ISSN: 1696-8352

Comparative Analysis of the Effects of Different Substrates on Protease Production by *Penicillium* spp. Isolated from the Amazon

Análise Comparativa dos Efeitos de Diferentes Substratos na Produção de Proteases por *Penicillium* spp. Isolados da Amazônia

Análisis Comparativo de los Efectos de Diferentes Sustratos en la Producción de Proteasas por *Penicillium* spp. Aislados de la Amazonía

DOI: 10.55905/oelv22n11-154

Receipt of originals: 10/17/2024
Acceptance for publication: 11/14/2024

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ISSN: 1696-8352

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ABSTRACT
The genus *Penicillium* is widely recognized for its ability to produce a diverse range of enzymes, including proteases, which play crucial roles in various biotechnological applications. This study investigated the influence of three distinct inducers (skim milk, gelatin, and a combination of skim milk and gelatin) on protease production by different *Penicillium* species, using the cup-plate method—a qualitative technique that detects proteolytic activity in solid media. The proteolytic activity of *Penicillium* species was assessed by measuring degradation halos in culture media supplemented with these inducers. The results demonstrated that skim milk was the most effective inducer, yielding the highest Enzyme Index (EI) values. The highest EI was observed for *P. citrinum* 414 (3.6), followed by *P. oxalicum* and *P. sumatraense*, both with EIs of 3.3. The strains *P. citrinum* 409, *P. paxilli*, *P. citrinum* 457, and *Penicillium* spp. 5 and 546 exhibited EIs ranging from 3.0 to 3.2. The analysis of degradation halos highlighted that both substrate type and cultivation conditions significantly influence protease production. This study enhances our understanding of the enzymatic potential of *Penicillium* species and underscores the importance of optimizing cultivation parameters to maximize protease production, which has broad potential applications in industrial processes.

Keywords: Proteolytic Activity, *Penicillium* spp., Qualitative Assays, Cup Plate.

RESUMO
O gênero *Penicillium* é amplamente reconhecido por sua capacidade de produzir uma variedade diversificada de enzimas, incluindo proteases, que desempenham papéis cruciais em diversas aplicações biotecnológicas. Este estudo investigou a influência de três indutores distintos (leite desnatado, gelatina e uma combinação de leite desnatado com gelatina) na produção de proteases por diferentes espécies de *Penicillium*, utilizando a técnica de placa de difusão em ágar — uma metodologia qualitativa que permite a

ISSN: 1696-8352

detecção de atividade proteolítica em meio sólido. A atividade proteolítica das espécies de *Penicillium* foi avaliada com base na formação de halos de degradação em meio de cultura suplementado com esses indutores. Os resultados demonstraram que o leite desnatado foi o indutor mais eficaz, promovendo os maiores valores de Índice Enzimático (IE). O maior IE foi observado para *P. citrinum* 414 (3,6), seguido por *P. oxalicum* e *P. sumatraense*, ambos com IE de 3,3. As cepas *P. citrinum* 409, *P. paxilli*, *P. citrinum* 457 e *Penicillium* spp. 5 e 546 apresentaram IE variando de 3,0 a 3,2. A análise dos halos de degradação destacou que tanto o tipo de substrato quanto as condições de cultivo influenciam significativamente a produção de proteases. Este estudo aprofunda o conhecimento sobre o potencial enzimático das espécies do gênero *Penicillium* e enfatiza a importância de otimizar os parâmetros de cultivo para maximizar a produção de proteases, com amplas aplicações potenciais em processos industriais.

Palavras-chave: Atividade Proteolítica, Gênero *Penicillium*, Ensaios Qualitativos, Técnica de Difusão em Placa.

