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# Full-factorial approach reveals key culture medium components enhancing growth and enzyme secretion in Amazonian *Penicillium*

Abordagem fatorial completa revela componentes-chave do meio de cultura que favorecem o crescimento e a secreção enzimática em *Penicillium* Amazônicos

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The biodiversity of fungi in the Amazon rainforest presents unexplored biotechnological potential, yet replicating environmental conditions for in vitro enzyme synthesis remains a challenge. This study assessed the impact of nutritional and physico-chemical factors on the growth and enzyme production of *Penicillium* species isolates from the Amazon. Using a full two-level factorial experiment, we examined the effects of carbon sources (glucose, mannose), pH (5, 9), nitrogen sources (peptone, yeast extract), and trace elements (Zinc, Magnesium) on mycelial growth and the production of protease, amylase, and cellulase in *Penicillium purpurogenum* and *Penicillium oxalicum*. Results showed that culture medium 15 (glucose, pH 9, yeast extract, magnesium) was optimal for growth and enzyme activity of *P. purpurogenum*, while medium 16 (mannose, pH 9, yeast extract, magnesium) was best for *P. oxalicum*. Medium 14 (mannose, pH 5, yeast extract, magnesium) enhanced protease production, and medium 15 favored amylase and cellulase synthesis. All tested conditions exhibited an enzyme index (EI) greater than 2 for both fungi, indicating their potential as effective hydrolytic enzyme producers. Our work demonstrates a fast method to investigate parameters of culture media composition beyond commercial options, highlighting the importance of it when prospecting the biotechnological potential of isolates from biodiverse environments.
Keywords: mycelial growth, nutritional factors, enzyme production.

A biodiversidade de fungos na floresta Amazônica apresenta potencial biotecnológico ainda não explorado, mas a replicação das condições ambientais para a síntese enzimática *in vitro* continua sendo um desafio. Este estudo avaliou o impacto de fatores nutricionais e físico-químicos no crescimento e na produção de enzimas por isolados de espécies de *Penicillium* da Amazônia. Utilizando um delineamento fatorial completo de dois níveis, foram examinados os efeitos de fontes de carbono (glicose, manose), pH (5, 9), fontes de nitrogênio (peptona, extrato de levedura) e elementos-traço (zinco, magnésio) sobre o crescimento micelial e a produção de protease, amilase e celulase em *Penicillium purpurogenum* e *Penicillium oxalicum*. Os resultados mostraram que o meio de cultura 15 (glicose, pH 9, extrato de levedura, magnésio) foi o mais adequado para o crescimento e atividade enzimática de *P. purpurogenum*, enquanto o meio 16 (manose, pH 9, extrato de levedura, magnésio) foi o melhor para *P. oxalicum*. O meio 14 (manose, pH 5, extrato de levedura, magnésio) favoreceu a produção de protease, e o meio 15 promoveu a síntese de amilase e celulase. Todas as condições testadas apresentaram índice enzimático (IE) superior a 2 para ambos os fungos, indicando seu potencial como produtores eficientes de enzimas hidrolíticas. Nosso trabalho demonstra um método rápido para investigar parâmetros da composição de meios de cultura além das opções comerciais, destacando sua importância na prospecção do potencial biotecnológico de isolados provenientes de ambientes biodiversos.
Palavras-chave: crescimento micelial, fatores nutricionais, produção enzimática.

## 1. INTRODUCTION

The Amazon rainforest is a hotspot of microbial biodiversity [1, 2]; the variety of ecological niches within the forest ensures multiple genetic profiles and an enriched source of biomolecules

with great economic potential [1]. Among these microorganisms, fungi are among the most diverse groups, due to their fundamental roles in nature as decomposers, mutualists, or pathogens [3]. An important genus from the Ascomycota phylum, the *Penicillium* presents a worldwide distribution and is ubiquitous to several environments, such as soil, water, air, and even extreme conditions regarding pH, salinity, and temperature [3-6]; its main function in the ecosystem is the decomposition of organic materials [5]. The function and the variety of habitats highlight the great potential of *Penicillium* sp. for biotechnological use [4, 5]; currently, penicillin, the first isolated antibiotic, is the most famous product of the genus [4], but several other compounds have been discovered and are used for a wide range of applications - from food to medicine [5-7]. Two relevant species of the *Penicillium* genus are *P. purpurogenum* and *P. oxalicum*: *P. purpurogenum* is known for producing a visible red pigment in solid culture media [8] and numerous enzymes, such as α-1,3-glucanase [9] and β-glucosidases, associated with cellulase activity [8-10]. The fungus *P. oxalicum*, named for its production of oxalic acid, is also known for producing metabolites and pigments, such as anthraquinone and red dye [11-13].

