*Bioengineering*

AIMS Bioengineering, 12(1): 162–176.
DOI: 10.3934/bioeng.2025007
Received: 28 October 2024
Revised: 18 February 2025
Accepted: 25 February 2025
Published: 12 March 2025

https://www.aimspress.com/journal/Bioengineering

*Research article*

# Exploring the potential of fungal proteases in aquatic environments of the Tapajós Basin, Brazil

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**Abstract:** In this study, we focused on aquatic environments and explored the fungal biodiversity and protease production in the Tapajós River Basin in Pará, Brazil. Our objectives were to identify and characterize fungal genera with potential protease activity and evaluate their biotechnological

applications. Variations in the enzymatic activity index, colony diameters, and halo sizes were documented. For molecular identification, genomic DNA was isolated and analyzed using both the ITS region and the 28S rRNA region, which provided additional resolution for the most promising isolate. *Penicillium citrinum* UFOPA-MI0019 exhibited significant protease activity, suggesting potential applications in biotechnological processes. We also included the kinetics of protease production of *P. citrinum* UFOPA-MI0019 under solid-state fermentation using wheat bran, indicating a peak (340 ± 30 UI/mL) in enzyme activity at 72 hours of fermentation at an optimal temperature of 30 °C. These findings highlight the ecological role and industrial potential of Amazonian fungi, contributing to sustainable waste transformation.

**Keywords:** fungal biodiversity; protease production; Tapajós River Basin; enzyme kinetics; solid-state fermentation; aquatic fungi; *Penicillium citrinum*; biotechnological applications

## 1. Introduction

Proteases are essential enzymes in industrial processes and play pivotal roles in the biochemical sector, including applications in detergents, pharmaceuticals, and food industries [1–2]. Their ability to catalyze the breakdown of proteins into peptides and amino acids makes them invaluable in processes such as waste management, protein recovery, and the manufacturing of leather goods [3]. Given their broad utility, there is a continuous demand for proteases that are more efficient, stable, and adaptable to harsh industrial conditions [4]. This demand underscores the necessity for ongoing discovery and development of novel proteases that can meet or exceed the performance of available enzyme preparations [5]. The Amazon region, characterized by its vast biodiversity and dynamic ecological processes, presents a largely untapped reservoir for novel bioactive compounds, including enzymes [6]. The dense and diverse microbial life fostered by the unique environmental conditions of the Amazon suggests a high potential for the presence of fungal species capable of producing distinctive proteases with possibly superior characteristics [7]. Despite this potential, the region remains underexplored in enzymatic studies focused on industrial applications [8]. The exploration and characterization of Amazonian fungal biodiversity for protease production are essential not only for expanding the global enzyme catalog but also for leveraging region-specific resources, which could drive innovations in enzyme technology [9].

In industrial contexts, the discovery of new fungal-derived proteases from the Amazon could revolutionize existing production paradigms by introducing enzymes with novel properties such as enhanced thermal stability, altered pH optima, or increased substrate specificity [10]. These traits are particularly valuable for industries operating under extreme conditions where traditional proteases may fail [11]. For instance, proteases with higher thermal stability can be integral to industries requiring high-temperature processes, such as in the manufacturing of bioethanol, where they can significantly improve the efficiency of biomass conversion [12]. Here, we propose the hypothesis that fungi from the Tapajós River Basin may produce proteases with significant biotechnological applications. Our objectives were to identify fungal isolates from aquatic environments, assess their protease production, and analyze their enzymatic kinetics under solid-state fermentation. By leveraging the region's

biodiversity, this research provides a foundation for exploring the ecological and industrial relevance of Amazonian fungi.

## 2. Materials and methods

### *2.1. Fungal strains*

The fungal isolates used in this study were collected from water samples in the Tapajós River Basin (western Pará state, Brazil) at coordinates (2.628225613108789, -55.085873686435995). This collection was supported by The Nature Conservancy's Tapajós Waters project in collaboration with the Federal University of Western Pará. These isolates were stored in the didactic collection of the Mycology and Bioassays Laboratory. Strain screening based on mycelial growth led to the selection of 14 strains, which were reactivated on potato dextrose agar - PDA (Kasvi®) and subsequently used in the following stages in this study.

### *2.2. Wheat bran*

The wheat bran used in this study was obtained as a by-product of the industrial milling process provided by Bunge Food S.A., located in São Paulo, Brazil. The wheat bran adhered to stringent quality specifications, including a maximum moisture content of 14%, minimum crude protein content of 14%, maximum crude fiber content of 8%, maximum ash content of 5%, and maximum fat content of 4%. Furthermore, the particle size distribution indicated that 90% of the bran passed through a 20-mesh sieve. These specifications ensured the consistency and reliability of the wheat bran used in the experimental procedures.

### *2.3. Conventional and molecular identification of isolates*

The conventional identification of fungal isolates was conducted through microcultures, focusing on the observation and identification of vegetative and reproductive structures. We utilized identification keys referenced from established literature sources [13,14]. For molecular identification, genomic DNA isolation was performed following a previously described protocol. A portion of the fungal mycelium was carefully excised and cryogenically macerated using liquid nitrogen to facilitate cell lysis. Genomic DNA was then extracted and purified using the Wizard® Genomic DNA Purification Kit (Promega), following the manufacturer's standardized protocol to ensure high-yield and high-purity DNA for subsequent molecular analyses.