RESUMEN

El género *Penicillium* es ampliamente reconocido por su capacidad para producir una variedad diversa de enzimas, incluidas las proteasas, que desempeñan un papel crucial en diversas aplicaciones biotecnológicas. Este estudio investigó la influencia de tres inductores distintos (leche descremada, gelatina y una combinación de leche descremada con gelatina) en la producción de proteasas por diferentes especies de *Penicillium*, utilizando la técnica de placa de difusión en agar, una metodología cualitativa que permite la detección de actividad proteolítica en medio sólido. La actividad proteolítica de las especies de *Penicillium* se evaluó mediante la formación de halos de degradación en medios de cultivo suplementados con estos inductores. Los resultados demostraron que la leche descremada fue el inductor más eficaz, promoviendo los valores más altos de Índice Enzimático (IE). El IE más alto se observó para *P. citrinum* 414 (3,6), seguido de *P. oxalicum* y *P. sumatraense*, ambos con un IE de 3,3. Las cepas *P. citrinum* 409, *P. paxilli*, *P. citrinum* 457 y *Penicillium* spp. 5 y 546 presentaron IE que varían de 3,0 a 3,2. El análisis de los halos de degradación destacó que tanto el tipo de sustrato como las condiciones de cultivo influyen significativamente en la producción de proteasas. Este estudio amplía nuestro conocimiento sobre el potencial enzimático de las especies de *Penicillium* y subraya la importancia de optimizar los parámetros de cultivo para maximizar la producción de proteasas, con amplias aplicaciones potenciales en procesos industriales.

Palabras clave: Actividad Proteolítica, Género *Penicillium*, Ensayos Cualitativos, Técnica de Difusión en Placa.

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1 INTRODUCTION

The biodiversity of the Amazon is among the richest in the world, harboring an immense variety of organisms, including a vast diversity of fungi. Studies have shown that the Amazon rainforest contains a wide array of fungal species, many of which remain underexplored in terms of their biotechnological potential. These fungi are considered promising sources of bioactive compounds and enzymes with applications across various industrial sectors (Martins *et al*., 2021). Among the species found, the genus *Penicillium* stands out due to its wide distribution and its ability to produce secondary metabolites of biotechnological interest, as well as its significant potential for the production of industrial enzymes.

Enzymes are specialized proteins that catalyze chemical reactions, playing vital roles in both biological and industrial processes. Fungal enzymes, in particular, have garnered significant market attention due to their suitability for applications in the food, textile, pharmaceutical, and biofuel industries (Souza *et al*., 2020). Among fungi, the *Penicillium* genus stands out for its ability to produce hydrolytic enzymes, such as amylases, lipases, cellulases, and proteases, all of which hold considerable industrial value (Ferreira *et al*., 2019).

Fungal proteases, particularly those produced by *Penicillium* species, have been shown to be highly efficient, with the added advantage of being produced in large quantities and at low cost through fermentation processes (Gomes *et al*., 2022). These enzymes are applicable across various industrial sectors. In the food industry, they are utilized in meat processing, cheese production, and the hydrolysis of vegetable proteins to create protein hydrolysates, which are widely used as food additives (Costa *et al*., 2021).

Additionally, proteases are employed in the production of biological detergents, where they aid in the removal of protein stains with low concentrations of surfactants, offering a more sustainable and efficient alternative to conventional detergents (Silva *et al*., 2020). In the pharmaceutical sector, fungal proteases have been explored for drug production, particularly in the synthesis of bioactive peptides and the treatment of

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digestive disorders (Oliveira *et al*., 2019). Furthermore, they are applied in the textile industry, where they assist in fabric processing to improve fiber quality, removing impurities and modifying textile surfaces.

Species of *Penicillium* stand out not only for their capacity to produce enzymes but also for the versatility of the proteases they generate. Recent studies have explored the potential of these species in the Amazon, aiming to identify strains with unique enzymatic characteristics. These studies focus on uncovering the biotechnological potential of these fungi, as the environmental conditions of the rainforest may drive the evolution of enzymes with distinct properties (Martins *et al*., 2021).

This study aims to investigate the potential for protease production by various *Penicillium* species isolated from the Amazon region, utilizing methodologies that efficiently assess the enzymatic index. In doing so, this work seeks to enhance the understanding of the proteolytic capabilities of these species, expanding knowledge of the Amazon's microbial biodiversity and its biotechnological potential.

2 METHODOLOGY

2.1 REACTIVATION OF FUNGAL STRAINS

Seventeen fungal strains from the collection of the laboratory of bioassays and microorganisms of the amazon (labmicra) were preserved in 20% glycerol and castellani solution, and were reactivated on potato dextrose agar (pda) plates, following the protocol of souza *et al*. (2002). The plates were incubated in a bod chamber at 28°c for 10 days. after reactivation, a spore suspension was prepared, using the mcfarland 10 standard as a reference. This suspension was employed in all subsequent experimental procedures.