The industrial application of this genus species is mainly related to their enzyme synthesis capabilities [14]. The production of enzymes such as proteases, lipases, amylases, cellulases, and dextranases permits to prepare honey artificially, improves the flavors of dairy products, the processing of animal feed and soybeans, as well as improves the recovery of sugar from sugar cane [14-17]. Enzymes are biomolecules that catalyze a chemical or biological reaction accelerating the process of product acquisition [14]. Their global market is projected to reach US$ 11.2 billion by 2029 [18], and these numbers reflect the commercial value of enzymes that can be alternatives to chemical products in various industrial sectors [19]. One of the challenges for enzyme manufacturing is replicating in vitro the proper conditions for the microorganism to produce the biomolecule. The diversity and quantity of enzymes vary mainly due to changes in the fungi's growth environment; nutritional conditions - carbon, nitrogen source, micronutrients, pH, etc. - are vital parameters that can favor metabolic routes otherwise silenced under standard cultivation conditions [20, 21]. Studies evaluating the effect of culture media composition on germination, mycelial growth, and enzyme synthesis have been performed [19-25]; to investigate multiple combinations of factors and to select the most relevant ones, with as few experiments as possible and in an organized manner, these studies used tools such as factorial design (DOE - Design of Experiments), resulting in savings of time and financial resources [22, 23]. However, most studies observed solely commercial culture media composition, which restrains the combinatorial possibilities being surveyed [22-25].

Considering the added value for hydrolytic enzymes in industrial sectors and using a full factorial design to evaluate non-commercial culture media combinations, this work aimed to examine the influence of nutritional and physico-chemical factors on mycelial growth and the production of protease, amylase, and cellulase enzymes by strains of *Penicillium oxalicum* and *Penicillium purpurogenum* isolated from the Amazon region.

## 2. MATERIALS AND METHODS

### 2.1 Fungal strains and monosporic culture

The strains of fungi *Penicillium purpurogenum* (CFAM 0214) and *Penicillium oxalicum* (CFAM 1311) used in this study were kindly provided by the Instituto Leônidas & Maria Deane (ILMD), Coleção de Fungos da Amazônia (CFAM), Manaus-AM, Brazil. These strains were isolated from the drinking water of the Lago do Limão community, Iranduba-AM [26]; their reactivation was performed by the collection curator, following the protocol described by Pitt (2000) [27]. To obtain a culture from a single spore, the microorganisms were subjected to the serial dilution technique [28].

## 2.2 Study Design

We designed a non-randomized full four-factor factorial experiment with two levels ($2^4$) using the Minitab 19.1 software to define the different culture medium compositions (Table 1). The factors considered were: 20 $g.L^{-1}$ of carbon source (D-glucose or Mannose), 10 $g.L^{-1}$ of nitrogen source (Peptone or Yeast Extract), pH (5 or 9), and 150 µM trace elements (Zinc Sulphate [Zn] or Magnesium Chloride [Mg]). The four factors were divided into lower (-1) and upper (+1). We carried out 16 experiments with three replicates, totaling 48 runs for each fungus. The pH was adjusted with 1M sodium hydroxide or hydrochloric acid solutions.

*Table 1: Organization of the variables and their levels for the experiment's design.*

| Code | Factor | Levels | |
|---|---|---|---|
| | | **Lower (-1)** | **Upper (+1)** |
| A | Carbon source | Glucose | Manose |
| B | pH | 5 | 9 |
| C | Nitrogen source | Peptone | Yeast extract |
| D | Trace element | Zinc | Magnesium |

## 2.3 Mycelial growth

To obtain mycelial samples, we grew the fungal strains in Malt Extract Agar (MEA) culture medium for 48 hours at 28 °C. Afterward, a germinated disc (Ø = 0.9 cm) was extracted and transferred to a Petri dish containing the culture media proposed by the factorial design combination. For this assay, all culture media composition was supplemented with agar (20 $g.L^{-1}$) to solidify. The plates were incubated in a Biochemical Oxygen Demand (BOD) chamber for 15 days at 28°C. Morphological growth was assessed every 3 days by observing and measuring the diameter of the macro-colony using a millimeter ruler.

