EXPLORING THE POTENTIAL OF PROTEOLYTIC ENZYME PRODUCTION BY FILAMENTOUS FUNGUS

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ABSTRACT

Microorganisms are capable of synthesizing a wide variety of secondary metabolites, including antibiotics, pigments, enzymes and other molecules. Among these, proteolytic enzymes that can break down complex molecules into smaller components, facilitating the transformation of biomass into value-added products, are driving the biotechnology market. To assess the feasibility of producing proteolytic enzymes by filamentous fungi, this capacity was evaluated in 10 fungal strains. Protease activity was determined using crude extracts (0.05 mL) that were subjected to quantitative evaluation using 1% casein (0.25 mL) as substrate in 0.15 M phosphate buffer and incubated at 35 °C for 30 minutes. The reaction was stopped using 0.4 M TCA (0.5 mL), followed by centrifugation for 5 minutes at 10,000 rpm. Enzymatic tests revealed different activities in each class tested, with varying growth patterns throughout the incubation period (24 to 168 h). Among the fungi evaluated, protease production peaked at 96 hours of incubation, in a stationary system, followed by a considerable drop in the following periods. The best producer was the fungus PA2A1TV (231 U/mL) on the fourth day. From these results we understand that filamentous fungi, especially thermophilic ones, are effective producers of proteolytic enzymes.

Keywords: Enzyme, Protease, Filamentous Fungus, Biotechnology.

1 INTRODUCTION

Microorganisms are capable of synthesizing a wide variety of secondary metabolites, including antibiotics, antifungals, antivirals, antitumor agents, antiparasitics, pigments, enzymes, organic acids, and other bioactive molecules. These compounds can be naturally produced by bacteria, fungi, and other microorganisms as part of their life cycle and in response to specific environmental stimuli[1,4]. In recent years, many of these secondary metabolites have been isolated and characterized through advanced analytical techniques and molecular biology. This characterization involves identifying the chemical structure of the metabolite, its biological activity, and its potential industrial applications. Thus, microorganisms have become valuable sources of bioactive compounds with great potential in the pharmaceutical, cosmetics, food, agricultural, and chemical industries[2,3].

The application of microorganisms for the production of biotechnologically relevant enzymes is widely utilized in industry, as their enzymes can modify the chemical composition of raw materials and consequently influence the final composition of products. Among the microorganisms used in the production of biotechnological products, fungi have played a significant role. These fungi possess unique metabolic characteristics, including a remarkable ability to secrete enzymes such as amylases, proteases, and cellulases, which are essential in many biotechnological processes[4]. However, it is important to note that the manipulation and use of these fungi require appropriate precautions to ensure biological safety and prevent risks of contamination and unwanted dissemination[5].

Proteases (EC 3.4.21-24 and 99), represent a fundamental class of enzymes that play a crucial role in the hydrolysis of peptide bonds (CO-NH), promoting the degradation of proteins and resulting in the formation of peptides or free amino acids. These enzymes are classified as peptidic hydrolases, constituting a vast family with distinct functions[6]. They are complex biomolecules, and their conversion rates depend on their chemical transformations, resulting in facilitated reactions with shorter times and lower production costs due to their specificity. This specificity allows them to have a positive impact on the environment and production lines, as they are natural substrates and do not have intensely harmful effects on the environment and people handling them. There is no need for costly safety precautions and equipment, as is necessary with harmful chemicals and acids[2,6 8].

Thus, it is understood that the hydrolytic enzyme secreted by filamentous fungi have significant potential for various biotechnological applications. These enzymes can break down complex molecules, such as polymers and organic compounds, into smaller components, facilitating the transformation of biomass into value-added products. Additionally, filamentous fungi are highly efficient microorganisms in the production and secretion of these enzymes, making them ideal candidates for exploring this biotechnological potential. This fact is driving the search for sustainable and efficient production alternatives, providing an intuitive understanding of the diversity of fungi and their enzymatic capabilities. Therefore, the present study aims to evaluate the proteolytic enzyme production capacity of filamentous fungi.