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2.2 PRODUCTION OF ENZYMATIC EXTRACTS BY SUBMERGED FERMENTATION WITH DIFFERENT INDUCING SUBSTRATES

Enzymatic extracts were produced from the fungal strains that exhibited enzymatic halos in the qualitative test on milk agar. In 50 mL Erlenmeyer flasks containing 15 mL of modified Manachini liquid medium (Manachini *et al.*, 1987), the composition was: 2 g potassium phosphate (KH_2PO_4), 0.9 g sodium phosphate ($Na_2HPO_4.2H_2O$), 1 g ammonium sulfate ($(NH_4)_2SO_4$), 0.1 g magnesium sulfate ($MgSO_4.7H_2O$), 1 g yeast extract, and 1 L distilled water, with the addition of three different inducing substrates: skim milk (1% w/v), skim milk + gelatin (1% w/v), and gelatin (1% w/v). The medium was autoclaved at 121°C for 15 minutes. After sterilization, 15 µL of spore suspension from each strain was inoculated in triplicate into the flasks and incubated in a shaker at 120 rpm for 120 hours at 28°C. After cultivation, the samples were transferred to 50 mL Falcon tubes, centrifuged at 4000 rpm for 20 minutes, and filtered through Whatman No. 1 filter paper followed by a 0.22 µm Millipore membrane. The crude enzymatic extract from each strain was then obtained for application in the cup plate assay.

2.3 QUALITATIVE ASSESSMENT OF PROTEOLYTIC ACTIVITY USING THE CUP PLATE METHOD

For this qualitative assay, 100 µl aliquots of each enzymatic extract were placed in petri dishes with three 6 mm diameter wells in the surface of the medium. The medium composition was as follows: 1 l distilled water, 5 g peptone, 0.4 g gelatin, 1 g sodium chloride (NaCl), 0.2 g magnesium sulfate ($MgSO_4.7H_2O$), 3 g yeast extract, 18 g agar, and 10 g skimmed milk, as described by hankin and anagnostakis (1975), with modifications. All components, except skimmed milk, were dissolved in 900 ml of distilled water and autoclaved at 121°C for 15 minutes, while the milk was dissolved in 100 ml of distilled water and sterilized in flowing steam for 15 minutes. The two solutions were mixed until fully homogenized before pouring into the plates. A control plate, with

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the medium but without fungal inoculation, was incubated under the same conditions. Plates were incubated at 28°c for 24 hours and revealed with 2% methyl red (0.2 g methyl red, 60 ml 96% alcohol, and 40 ml distilled water).

Enzyme production was indicated by the formation of halos resulting from enzymatic hydrolysis. Photos were taken, and the halo diameters were measured to determine the enzymatic activity index (eai) and for statistical analysis. The test was performed in triplicate, and the eai was calculated by dividing the mean halo diameters by the mean well diameters (6 mm).

2.4 CALCULATION OF ENZYMATIC INDEX AND STATISTICAL ANALYSIS

To calculate the Enzymatic Index (EI) in the milk agar experiments, the ratio between the average total diameter of the hydrolysis halos (D) and the diameter of the inoculated colonies (d) was used. For the results from the cup plate tests, the ratio between the average hydrolysis halo diameter (D) and the well diameter (d) was considered (Bhuyan *et al*., 2020). This methodology is widely employed to quantify the proteolytic activity of microorganisms. Higher EI values indicate greater enzymatic activity, reflecting the efficiency of proteases in hydrolyzing the surrounding substrate (Singh, Kumar, and Mittal, 2021; Oliveira *et al*., 2022).

Data analysis was conducted using R software version 4.4.1, a programming language well-known for its robustness in statistical and graphical analysis (R Core Team, 2024). Analysis of Variance (ANOVA) was applied to determine statistical significance between the different experimental groups, with p-values less than 0.05 considered significant. R was chosen for its capability to handle complex datasets and the availability of specialized packages that facilitate detailed statistical analysis. The 'stats' and 'ggpubr' packages were used to create the graphs and figures.