## 2.4 Submerged culture

We carried out submerged cultures containing an inoculum at $1x10^6$ $conidia.mL^{-1}$ concentration for 15 days at 28 °C, the medium followed the composition defined according to the factorial design. Afterward, the secretomes were vacuum filtered using a cellulose ester membrane. The filtered secretomes were inoculated into cup plates (0.9 cm diameter) on Petri dishes containing the culture medium specific for each enzyme assay.

## 2.5 Protease enzyme assay

For the protease evaluation, we utilized a milk-agar culture medium containing milk (5%), agar (90%), and gelatine (5%) [29]. The agar and gelatine were sterilized for 15 minutes at 121 °C, while the milk was sterilized by flowing steam for 5 minutes. After inoculating the secretomes, the samples were incubated for 48 hours at 28 °C. A clear halo around the cup plate was observed and measured with a millimeter scale when positive for protease activity.

## 2.6 Amylase enzyme assay

For the amylase assay, we used a starch agar culture medium composed of soluble starch (1%) and nutrient agar (1.8%), sterilized for 15 minutes at 121°C. After inoculating the secretomes, the plates were incubated for 7 days at 28°C [29]. Afterward, we applied a 1% iodine solution onto the plate. A clear halo around the cup-plate was observed and measured with a millimeter scale when positive for amylase activity.

### 2.7 Cellulase enzyme assay

For the cellulase assay, we used cellulose agar medium composed of sodium nitrate (20 $g.L^{-1}$); sodium phosphate dibasic dihydrate (1 $g.L^{-1}$); magnesium sulfate (0.5 $g.L^{-1}$); potassium chloride (0.5 $g.L^{-1}$); carboxymethyl cellulose (2 $g.L^{-1}$); peptone (0.2 $g.L^{-1}$) and agar (17 $g.L^{-1}$), and sterilized for 15 minutes at 121 °C. After inoculating the secretomes, we stored the plates for 7 days at 28 °C [30]. Afterward, we applied a solution of 0.1% Congo red for 30 minutes and washed with a 1M sodium chloride solution for 5 minutes; a clear halo around the cup plate was observed and measured with a millimeter scale when positive for cellulase activity.

### 2.8 Enzyme index calculus

The Enzymatic Index (EI) was calculated by dividing the total diameter of the degradation halo by the diameter of the cup plate [24, 29], according to the equation below:

$$EI = \frac{Total\ diameter\ of\ the\ degradation}{Diameter\ of\ the\ cup\ plate}$$

### 2.9 Statistical analysis

The data statistical analysis was carried out using Minitab 19.1 software. We applied a Tukey test to compare the means, considering a significance level of 5%. The graphs were generated using Minitab software version 19.1.

## 3. RESULTS

### 3.1 Culture media composition effect on mycelial growth of *P. purpurogenum* and *P. oxalicum*

Regarding the effect of culture media composition on mycelial growth, the optimum culture media considering the largest growth diameters (cm) were: for *P. purpurogenum*, media 7 (glucose, pH 9, yeast extract, Zinc; 6.97 ± 0.0580), 15 (glucose, pH 9, yeast extract, Mg; 6.7 ± 0.265), and 16 (mannose, pH 9, yeast extract, Mg; 6.7 ± 0.361); these statistically showed no difference (Figure 1: A). For the *P. oxalicum*, culture medium 16 (7.72 ± 0.208) displayed the most significant results for this strain development (Figure 1: B). In overview, our results indicate that the main factors influencing mycelial growth are an alkaline pH (9) and yeast extract as a nitrogen source. In all the culture compositions evaluated, both species showed mycelial growth greater than 2 cm after 15 days of incubation at 28 °C. Additionally, the period of fastest growth occurred between 6 and 9 days for both strains (Figure 1).