The internal transcribed spacer (ITS) region and the 28S ribosomal RNA (rRNA) gene were targeted for sequence analysis. The ITS region was amplified using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), while the D1/D2 domain of the 28S rRNA gene was amplified using the primer pair NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') [15,16]. PCRs were performed using the following reagent concentrations: 3.0 µL $MgCl_2$ (20 mM), 5.0 µL of the 10× buffer, 3.0 µL dNTP mix (20 mM each), 0.6 µM of each primer, 2 U of the Taq DNA polymerase

(Cellco), 1 µl of template DNA, and the total PCR volume adjusted to 50 µl with sterile ultrapure water. The amplification conditions were 95 °C for 4 min and 35 cycles at 95 °C for 1 min, 52 °C for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 5 mins in a ProFlex TM 3 × 32-Well PCR System thermocycler (Thermo Fisher Scientific). Amplicons were visualized on a 1.0% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 60 V for 60 min, stained with Blue Green dye, and visualized on an LT blue light transilluminator BLUE (Loccus). The PCR products for sequencing were dried in an oven at 60 °C before being sent to the company ACTGene Molecular Analysis (https://actgene.com.br) for sequencing. The nucleotide sequences of D1/D2 and ITS generated from the yeast isolates were checked, compiled, and edited using the BioEdit 7.0.9.0 program [15]. The DNA sequences from related species that were retrieved from the National Center for Biotechnology Information (NCBI) database using BLASTn [16] were compared to those obtained and considered when values were above 99% similarity.

### *2.4. Screening of fungi for protease production*

For the induction of protease enzyme, the solid agar-milk culture medium was prepared [17], comprising 5.0 g of skim milk, and 1 g of nutrient agar in 100 mL of distilled water. The medium was sterilized and poured into 90 mm diameter Petri dishes, where two approximately 5 mm mycelial discs were inoculated onto the surface. Plates were then incubated in a Biological Oxygen Demand (BOD) incubator at 28 ± 2 °C for five days, with the formation of translucent enzymatic halos observed every 24 hours without the need for revealing substances. Upon formation, the diameters of both the enzymatic halos and the colonies were measured using a ruler. The Enzymatic Activity Index (EAI) was determined using Equation 1 as described by Florencio et al., [18], whereby a microorganism is considered a proficient enzyme producer in solid medium when the EAI value equals or exceeds 2.0 [18].

Equation 1: EAI = diameter of halo (mm) / diameter of colony (mm).

### *2.5. Kinetics of protease production by Penicillium citrinum Thom UFOPA-MI0019*

For inoculum preparation, the selected strain for solid-state fermentation was subcultured on PDA at 30 ± 2 °C for five days in a BOD incubator. Following growth, mycelium from the culture was scraped using an inoculation loop and homogenized in test tubes containing 5 mL of sterile 0.85% saline solution with the aid of an orbital shaker for 15 seconds. Subsequently, 10 µL of the suspension was pipetted onto a Neubauer chamber, and the number of fungal cells was counted by observing three random squares in the central quadrant using an optical microscope, aiming to achieve a final solution of $1 \times 10^6$ spores.

Solid-state fermentation (SSF) was conducted using wheat bran agro-industrial residue. In 125 mL Erlenmeyer flasks, 5.0 g of moistened wheat bran was supplemented with 10 mL of nutrient solution containing 0.1% (w/v) each of $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, and $NH_4NO_3$. The material was autoclaved at 121 °C for 40 minutes [19]. Inoculation with $1 \times 10^6$ spores per 5 g of wheat bran was performed according to [20]. Fermentation proceeded for 96 hours at 30 °C and 25 °C in a BOD Incubator, with samples taken every 24 hours and solubilized in 40 mL of deionized water at 4 °C. The fermentation process was conducted for 96 hours at 25 °C and 30 °C in a BOD incubator. The selection

of these temperatures was guided by the findings of Haq et al. (2005) [21]. Homogenized samples were filtered through Whatman No. 1 filter paper and subsequently centrifuged at 5,000 x g for 20 minutes at 4 °C. The supernatant from crude extracts was stored for proteolytic activity quantification and subsequent spectrophotometric analysis.

### *2.6. Protease quantification*

The protease activity was determined through an enzymatic assay using Sigma-Aldrich Azocasein as a substrate, following the colorimetric methodology described in the literature [22] Leighton. The enzymatic reaction involved the hydrolysis of Azocasein, releasing amino acids and peptide fragments that absorb UV light at a wavelength of 280 nm. The reaction mixture consisted of a 0.5% (w/v) Azocasein solution in Tris-HCl buffer, pH 7.2. A reaction mixture containing 150 µL of enzyme extract and 250 µL of Azocasein was prepared and incubated in Eppendorf microtubes with a capacity of 2.0 mL at 25 °C in the absence of light. After one hour, the reaction was terminated by adding 1.2 mL of 10% (w/v) Trichloroacetic acid (TCA), followed by centrifugation at 8,000 x g for 10 minutes at 4 °C. From the recovered supernatant, 800 µL was withdrawn for homogenization in 1.2 mL of 1M NaOH. A blank sample was prepared under the same conditions as the test sample.

The protease activity was expressed in U/mL, defined as the amount of enzyme required to release 1 µmol of Tyrosine per minute under the assay conditions, with the absorbance standard of Tyrosine set at 0.1. The reaction was monitored for 60 minutes at a wavelength of 280 nm using a UV-Vis spectrophotometer (Shimadzu UV-1900).