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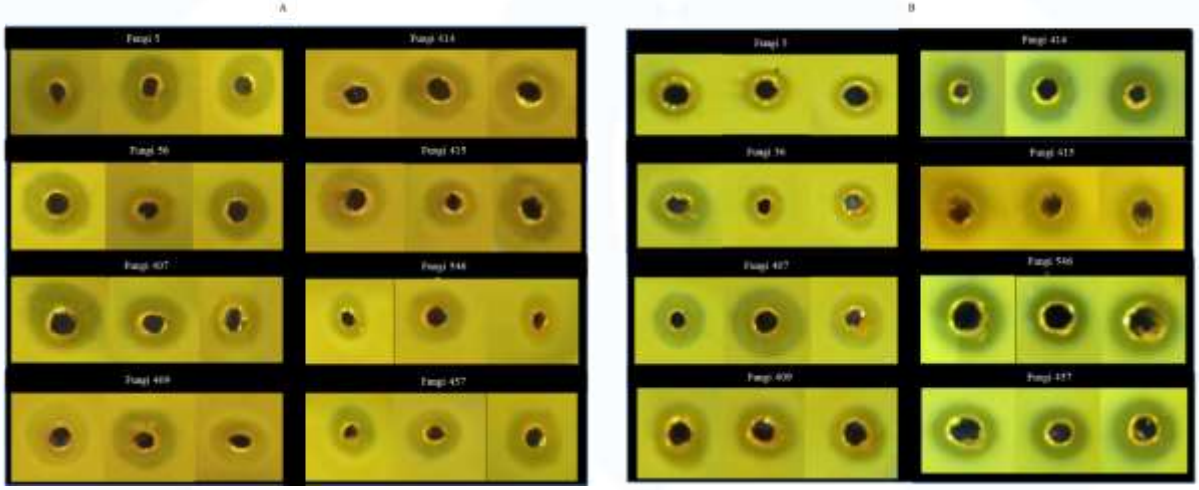
3 RESULTS AND DISCUSSIONS

3.1 EVALUATION OF PROTEOLYTIC ACTIVITY USING THE CUP PLATE METHOD

Of the seventeen reactivated strains, eight showed positive results, evidenced by the formation of halos around the wells.

Figure 1: Enzymatic Hydrolysis Halos in Different Substrates. A: 1% Gelatin; B: 1% Skim Milk.



Source: Author, 2024.

Figure 1 presents the comparative results of degradation halos observed in two distinct substrates: gelatin (group A) and skimmed milk (group B). All fungi evaluated in the study demonstrated the ability to form degradation halos in both substrates, highlighting the proteolytic activity of the Penicillium genus. Based on these results, the enzymatic indexes for each sample were calculated, followed by statistical analysis to compare performance and identify significant differences. The results from the induction with the combination of gelatin and skimmed milk were excluded from the final analysis, as they did not demonstrate statistically significant relevance in terms of enzymatic performance.

The genus *Penicillium* is widely recognized for its effectiveness in producing proteases with diverse industrial applications. The literature indicates that these species

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are highly efficient in obtaining proteases, and the cup plate technique is commonly used to qualitatively evaluate this activity. In this method, halo formation indicates casein degradation by the proteases produced by the fungi (Li *et al*., 2022; Wang *et al*., 2021).

In the study conducted by Silva *et al*. (2019), it was observed that protease production by the *Penicillium* genus using the cup plate method resulted in significant degradation halos. The results indicated that the substrate used effectively promoted enzyme expression, with halo diameters ranging from 10 to 15 mm after 48 hours of incubation.

In another study using the same methodology, enzyme production screening of *Penicillium* strains was conducted. The results demonstrated that the presence of degradation halos around the inoculated wells indicated proteolytic activity, with halo diameters reaching up to 8 mm (Souza *et al*., 2018).

According to Rahayu *et al*. (2021), *P. sumatraense* produced a diverse set of enzymes tested under various conditions to evaluate their activity and efficiency. The study revealed a robust enzymatic arsenal, including proteases such as dipeptidyl- and aminopeptidases, as well as β-1,3-glucanases, which were effective in the decomposition of algal biomass. These findings suggest that this species may be a promising tool for algal biorefinery, a process that aims to convert algal biomass into valuable products such as biofuels, chemical compounds, and food.