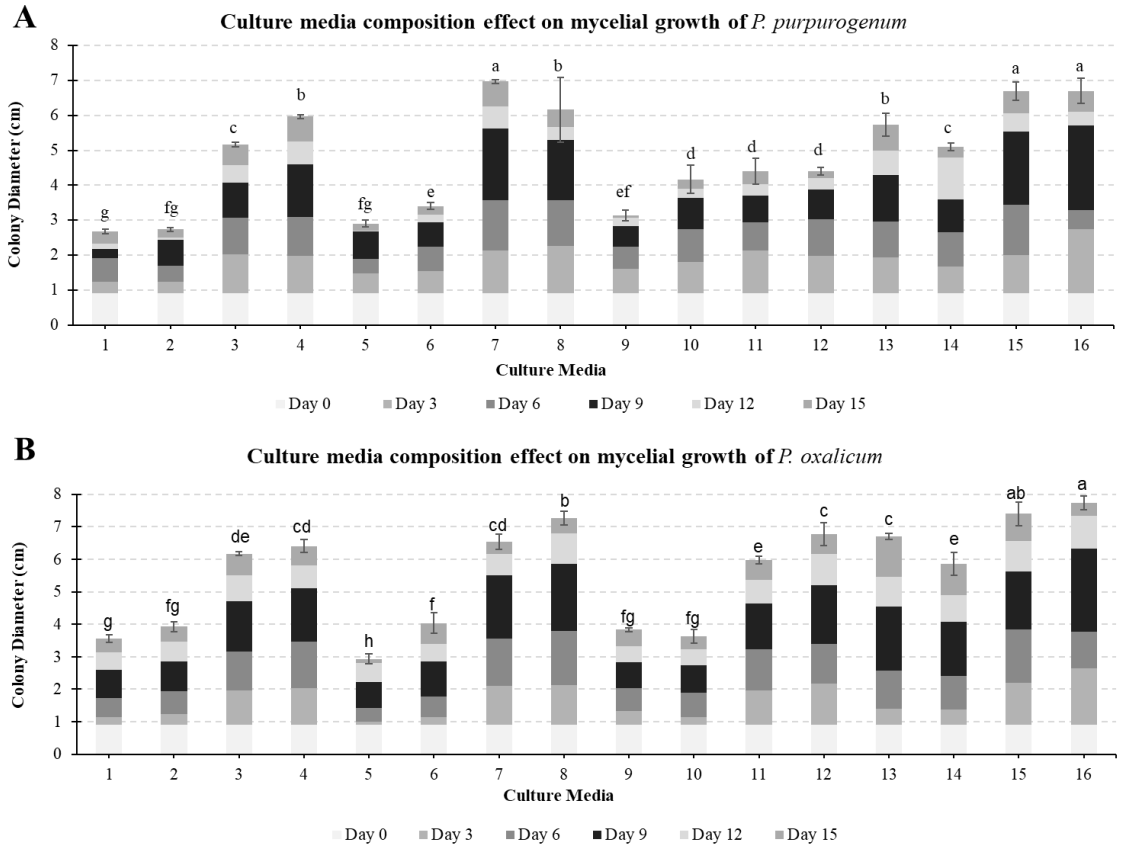


*Figure 1: Mycelial growth of Penicillium purpurogenum (A) and Penicillium oxalicum (B) in different culture media. Values represent the mean of three replicates with accumulated standard deviation. Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$).*

### 3.2 Influence of nutrients on the development of *P. purpurogenum* and *P. oxalicum*

Analyzing the influence of carbon and nitrogen sources, pH and trace elements on the fungi development, we found pH and nitrogen source as the most significant variables for *P. purpurogenum* and for *P. oxalicum* (Figure 2). Following these, in an isolated manner or combination with another variable, trace element was another relevant factor for mycelial growth in both strains. As for the carbon source, it was not a significant factor in the development of *P. purpurogenum* (Figure 2A), showing a small influence for *P. oxalicum* (Figure 2B). Observing the interaction between factors, the combination of nitrogen and trace elements showed the most positive effect for both strains; for *P. purpurogenum*, the combination of pH and micronutrients was also relevant for growth.