### *2.7. Statistical analysis*

The experimental design followed a completely randomized structure with three replicates for each condition tested. Arithmetic means of triplicate values were calculated and plotted using Excel version 365. All experimental data underwent analysis of variance (ANOVA), and the means were compared using the Tukey test ($P < 0.05$) for significance analysis. T-tests analyzed differences in protease activity across temperatures. Statistical analyses were performed using Past software (version 4.03). Enzyme activity was calculated based on tyrosine equivalents released per minute.

## 3. Results

### *3.1. Strain identification*

We focused on isolates with potential protease production capabilities. From the collected samples, 14 fungal isolates were selected based on specific criteria, including distinct morphological features such as pigmentation, colony texture, and growth patterns, as well as evidence of protease activity through agar-milk assays (Table 1). The selection process aimed to prioritize isolates demonstrating enzymatic potential rather than comprehensively representing the region's fungal diversity. As a result, five genera, *Curvularia* Boedijn, *Aspergillus* P. Micheli ex Haller, *Trichocladium* Harz, *Talaromyces* C.R. Benj., and *Penicillium* Link, were identified among the selected isolates and subsequently analyzed for their protease production capacity.

**Table 1.** Isolated fungal strains from aquatic environments in the Tapajós River Basin, Brazil: NCBI isolate accession numbers, % identity/similarity and reference accession numbers.

| Strain code | NCBI best blast hits | Identity % | Ac. number NCBI ITS | Ac. number NCBI 28S |
|---|---|---|---|---|
| UFOPA-MI0005 | *Aspergillus sydowii* | 100 | MN413179.1 | - |
| UFOPA-MI0054 | *Curvularia lunata* | 100 | GQ169765.1 | - |
| UFOPA-MI0019 | *Penicillium citrinum* | 99.8 | LC514694.1 | DI188008.1 |
| UFOPA-MI0008 | *Talaromyces* sp.1 | 99.8 | MK359011.1 | - |
| UFOPA-MI0039 | *Trichocladium arxii* | 100 | MN733136.1 | - |

The molecular identification of the fungal strains tested was performed through ITS sequencing, enabling precise taxonomic classification. Sequences alignments revealed that the 14 isolates belonged to five distinct genera: *Curvularia, Aspergillus, Trichocladium, Talaromyces,* and *Penicillium*. The internal transcribed spacer (ITS) region was used to molecularly identify all five tested genera, while the strain *P. citrinum* MI0019, which exhibited the highest enzymatic activity, was further analyzed using the 28S ribosomal RNA (rRNA) gene to achieve higher taxonomic resolution. *P. citrinum* UFOPA-MI0019 was identified with 99.8% sequence identity to reference strains. These results demonstrate the effectiveness of combining molecular techniques with morphological screening to identify fungal taxa with biotechnological potential. Figure 1 succinctly illustrates the macro and micromorphology of these isolates, providing valuable insights into their structural characteristics.