Dossis *et al*. (2013) aimed to evaluate the enzymatic potential of different *Penicillium* species, with an emphasis on proteolytic enzyme production. Among the species analyzed, *P. oxalicum* stood out for its effectiveness in producing enzymes with potential applications in the food industry, particularly in meat maturation processes and the production of fermented foods.

P. citrinum is a fungal species widely studied for its ability to produce a variety of enzymes, including proteases. Several studies indicate that this species has significant potential in protein degradation, making it valuable in various industries, particularly the food industry. In addition to its milk-clotting action, as noted by Nishihara *et al*. (1972), other research suggests that the proteases produced by *P. citrinum* are effective under

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specific pH and temperature conditions, characteristics that make it promising for industrial applications such as cheese production and fermented food processing.

The literature suggests that *P. paxilli* has potential biological activity (Palmquist *et al*., 2015). In this study, the formation of degradation halos observed in the assays indicates the ability of this species to produce active proteases. This result is highly relevant, as there are currently no scientific reports describing the proteolytic activity of *P. paxilli*. The absence of previous studies on this characteristic in the species suggests that this work could contribute in an unprecedented way to the field, opening new perspectives for future investigations into the use of this fungus for the production of proteolytic enzymes with biotechnological potential.

3.2 DETERMINATION OF ENZYMATIC INDEX (EI) IN DIFFERENT SUBSTRATES

The *Penicillium* species examined in this study are presented in Figure 2. The highest Enzymatic Index (EI) was observed for P. citrinum 414 (3.6), followed by *P. oxalicum* and *P. sumatraense*, both with an EI of 3.3. The strains *P. citrinum* 409, *P. paxilli*, *P. citrinum* 457, *Penicillium* spp. 5, and 546 exhibited EI values ranging from 3.0 to 3.2. On the gelatin substrate, EI values varied between 2.4 and 2.9. *P. citrinum* 409 and *Penicillium* spp. 5 showed the highest index (EI of 2.9), while *P. oxalicum* displayed the lowest index (2.4), suggesting a lower efficiency in gelatin degradation compared to skim milk.

These results indicate that the efficiency of protease production varies between species and the substrates used, with skimmed milk leading to higher enzyme levels in most of the species tested.

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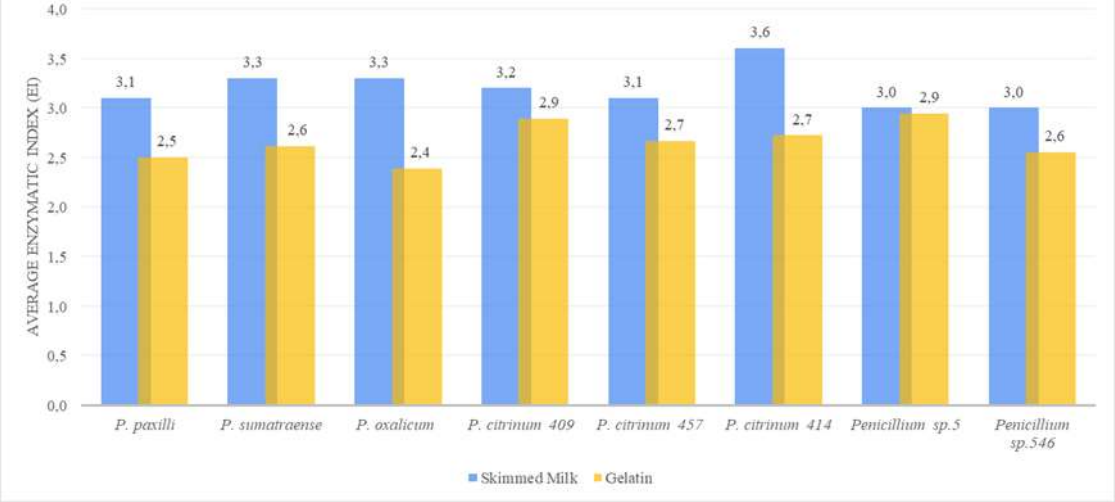
Figure 2: Comparison of Enzymatic Index between Skim Milk and Gelatin Substrates.