2 MATERIAL & METHODS

To evaluate enzymatic viability, 10 fungal strains were isolated from soil samples collected in a fragment of Atlantic Forest located in the municipality of Nova Aurora, Paraná, Brazil, between 24° 32' 00" South and 53° 15' 10" West, at an altitude of 520 meters

above sea level, and they are available at the Microorganism Biochemistry Laboratory of the Western Paraná State University were assessed. The preparation of inoculum was a crucial step in the study's development, as the prepared inoculum was used for culture and enzyme production throughout the process. Initially, test tubes containing BDA medium (Potato Dextrose Agar) – Glucose (1.5%), Agar-Agar (2%), and Potato (20 g) were prepared. After 6 days of incubation, inocula were obtained from the established cultures. For this purpose, a solution containing 5 mL of 0.8% NaCl and 0.05% Tween 80 was added to the cultures. The spores were then resuspended using a rod, promoting their uniform dispersion in the solution.

Protease activity was determined using the method described by Fleuri and Sato (2008)[7], with some modifications. The crude extracts were subjected to quantitative proteolytic evaluation using casein as a substrate in 0.15 M phosphate buffer. A volume of 0.05 mL of the extracts was added to 0.25 mL of casein (1.0%), prepared in 0.15 M phosphate buffer, pH 7.5. Subsequently, the enzymatic solution was incubated at 35 °C for 30 minutes. The reaction was stopped by adding 0.5 mL of 0.4 M trichloroacetic acid (TCA) solution, followed by refrigerated centrifugation for 5 minutes at 10,000 rpm. One unit of proteolytic activity was defined as the amount of enzyme capable of producing an increase in absorbance of 0.01 in 1 hour at 280 nm, expressed in U/mL, according to Kirsch *et al.* (2011)[9]. All assays were performed in triplicate. For the reaction blank, all compounds were added as in the reaction, except that the TCA to stop the reaction was added before the crude extract.

To optimize the production process and evaluate the biotechnologically relevant enzymes produced by the microorganisms, the enzymatic extracts were inoculated into Czapeck culture medium, supplemented with soybean fiber and orange peel (1:1) at 1.5%, for 7 days, in a stationary and rotating system at 120 rpm.

3 RESULTS & DISCUSSION

The enzymatic tests revealed different activities in each class tested, with varying growth patterns over the incubation periods (24, 48, 72, 96, 120, 144 and 168 hours). The results of the proteolytic activity across different cultivation systems and submerged fermentation conditions showed a significant difference ($p<0.05$) in protease production among the 10 species evaluated. Most of the systems analyzed showed greater production in submerged fermentation under agitation, which proved to be the best format for allowing nutrients to come into contact with the hyphae, thereby increasing protease activity. However, in the case of the PA2A1TV fungus, the correlation between agitation and increased activity was not corroborated, as activity decreased significantly (Figure 1).

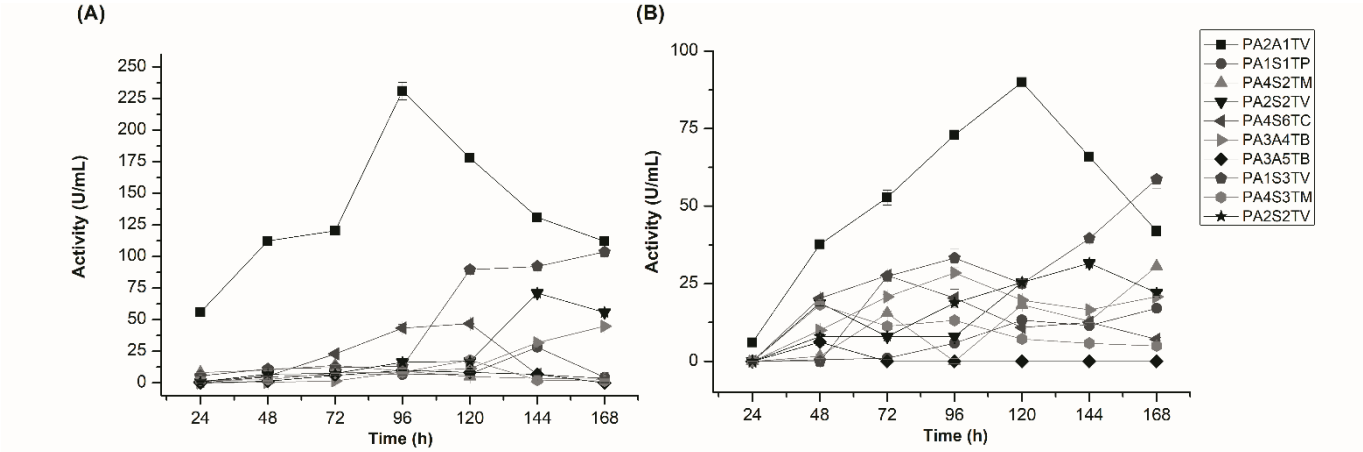

Figure 1 Quantification of the proteolytic activity of the 10 fungal strains, was conducted in Czapek culture medium, supplemented with soybean fiber and orange peel (1:1) at 1.5%, over 7 days, in two systems; (A) stationary; and (B) 120 rpm rotational.