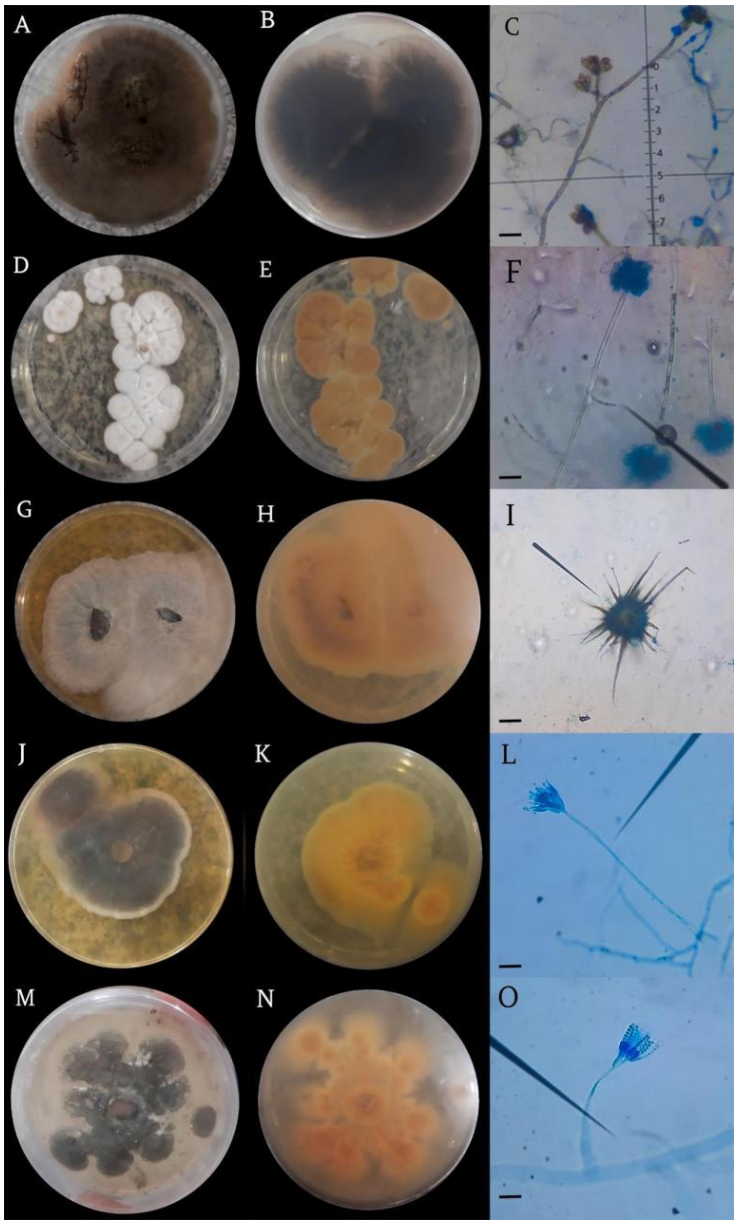


**Figure 1.** Macromorphology and Micromorphology of Filamentous Fungi Isolated from Aquatic Environments in Santarém, Pará, Brazil. Details of cultures on Potato Dextrose Agar (PDA) medium and micromorphological observations at 40x magnification: A-C: *Curvularia lunata* UFOPA-MI0054 (top and bottom views of the colonies); D-F: *Aspergillus* sp. UFOPA-MI0017 (top and bottom views of the colonies); G-I: *Trichocladium arxii* UFOPA-MI0039 (top and bottom views of the colonies); J-L: *Talaromyces* sp. UFOPA-MI0002 (top and bottom views of the colonies); and M-O: *P. citrinum* UFOPA-MI0019 (top and bottom views of the colonies). Scale bars:100 μm.

We aimed to identify the most proficient protease-producing fungi in aquatic environments of Santarém, Pará, Brazil. Therefore, measurements of degradation halo diameter, colony diameter, and enzymatic activity index (EAI) were performed for various fungal isolates. Table 2 presents a comprehensive summary of these measurements.

**Table 2.** Enzymatic Activity Index (EAI), halo and colony diameters for fungal isolate from aquatic environments in Santarém, Pará, Brazil.

| Specie | Isolate code | Halo diameter (mm) | Colony diameter (mm) | Enzymatic activity index (EAI) |
|---|---|---|---|---|
| *P. citrinum* Thom | UFOPA-MI0019 | 12.0 ± 0.1 | 5.0 ± 0.5 | 2.45 ± 0.1 |
| *Penicillium javanicum* J.F.H. Beyma | UFOPA-MI0001 | 20.0 ± 0.2 | 9.0 ± 0.1 | 2.22 ± 0.2 |
| *Talaromyces* sp.1 | UFOPA-MI0008 | 15.0 ± 0.1 | 7.0 ± 0.1 | 2.14 ± 0.2 |
| *Aspergillus sydowii* (Bainier & Sartory) Thom & Church | UFOPA-MI0005 | 19.1 ± 0.2 | 11.0 ± 0.1 | 1.75 ± 0.2 |
| *Trichocladium arxi* (Benny) X. Wei Wang & Houbraken i | UFOPA-MI0039 | 15.1 ± 0.1 | 10.0 ± 0.1 | 1.5 ± 0.2 |
| *Penicillium javanicus* J.F.H.Beyma | UFOPA-MI0047 | 27.0 ± 0.3 | 20.0 ± 0.2 | 1.35 ± 0.1 |
| *Penicillium* sp.1 | UFOPA-MI0041 | 2.0 ± 0.1 | 1.6 ± 0.1 | 1.27 ± 0.1 |
| *Curvularia lunata* (Wakker) Boedijn | UFOPA-MI0054 | 40.0 ± 0.4 | 34.0 ± 0.2 | 1.25 ± 0.2 |
| *Talaromyces* sp.2 | UFOPA-MI0002 | 14.0 ± 0.1 | 12.0 ± 0.1 | 1.21 ± 0.2 |
| *Aspergillus* sp.1 | UFOPA-MI0007 | 20.0 ± 0.2 | 16.0 ± 0.1 | 1.19 ± 0.1 |
| *Penicillium* sp.2 | UFOPA-MI0038 | 0 | 5.0 ± 0.1 | 0.00 |
| *Aspergillus* sp.2 | UFOPA-MI0017 | 0 | 5.0 ± 0.1 | 0.00 |
| *Aspergillus* sp.3 | UFOPA-MI0015 | 0 | 5.0 ± 0.1 | 0.00 |
| *Aspergillus* sp.4 | UFOPA-MI0045 | 0 | 5.0 ± 0.1 | 0.00 |

Among the isolates, *P. citrinum* UFOPA-MI0019 exhibited a degradation halo diameter of 12 mm, colony diameter of 5 mm, and a the highest EAI (2.45). Similarly, *P. javanicus* UFOPA-MI0001 and *Talaromyces* sp.1 UFOPA-MI0008 demonstrated substantial enzymatic activity, with EAI values of 2.2 and 2.14, respectively. Conversely, some isolates, such as *Aspergillus* sp.2 UFOPA-MI0017, 0015, 0045, exhibited low protease activity.

Figure 2 illustrates the kinetics of protease production by the isolate *P. citrinum* UFOPA-MI0019 under solid-state fermentation using wheat bran as an agro-industrial substrate. At 25 °C, the enzymatic activity of *P. citrinum* UFOPA-MI0019 fluctuated over time, peaking at 72 hours before declining at 96 hours. The activity ranged from 158.0 U/mL at 48 hours to a peak of 227.6 U/mL at 72 hours. The variability in measurements was reflected in standard deviation values, which ranged from 19.88 U/mL at 48 hours to 25.80 U/mL at 72 hours, indicating consistency in the experimental setup. Statistical analysis using ANOVA revealed significant differences among time points ($F = 5.8077$, $p = 0.021$). Tukey's post-hoc test identified significant differences specifically between 48 and 72 hours ($p < 0.05$), suggesting a sharp increase in enzymatic activity during this period. At 30 °C, enzymatic activity was consistently higher across all time points compared to 25 °C, with a notable peak at 72 hours. Activity ranged from 224.6 U/mL at 24 hours to a maximum

of 341.6 U/mL at 72 hours, followed by a decline to 256.0 U/mL at 96 hours. The standard deviation increased over time, from 14.97 U/mL at 24 hours to 28.80 U/mL at 96 hours, reflecting greater variability in measurements at later stages of fermentation. Statistical analysis using ANOVA confirmed significant differences among time points ($F = 12.6917$, $p = 0.0026$). Tukey's post-hoc test revealed significant increases in enzymatic activity between 24 and 72 hours ($p < 0.01$), highlighting the progressive enhancement in protease production. Additionally, a significant decline in activity was observed between 72 and 96 hours ($p < 0.05$), indicating the end of the peak production phase.