Source: Author, 2024.

The results of the present study corroborate findings in the literature regarding the variability in protease production across different substrates. Silva *et al*. (2022) demonstrated that the interaction between proteases and protein substrates, such as casein in milk, is a crucial factor in determining the efficiency of enzymatic activity. Additionally, Silva *et al*. (2021) emphasized that the choice of substrate directly influences enzymatic yield, noting that more complex substrates can lead to greater variability in the enzymatic index. In comparison with our results, it is evident that skim milk promotes greater efficiency in protease production by *Penicillium*, while gelatin exhibited lower efficiency, possibly due to its simpler protein structure.

The findings of this study were compared with those of Souza *et al., (2015), who also used the cup* methodology *plate* to evaluate proteolytic activity in endophytic fungi. Although the substrates used in both studies were different, the investigations corroborate the effectiveness of the method in detecting proteolytic activity in different fungal species. Silva *et al* . (2012) also used gelatin as a substrate and observed results that highlight the influence of the type of substrate on the variation of the Enzyme Index (IE) between species. These findings are consistent with the present study, in which *P. citrinum* demonstrated greater efficiency in the production of proteases when gelatin was used as a substrate.

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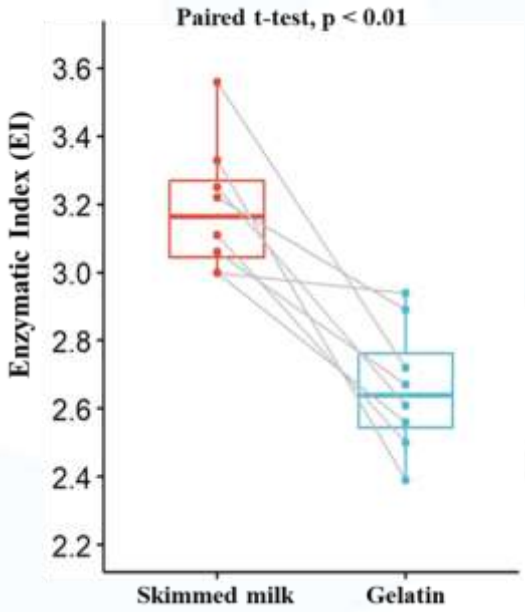
3.3 STATISTICAL COMPARISON OF ENZYMATIC INDEX AMONG STRAINS

A one-way ANOVA with repeated measures showed that the type of substrate has a significant effect on the Enzymatic Index [$F(2, 14) = 8.24$; $p < 0.01$]. Figure 3 presents the pairwise comparisons using the paired t-test with false discovery rate (FDR) correction, indicating that cultures with 'Skimmed Milk' result, on average, in higher Enzymatic Index values compared to cultures with 'Gelatin' ($p < 0.01$). No statistically significant differences were found between 'Milk with Gelatin' and 'Skimmed Milk' cultures, indicating that both substrates produce similar Enzymatic Index values.

Figure 3: Comparison of enzymatic indexes between 1% skimmed milk and 1% gelatin cultures.



Source: Author, 2024.

4 CONCLUSION

During this study, which focused on prospecting proteases produced by *Penicillium* species using the cup plate method, a significant gap was identified in the available literature regarding specific reports on *Penicillium paxilli*, *P. oxalicum*, *P. citrinum*, and *P. sumatraense*. This presented a substantial challenge in supporting the discussion of the

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results. This gap highlights the need for more in-depth and targeted research on these species. Consequently, this study aims to contribute to expanding the understanding of the proteolytic capabilities of these *Penicillium* species, offering new insights that may serve as a foundation for future research, with the goal of consolidating and comparing findings in a more comprehensive manner.

ACKNOWLEDGEMENTS

The authors express their gratitude to God for providing strength and guidance throughout this work. We would like to thank Prof. Dr. Antonia Queiroz Lima de Souza for her mentorship and invaluable contributions. We are also grateful to our colleagues for their scientific support and collaboration. Special thanks are extended to UFAM and UEA for the infrastructure and equipment provided. Finally, we acknowledge the financial support from FAPEAM and CAPES, which was essential for the development of this research.

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