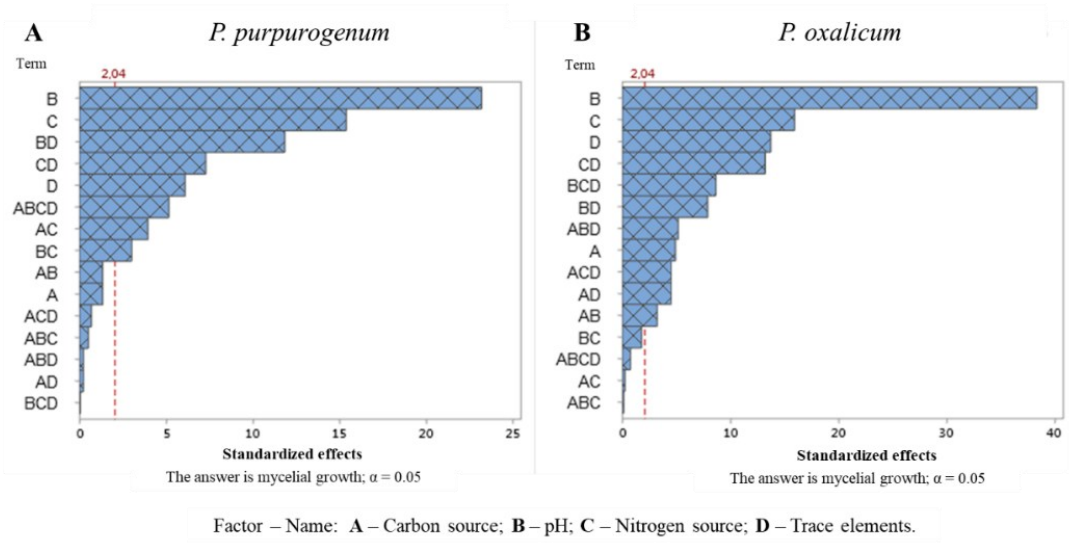


*Figure 2: Pareto chart of the effect of culture medium components on mycelial growth: (A) Penicillium purpurogenum; (B) Penicillium oxalicum. Factors crossing the vertical reference line are statistically significant at the 0.05 level.*

### 3.3 Production of hydrolytic enzymes by *P. purpurogenum* and *P. oxalicum* in different culture media

We tested the fungal secretomes according to culture media composition for protease, amylase, and cellulase activity. On *P. purpurogenum* assays, the culture media 15 was optimum for all proteins evaluated, presenting the highest enzyme index for all essays performed of proteases (EI= 3.222 ± 0.000), amylases (EI = 4.111 ± 0.112) and cellulases (EI = 5.259 ± 0.064). Additionally, the enzymatic activity observed was statistically different from all the other culture media tested (Figure 3A). Concerning *P. oxalicum*, 14 was the best culture media for proteases production, (EI = 3.407 ± 0.170) meanwhile medium 15 reported the highest enzyme indexes for amylase (EI = 2.519 ± 0.064) and cellulase (EI = 5.926 ± 0.064) (Figure 3B). In overview, the highest enzyme levels were reported for cellulase activity, being this the only enzyme produced by both fungi in the 16 different culture media. In contrast, the secretome of culture medium 3 showed no proteolytic activity for both fungi, suggesting the combination of glucose, pH 9, peptone and Zinc interfered with these enzyme productions or activity. Additionally, on *P. oxalicum* assays, amylase was detected only in six secretomes (i.e. 2, 3, 6, 14, 15, 16), while four culture media showed exclusively cellulase activity (i.e. 3, 7, 8, 9).