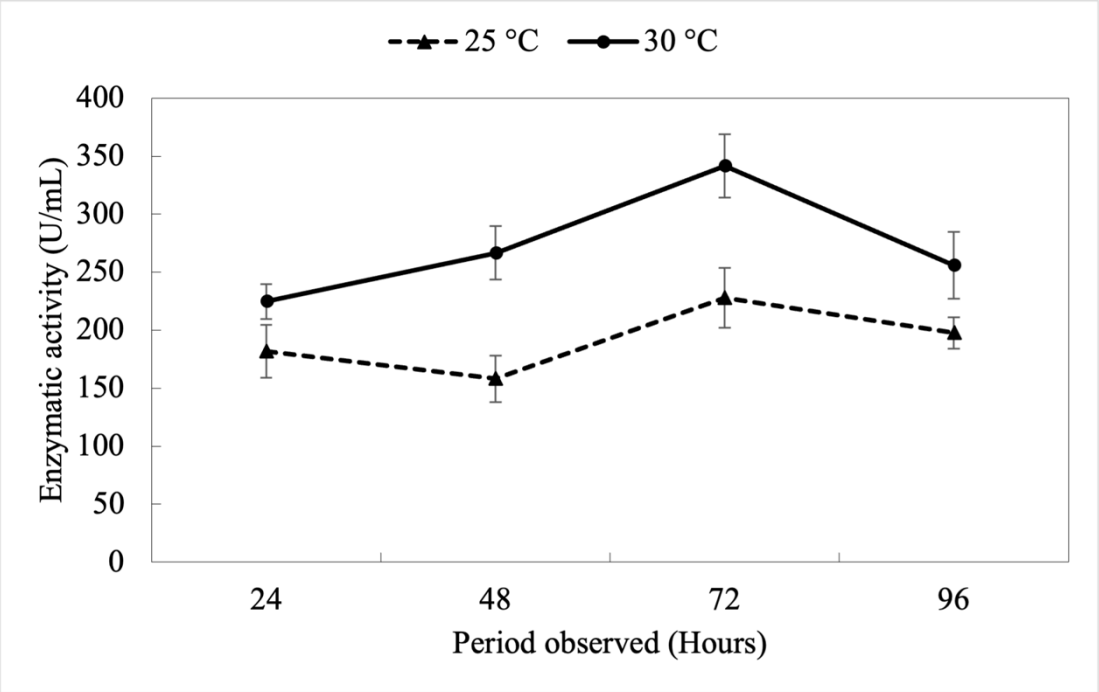


**Figure 2.** Kinetic profile of protease production by *P. citrinum* UFOPA-MI0019 isolated from the Tapajós River Basin, using wheat bran as a solid-state fermentation substrate.

*P. citrinum* UFOPA-MI0019 was identified through sequencing of the ITS region, exhibiting 99,8% similarity with the type strains and confirmed by the 28S region, exhibiting 99% similarity with the type strain. Additionally, a detailed micromorphological study was conducted for species identification, revealing distinctive characteristics. Microscopic examination unveiled septate, hyaline (non-pigmented) hyphae, and smooth-walled conidiophores featuring rather long stipes (100–300 µm) and a biverticillate structure. Metulae, arranged in whorls of 3–5 divergent structures, measured approximately 12–15 µm in length (Figure 1). These microscopic features are consistent with the identification of *P. citrinum* TYPE with accession numbers for the sequence being LC514694.1 (ITS) and DI188008.1 (28S).

Notably, the maximum production of proteases (340 ± 30 U/mL) was observed at 72 hours of fermentation time, suggesting a critical time point for optimal enzyme synthesis under the specified conditions. Moreover, our findings underscore the pronounced influence of temperature on protease production, with higher temperatures (30 °C) generally correlating with elevated enzyme activity

levels compared to lower temperatures (25 °C), highlighting the temperature sensitivity of *P. citrinum* UFOPA-MI0019 in enzyme synthesis.

## 4. Discussion

The fungal genera identified, *Curvularia*, *Aspergillus*, *Trichocladium*, *Talaromyces*, and *Penicillium*, are well-documented for their roles in decomposing vegetative matter in aquatic systems [23–25]. Researchers confirm the presence of these genera in similar ecological niches, highlighting their ability to thrive in transient spore states while seeking suitable substrates for colonization [26,27]. Our findings align with the literature, reinforcing the expected ecological roles of these fungi as decomposers and transient colonizers in riverine environments [28].