Among the fungi evaluated, protease production peaked was at 96 hours of incubation, under a stationary system, followed by a considerable drop in the subsequent periods. The highest activity was shown by the fungus PA2A1TV (231 U/mL) on the fourth day, followed by PA1S3TV (103.6 U/mL) on the seventh day and PA1S5TV (84.1 U/mL) on the fifth day, generally outperforming the other fungi.

The noticeable quantitative difference in protease production between the fungi studied may be due to the physiological differences present in the microorganisms under the different cultivation conditions used. This is corroborated by the studies of Merheb *et al.* (2007)[10] and Fernandes *et al.* (2012)[11] which show that the production of proteases by fungal cultures varies depending on the medium. It can be understood that the variations in cultivation conditions can either increase or decrease proteolytic activity exponentially.

Proteolytic enzymes play a crucial role in the metabolism of microorganisms, enabling them to utilize proteins as sources of carbon and nitrogen. However, the efficacy of production and secretion of these enzymes can be highly variable, influenced by factors such as the conditions of the culture medium and the nature of the protein substrate. Thus, standardizing a specific culture medium becomes a necessary challenge to allow precise and comparative evaluation of enzymatic capacity among different fungal species. This standardization would facilitate a better understanding of enzymatic degradation profiles and the optimal conditions for protease production, thereby advancing biotechnology and industrial processes reliant on these enzymes.

The genera Aspergillus and Penicillium are among the most economically significant fungi, known for their extensive contributions to diverse industries. Belonging to the Aspergillaceae family, these fungi are prolific producers of a variety of metabolites and enzymes that hold substantial biotechnological value, including cellulases, amylases and proteases[12]. Their ability to produce these enzymes makes them invaluable in processes such as breaking down complex carbohydrates and proteins, which are essential in countless industrial applications, ranging from food production to pharmaceuticals.

These fungi are classified as GRAS (Generally Recognized as Safe) due to their low toxicity, making them suitable for use in the food and pharmaceutical industries without posing significant health risks. In addition to their enzymatic capabilities, Aspergillus and Penicillium are also known for producing a wide range of bioactive substances. These bioactive compounds have applications in medicine, agriculture and biotechnology, further increasing the economic importance of these genera. Their proficiency in the production of hydrolytic enzymes, particularly proteases, highlights their role in biotechnological advances. Proteases are crucial to several industrial processes, including the production of detergents, the processing of food products, and the development of therapeutic agents. The ability of Aspergillus and Penicillium to efficiently produce these enzymes underscores their potential as sustainable and effective resources for biotechnological innovation[12,13,14].

Thus, it is understood that there is no culture medium that is 100% effective in evaluating the production of these enzymes excreted by different microorganisms. Therefore, the development of a standardized medium is essential for differentiating fungal species that produce proteases and for assessing the various substrate degradation profiles used in enzymatic evaluation tests[3].

The catalytic properties of proteases vary according to the strain of the microorganism, the bioprocess used to obtain the enzyme, the type of protein substrate that may inhibit or stimulate protein synthesis, and the microorganism's ability to adapt to the culture medium and secrete enzymes to obtain carbon and nitrogen sources. Additionally, the microorganism may secrete proteases that are incapable of degrading the proteins present in the culture medium[10]. This characteristic is related to the presence of specific cleavage sites in the protein used in the in vitro test[15].

4 CONCLUSION

With these results, we understand that filamentous fungi, particularly thermophiles, are effective self-producers of proteolytic enzymes, with the highest production observed in the PA2A1TV strain (231 U/mL). Additionally, we recognize that fungal physiology exhibits significant variation in the process of fungal optimization, indicating that changes in the composition of the cultivation system can substantially affect enzyme production capacity. This enzyme production system not only contributes to the economic system by providing alternative enzyme production methods for industry, thereby fostering a more sustainable economy, but also benefits the environment by reducing agro-industrial waste and generating sustainable alternatives for its use.

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