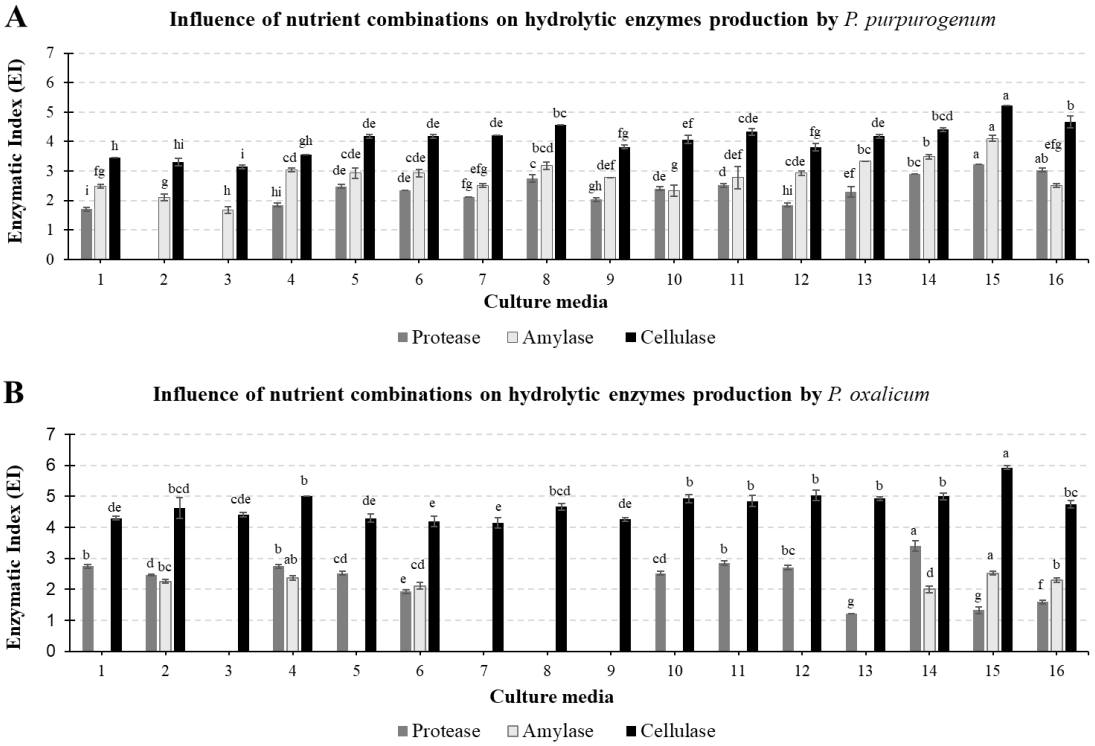


*Figure 3: Enzymatic index of protease, amylase, and cellulase halos from the secretome of Penicillium purpurogenum (A) and Penicillium oxalicum (B) in different culture media. Values represent the mean of three replicates. Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$).*

### 3.4 Influence of nutrient combinations on the production of hydrolytic enzymes by *P. purpurogenum* and *P. oxalicum*

We applied statistical analysis to observe the effect of culture media elements on hydrolases production, as isolated or combined variables (Figure 4). On *P. purpurogenum* assays, nitrogen source was the most significant variable for all enzyme production, with a t-value of 54.14 (protease), 15.24 (amylase), and 29.99 (cellulase). The trace element variable was also positively significant for enzyme production, as an isolated factor or in combination with pH and carbon source. In contrast, the carbon source as a single variable did not significantly influence the production of amylase and cellulase (Figures 4C and 4E), showing a small effect on protease production. However, when in combination with other factors, especially the pH, it had a significant positive impact on all enzyme synthesis. For *P. oxalicum*, the factors varied for each enzyme evaluated. For protease production, the pH in combination with trace elements was the main positive factor (Figure 4B), while for amylase production, the nitrogen source in combination with trace elements (Figure 4D), and for cellulase, the trace element (Figure 4F).