The discussion on fungal identification methods provides strong support for the molecular approaches used in the present study [29]. The importance of the internal transcribed spacer (ITS) region as the primary DNA barcode for fungi was highlighted, particularly in metabarcoding and species-level identification. However, it is emphasized that the ITS alone may not always provide sufficient taxonomic resolution, advocating for the use of secondary DNA markers, such as 28S rRNA and other genomic regions, for a more precise classification. Our findings align with this recommendation, as the strain *P. citrinum* UFOPA-MI0019, which exhibited the highest enzymatic activity, was further analyzed using the 28S ribosomal RNA (rRNA) gene, achieving 99.8% sequence identity with reference strains. This multi-locus approach ensured a more accurate taxonomic classification, reinforcing the need for integrative methods that combine sequence alignment and multiple genetic markers to enhance fungal identification.

It was possible to identify and quantify protease production among fungal isolates from the Tapajós River Basin, highlighting significant variability in enzyme activity. Notably, *P. citrinum* UFOPA-MI0019 emerged as a standout performer, exhibiting an Enzymatic Activity Index (EAI) of 2.4 ± 0.1, indicative of its potent protease production capabilities [30]. This high level of enzyme activity underscores the potential of selected fungal strains to contribute to biotechnological applications, particularly in the degradation of protein-rich substrates like wheat bran [31].

Among the strains tested, *P. citrinum* UFOPA-MI0019 demonstrated the highest protease production. Comparatively, researchers have identified other prolific protease producers, such as *Bacillus* species [32]. The comparison of protease indices is complicated by varying methodologies across studies, which affects the direct comparability of enzymatic activity levels. Nonetheless, the EAI observed in our study is notably high, suggesting an efficient protease production by *P. citrinum* UFOPA-MI0019 under our experimental conditions [31].

Protease production peaked at 72 hours for *P. citrinum* UFOPA-MI0019, a finding consistent with the literature, which suggests that early enzyme production is crucial for the initial stages of substrate degradation [33]. This optimal timeframe aligns with the degradation requirements for wheat bran, a protein-rich, economically viable substrate for solid-state fermentation.

The selection of 25 °C and 30 °C as incubation temperatures provided critical insights into the thermal dependence of protease production by *P. citrinum* UFOPA-MI0019. The higher activity observed at 30 °C aligns with earlier reports, such as those by Haq et al. [21], indicating that fungal proteases typically exhibit peak production at temperatures around 30 °C. This suggests that metabolic

processes involved in enzyme synthesis are most efficient at this temperature. Conversely, the reduced activity at 25 °C highlights the temperature sensitivity of the protease production process, emphasizing the importance of maintaining optimal conditions for industrial applications.

The maximum protease production at 30 °C highlights the importance of optimizing temperature conditions in enzymatic processes [33]. This result is consistent with the broader search for thermotolerant proteases that can operate efficiently under varying industrial conditions. Our findings contribute to this ongoing research, emphasizing the need for enzymes that maintain stability and activity at higher temperatures [34].

The results of statistical analyses reinforce the reliability of the measures obtained. The application of the Tukey test allowed to identify significant differences between the conditions tested, providing greater robustness to the conclusions about the proteolytic activity of the demonstrated strain.

The limitations of this study include the low number of species tested, which does not fully represent the environmental diversity, and the challenges associated with comparing our results with other studies due to the absence of standard enzymes for industrial potential comparison [35,36]. To establish *P. citrinum* UFOPA-MI0019 as a definitive candidate for industrial applications, researchers should focus on large-scale fermentation trials, stability assays under industrial conditions, and testing across a broader range of substrates. These steps will provide the necessary statistical and empirical support to validate its suitability for industrial use [37–39]. By addressing these gaps, future research will contribute significantly to leveraging Amazonian biodiversity for innovative enzyme technologies.

## 5. Conclusions

We successfully isolated and identified 14 fungal strains from aquatic environments in the Tapajós River Basin, representing five genera: *Curvularia, Aspergillus, Trichocladium, Talaromyces*, and *Penicillium*. Among these, five isolates exhibited protease production, with *P. citrinum* UFOPA-MI0019 demonstrating the highest enzymatic activity. The protease production kinetics of *P. citrinum* UFOPA-MI0019 under solid-state fermentation showed peak activity at 340 ± 30 U/mL after 72 hours at 30 °C, followed by a decline at 96 hours. This pattern indicates that enzymatic activity is influenced by fermentation time and temperature, with 30 °C supporting significantly higher production than 25 °C. The key factors influencing protease production in the identified species include their ability to utilize wheat bran efficiently, an agro-industrial substrate, and their capacity for high enzymatic yield under optimized conditions. These findings underscore the biotechnological potential of Amazonian fungi for enzyme production and sustainable organic waste biotransformation. To establish *P. citrinum* UFOPA-MI0019 as a viable candidate for industrial applications, researchers should focus on scaling up fermentation processes, evaluating enzyme stability under industrial conditions, and utilizing additional molecular markers to enhance species identification and assess the genetic basis of protease synthesis.

## Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article

## Acknowledgments

We express our deep gratitude to The Nature Conservancy for supporting the collection of samples through the Tapajós Waters Project on behalf of Dr. Dávia Talgatti and Fernando Abreu. Our thanks also go to the Federal University of Western Pará – Ufopa, for providing the necessary infrastructure and resources and for the financial support from Fapespa (Amazon Foundation for the Support of Studies and Research) and to the Interaction Genetics Laboratory - LGI for support in molecular biology procedures. The collaborative efforts and expert insights of our colleagues at INPA were invaluable to our research.

## Conflicts of interest

The authors declare no conflict of interest.

