROIANO, D.; ORSAT, V.; DUMONT, M. 2020. Status of filamentous fungi in integrated biorefineries. Renewable and Sustainable Energy Reviews, 117, 109472. 10.1016/j.rser.2019.109472

[5] MHLONGO, S.; EZEOKOLI, O.; ROOPNARIN, A.; NDABA, B.; SEKOAI, P.; HABIMANA, O.; POHL, C. 2021. The Potential of Single-Cell Oils Delivered From Filamentous Fungi as Alternative Feedstock Source for Biodiesel Production. https://doi.org/10.3389/fmicb.2021.637381

[6] RATUCHNE, A., KNOB, A. 2021. A new and unusual β-glucosidase from Aspergillus fumigatus: Catalytic activity at high temperatures and glucose tolerance, Biocatalysis and Agricultural Biotechnology, Volume 35. https://doi.org/10.1016/j.bcab.2021.102064.

[7] ALBUQUERQUE, M. F. G.; LEAL, T. F., ÁZAR, R. I. S. L.; MILAGRES, A. M. F.; GUIMARÃES, V. M.; REZENDE, S. T. 2021. Hydrothermal Pretreatment as a Strategy for the Improvement of Sugarcane Bagasse Saccharification by Fungal Enzyme Blend. Brazilian Archives of Biology and Technology, Volume 64, ISSN 1678-4324. https://doi.org/10.1590/1678-4324-2021200422

[8] El-GHONEMY, D. H. 2021. Optimization of extracellular ethanol-tolerant β-glucosidase production from a newly isolated Aspergillus sp. DHE7 via solid state fermentation using jojoba meal as substrate: purification and biochemical characterization for biofuel preparation, Journal of Genetic Engineering and Biotechnology, Volume 19, Issue 1, 45, ISSN 1687-157X. https://doi.org/10.1186/s43141-021-00144-z.

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