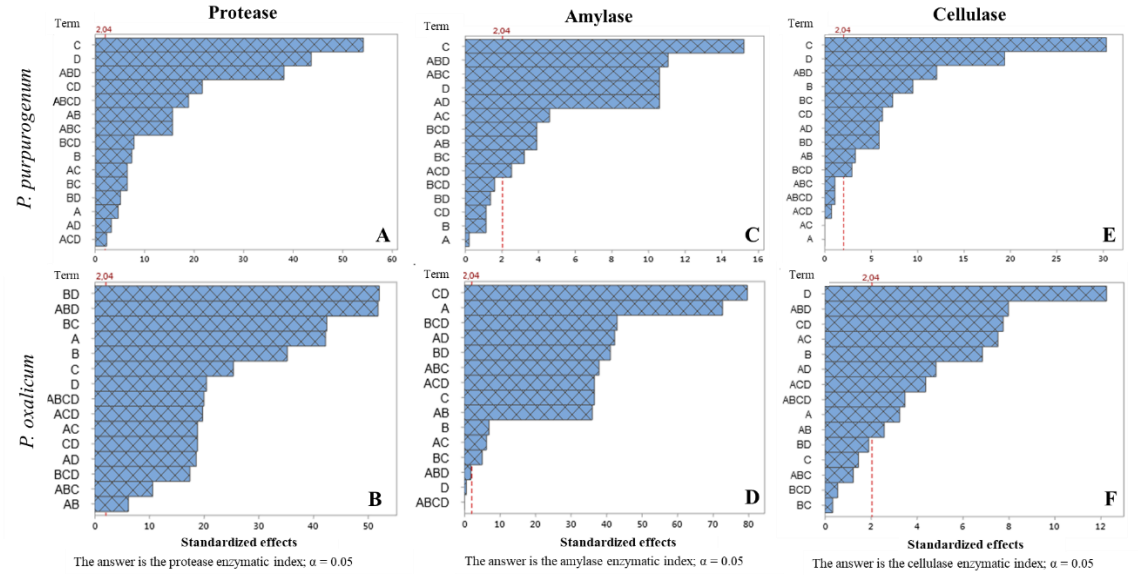


*Figure 4: Pareto chart of the effect of the culture medium components on the enzymatic degradation halo. Penicillium purpurogenum: (A) protease; (C) amylase; (E) cellulase. Penicillium oxalicum: (B) protease; (D) amylase; (F) cellulase. Factors that cross the vertical reference line are statistically significant at the 0.05 level.*

## 4. DISCUSSION

Culture medium composition affects germination, sporulation, mycelial growth, and even virulence of fungi [19, 20, 22, 30]. Thus, the nutritional requirements are important for the success of fungal in vitro cultivation and for optimizing industrial fermentation processes [31], especially when it comes to the genus *Penicillium*. Within this genus, we highlight *P. purpurogenum* and *P. oxalicum* for their biotech applications, especially the secretion of hydrolytic enzymes, such as proteases, invertases, lipases, amylases, cellulases, D-galactosidases, xylanases and dextranases [8-13, 15-17]. Studies assessing the growth of *Penicillium* sp. species on different commercial culture media have been carried out [32, 33]. However, research investigating nutritional factors individually or using non-commercial media combinations are scarce. Hence, we applied a full factorial design approach to evaluate the main nutrients influencing growth, and the enzyme secretion of *Penicillium purpurogenum* CFAM0214 and *Penicillium oxalicum* CFAM1311.

Considering the sixteen (16) culture media compositions tested, we found three optimum culture media for *P. purpurogenum* mycelial growth: glucose, pH 9, yeast extract, and Zinc (media 7), glucose, pH 9, yeast extract, and Mg (media 15), and mannose, pH 9, yeast extract, and Mg (media 16); the culture media 16 also presented the best results for the *P. oxalicum* mycelium development. These results suggest yeast extract as the nitrogen source, and an alkaline pH (pH 9) are the two main components influencing the growth of the two strains. Yeast extract has been reported as an optimum supplement for growth and metabolite production in bacteria and fungus cultivates [34-36]. Within studies focusing on *Penicillium* sp., Sharma and Pandey (2010) [32] observed *P. corylophilum* and *P. expansum* obtaining their maximum mycelial diameters when grown on CYA (Czapek Yeast Extract) and LCA (Lignocellulose Agar) media, with these ranging from 4.23 to 4.83 cm [32]; *P. janthinellum* and *P. duclauxii* also showed excellent growth on culture media such as YES (yeast extract sucrose) and Harrold's agar (malt extract, yeast extract and sucrose), as reported by Zain et al. (2009) [33]. Our findings supported these results with the nitrogen source and pH as the most significant positive factors for the growth of both fungi, with little influence from the carbon source being reported. This lack of impact from carbon sources on growth was found in research over *Bauveria bassiana*; as described by Bidochka et al. (1987) [37], the carbon source influencing initial germination, while nitrogen was required for mycelial growth. In another study, *Arthrinium saccharicola* grew twice as fast in

media with a high content of glucose, peptone, and yeast extract, as demonstrated by Miao et al. (2006) [38]; statistical analysis also showed that high concentrations of peptone and yeast extract, but not glucose, enhanced the mycelial production of this species, highlighting yeast extract as more relevant than peptone in supporting the fungus [38].