## Author contributions:

Aline Lima de Aguiar, João Vicente Braga de Souza and Eveleise Samira Martins Canto conceived and designed the study. Aline Lima de Aguiar, Ana Luiza Figueira da Silva, Rayane Bonfim Ferreira Xavier, Marcos Diones Ferreira Santana, Dávia Marciana Talgatti, Fernando Abreu Oliveira, Carlos Ivan Aguillar-Vildoso, Érica Simplício de Souza, Marcos Rodrigues Barreto, João Paulo Alves Silva, Clarice Maia Carvalho, Lívia Melo Carneiro, Joao Vicente Braga de Souza, and Eveleise Samira Martins Canto conducted experiments and organized the database. Aline Lima de Aguiar, João Vicente Braga de Souza and Eveleise Samira Martins Canto wrote the first draft of the manuscript. All authors wrote sections of the manuscript and contributed to manuscript revision, read, and approved the submitted version.

## References

1. Abdullah AA, Babu J (2020) Current prospective in using cold-active enzymes as eco-friendly detergent additive. *Appl Microbiol Biotechnol* 104: 2871–2882. https://doi.org/10.1007/s00253-020-10429-x
2. Lima ADDR, Farias VA, Oliveira HD (2018) Patents technological prospecting of proteases used in cheese making. *Prospect Noteb* 11: 1726. https://doi.org/10.9771/cp.v12i5.27619
3. Cardoso KBB, Nascimento TP, Oliveira VM, et al. (2022) Protease with fibrinolytic and collagenolytic activity produced by *Aspergillus ochraceus* URM604. *Res Soc Dev* 11: e15511225500. https://doi.org/10.33448/rsd-v11i2.25500
4. Climaco LR, Roder C, Olivo JE (2023) Production of proteases by fungi of the *Aspergillus* genus: the impact of cultivation conditions and their biotechnological implications. *Obs Lat Am Econ* 21: 17465–17481. https://doi.org/10.55905/oelv21n10-159
5. Paz Junior FB, Silva CRB, Freitas LR, et al. (2020) Evaluation of the proteolytic activity of *Penicillium chrysogenum* isolated from the IFPE - Campus Recife memorial room. *Braz J Dev* 6: 60502–60508. https://doi.org/10.34117/bjdv6n8-459

6. Santos BR, Maeda RN, Hamada N, et al. (2023) Screening of fungal strains isolated from Simuliidae (Insecta: Diptera) larvae to obtain enzymes of industrial interest in the Amazon. *Braz J Anim Environ Res* 6: 3350–3358. https://doi.org/10.34188/bjaerv6n4-021
7. Prado FB, Batista SCP, Martim SR, et al. (2021) Feasibility of protease production by Aspergillaceae species and screening of coagulants from bovine milk. *Braz J Dev* 7: 16356–16373. https://doi.org/10.34117/bjdv7n2-317
8. Oliveira Filho DC, Coelho KWSA, Costa VCA, et al. (2022) Synthesis of proteases by a species of anamorphic filamentous fungus for application in industrial processes. *Res Soc Dev* 11: e548111638526. https://doi.org/10.33448/rsd-v11i16.38526
9. Canto ESM, Costa DVS, Sousa LS, et al. (2023) Evaluation of enzymatic activity production by filamentous fungi collected from Amazonian soil. *Microb Biotechnol* 1: 142–156. Available from: https://downloads.editoracientifica.com.br/articles/230111840.pdf.
10. Benchaya GA, França EM, Silva NCCA, et al. (2022) Proteases from a species of the Trichocomaceae family for application in the food industry. *Res Soc Dev* 11: e547111638525. https://doi.org/10.33448/rsd-v11i16.38525
11. Batista Junior GP, Silva KND, Santiago PAL, et al. (2021) Enzyme prospection and antimicrobial activity of Penicillium species isolated from the Amazon Biome. *Braz J Dev* 7: 75011–75025. https://doi.org/10.34117/bjdv7n7-603
12. Kieliszek M, Pobiega K, Piwowarek K, et al. (2021) Characteristics of the proteolytic enzymes produced by lactic acid bacteria. *Molecules* 26: 1858. https://doi.org/10.3390/molecules26071858
13. Santos AF, Parisi JJD, Menten JOM (2023) Diversity of endophytic filamentous fungi in seeds of native forest species in southern Brazil. *Summa Phytopathol* 49: e257357. https://doi.org/10.1590/0100-5405/257357
14. Tsang CC, Tang JY, Lau SK, et al. (2018) Taxonomy and evolution of *Aspergillus, Penicillium* and *Talaromyces* in the omics era–Past, present and future. *Comput Struct Biotechnol J* 16: 197–210. https://doi.org/10.1016/j.csbj.2018.05.003
15. White TJ, Bruns T, Lee S, et al. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols* 18: 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1
16. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41: 95–98.
17. Chen L, Daniel RM, Coolbear T (2003) Detection and impact of protease and lipase activities in milk and milk powders. *Int Dairy J* 13: 255–275. https://doi.org/10.1016/S0958-6946(02)00171-1
18. Florencio C, Couri S, Farinas, CS (2012) Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by trichoderma strains. *Enzyme Res* 2012: 1–7. https://doi.org/10.1155/2012/793708
19. Merheb CW, Cabral H, Gomes E, et al. (2007) Partial characterization of protease from a thermophilic fungus, *Thermoascus aurantiacus*, and its hydrolytic activity on bovine casein. *Food Chem* 104: 127–131. https://doi.org/10.1016/j.foodchem.2006.11.010
20. Montoya S, Patiño A, Sánchez ÓJ (2021) Production of lignocellulolytic enzymes and biomass of *Trametes versicolor* from agro-industrial residues in a novel fixed-bed bioreactor with natural convection and forced aeration at pilot scale. *Processes* 9: 1–19. https://doi.org/10.3390/pr9020397

21. Haq I, Mukhtar H, Umber H (2006) Production of protease by *Penicillium chrysogenum* through optimization of environmental conditions. *J Agric Soc Sci* 2: 23–25. https://www.researchgate.net/publication/238728994
22. Leighton TJ, Doi RH, Warren RAJ, et al. (1973) The relationship of serine protease activity to RNA polymerase modification and sporulation in Bacillus subtilis. *J Mol Biol* 76: 103–122. https://doi.org/10.1016/0022-2836(73)90083-1
23. Nicoletti R, Vinale F (2018) Bioactive compounds from marine-derived Aspergillus, *Penicillium*, *Talaromyces* and *Trichoderma* species. *Mar Drugs* 16: 408. https://doi.org/10.3390/md16110408
24. Khan NA, Asaf S, Ahmad W, et al. (2023) Diversity, lifestyle, genomics, and their functional role of *Cochliobolus*, *Bipolaris*, and *Curvularia* species in environmental remediation and plant growth promotion under biotic and abiotic stressors. *J Fungi* 9: 254. https://doi.org/10.3390%2Fjof9020254
25. Kaur M, Peshwani H, Goel M (2024) *Penicillium*: a treasure trove for antimycobacterial and antioxidant metabolites, In: Deshmukh, S.K., Takahashi, J.A., Saxena, S., *Fungi Bioactive Metabolites: Integration of Pharmaceutical Applications,* Singapore: Springer Nature Singapore, 263–281. https://doi.org/10.1007/978-981-99-5696-8_9
26. Heo I, Hong K, Yang H, et al. (2019) Diversity of *Aspergillus*, *Penicillium*, and *Talaromyces* species isolated from freshwater environments in Korea. *Mycobiology* 47: 12–19. https://doi.org/10.1080/12298093.2019.1572262
27. Pangging M, Nguyen T, Lee H (2019) New records of four species belonging to Eurotiales from soil and freshwater in Korea. *Mycobiology* 47: 154–164. https://doi.org/10.1080/12298093.2018.1554777
28. You YH, Park JM, Park JH, et al. (2015) Diversity of endophytic fungi associated with the roots of four aquatic plants inhabiting two wetlands in Korea. *Mycobiology* 43: 231–238. https://doi.org/10.5941/MYCO.2015.43.3.231
29. Lücking R, Aime MC, Robbertse B, et al. (2020) Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? *IMA Fungus* 11: 1–32. https://doi.org/10.1186/s43008-020-00033-z
30. Queiroz C, Sousa ACB (2020) Production of hydrolytic enzymes by filament fungi in different solid substrates. *Braz J Dev* 6: 51849–51860. https://doi.org/10.34117/bjdv6n7-725
31. Ikhsanudin A, Emantis R, Ekowati CN (2019) Proteolytic activity of the entomopathogenic fungi (*Penicillium* sp.) of cockroaches (*Periplaneta americana*). *J IIm Biol EKspe Keanekaragaman Hayati* 6: 81–84. https://doi.org/10.23960/jbekh.v6i2.47
32. Harwood CR, Kikuchi Y (2022) The ins and outs of *Bacillus* proteases: activities, functions and commercial significance. *FEMS Microbiol Rev* 46: 1–20. https://doi.org/10.1093/femsre/fuab046
33. Sethi S, Gupta S (2015) Optimization of protease production from fungi isolated from soil. Available from: https://pesquisa.bvsalud.org/portal/resource/pt/sea-168920.
34. Behera BC, Bijay KS, Mohapatra S, et al. (2021) Bio-production of alkaline protease by *Trichoderma longibrachiatum* and *Penicillium rubidurum* using different agro-industrial products. *Novel Res Microbiol J* 5: 1241–1255. https://doi.org/10.21608/nrmj.2021.178300

35. Chapla VM, Biasetto CR, Araujo AR (2013) Endophytic Fungi: An unexplored and sustainable source of new and bioactive natural products. *Virtual Quim* 5: 421–437. https://doi.org/10.5935/1984-6835.20130036
36. Bogas AC, Cruz FPN, Lacava PT, et al. (2022) Endophytic fungi: an overview on biotechnological and agronomic potential. *Braz J Biol* 84: e258557. https://doi.org/10.1590/1519-6984.258557
37. Guillaume A, Thorigné A, Carré Y, et al. (2019) Contribution of proteases and cellulases produced by solid-state fermentation to the improvement of corn ethanol production. *Bioresour Bioprocess* 6: 1–12. https://doi.org/10.1186/s40643-019-0241-0
38. Figueiredo CCM, Granero FO, Silva LP, et al. (2024) Solid-state fermentation using wheat bran to produce glucose syrup and functional cereal bars. *Bioprocess Biosyst Eng* 47: 1081–1094. https://doi.org/10.1007/s00449-024-03032-1
39. Freire AFN, Canto ESM, Paiva MJM, et al. (2024) Scientific and technological prospecting of environmental yeasts regarding the production of hydrolytic enzymes of biotechnological interest. *Contrib Cienc Soc* 17: 1499–1512. https://doi.org/10.55905/revconv.17n.1-084