Regarding the pH, most fungi grow well in environments with a pH between 4 and 6. However, it's not uncommon to find fungus growing, albeit to a lesser extent, in more alkaline conditions - above pH 8 [31]. In our study, the two Amazonian strains achieved the maximum diameter of growth when cultivated in an alkaline medium, which implies these might be alkali-tolerant species. Some studies with *Phoma exígua* [39], *Penicillium roqueforti* [40], *Arthrinium saccharicola* [38], have revealed strains sensitive to pH higher than 7,5. In another study, the *P. citrinum* and *P. waksmanii* specimens were isolated from alkaline sediments and grew in a culture medium with a pH of 9 [41]. All the mechanisms of how pH influences microbial development have yet to be elucidated; current evidence shows a direct association of metabolic tolerance in distinct pH ranges with fungi functionality [31]. It's noteworthy to mention that the compounds used to adjust the pH may impact the growth, when the culture medium is adjusted with organic reagents (e.g. acetic acid) the growth is inhibited when compared to those adjusted with inorganic reagents (e.g. hydrochloric acid) [31]; in our experiments, the pH was adjusted with inorganic reagents which might have influenced the performance of both strains in alkaline media.

Concerning protein synthesis, the enzyme index (EI) method is the fastest and simplest parameter for determining enzyme activity in solid culture media [42, 43]. According to Hankin and Anagnostakis (1975) [43], fungi with an enzymatic index equal to or superior to two ($EI \geq 2$) can be considered good producers [44]. In our work, all conditions tested presented an EI higher than 2 for both fungi, reporting both strains as good enzyme producers of hydrolytic enzymes. When analyzing the media composition influence on the EI, we observed not all culture media stimulated the assessed enzymes. Cellulase was the only enzyme constantly produced in all assays, with *P. oxalicum* presenting more gaps in protease and amylase secretion. These findings diverge from the work of Batista Junior et al. (2021) [24] with *P. purpurogenum* CFAM214 and *P. oxalicum* CFAM 1311 which reported the absence of cellulase activity for these strains. We hypothesize this difference may derive from their use of commercial culture media in opposition to our multiple combinatorial composition approach, evidencing that changes in media components can alter metabolic routes [19, 20, 34].

In addition, the assays of *P. purpurogenum* report culture media 15 combination - glucose, pH 9, yeast extract, Mg - as optimum media composition for mycelial growth and enzyme production; since the Pareto analysis for this strain displays nitrogen source as the main factor for protein production, these results reinforce the previous suggestion of yeast extract as an important supplement for *P. purpurogenum* cultivation with a biotechnological purpose. Interestingly, *P. oxalicum* showed the best EI for proteases with secretomes from culture medium 14 (mannose, pH 5, yeast extract, Mg), and higher indexes for amylases and cellulases with media 15. This variability is reflected in the Pareto analysis where each enzyme presents a distinct main factor: protease is mostly affected by the combination of pH and trace element, whereas amylase's main factor is a combination of nitrogen source and trace element, and for cellulase, the trace element as an isolated variable; observing the pH of culture media 14 (pH 5), we suggest that acidic pH may favor protease production on this strain of *P. oxalicum*.

The divergence between optimum composition for biomass production and enzyme activity in fungal assays has been reported [45], as well as the influence of trace elements on protein secretion and cell metabolism [46-49]. The precise mechanisms by which trace elements affect enzyme activity are mostly unexplored; however, the existing research suggests their effect varies by the ion and the organism, also depending on concentration [47-49]. Unfortunately, our results were limited to evaluating the factors in a quality manner, but not in quantities. Considering that all culture media with significant results for *P. oxalicum* - 14, 15, and 16 - contained magnesium, we suggest this trace element as the most significant for this strain. Magnesium is the cofactor of several families of enzymes found in general metabolic pathways and in nucleic acid biochemistry, hence it is related to cell metabolism regulation [49]. In overview, $Mg^{2+}$ may be a coadjuvant on enzyme-substrate interaction by binding to the substrate to form a complex or may

bind directly to the enzyme altering its structure and enabling the interaction and/or catalysis [48, 49].

## 5. CONCLUSION

The results of this study demonstrate that culture medium composition significantly influences the mycelial growth and production of hydrolytic enzymes by *Penicillium purpurogenum* and *Penicillium oxalicum* isolates from the Amazon. Media containing alkaline pH, magnesium, and yeast extract enhanced the production of protease, amylase, and cellulase, with enzyme index values exceeding 2, indicating high biotechnological potential. The use of a factorial design efficiently identified non-commercial nutrient combinations with superior performance, highlighting the importance of optimized experimental strategies in the bioprospecting of microorganisms from biodiverse environments such as the Negro River basin.

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