Vol.68: e25250100, 2025
https://doi.org/10.1590/1678-4324-2025250100
ISSN 1678-4324 Online Edition

BABT
Brazilian Archives of Biology and Technology

Article - Biological and Applied Sciences

Investigation of the Enzymatic Activity of Fungicolous Fungi in Basidiomes of Hymenochaetaceae

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Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Paulo Vitor Farago

Received: 12-Feb-2025; Accepted: 25-Jun-2025

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HIGHLIGHTS

- Macrofungi are reservoirs of microorganisms with industrial applications.
- Genetically identical strains from different macrofungal origins produce distinct enzymes.
- The fungicolous fungi produce amylases, cellulases, proteases and oxidoreductases.
- Enzymatic production of fungicolous fungi varied significantly between PYD and Manachini.

Abstract: Fungi isolated from the basidiomes of Hymenochaetaceae are a promising source of hydrolytic enzymes and oxidases, which are of interest mainly due to the ease of obtaining these enzymes and their industrial applications. This study aims to detect the activity of amylases, cellulases, proteases, and oxidoreductases produced by fungi isolated from the basidiomes of Hymenochaetaceae, collected in the states of Acre and Amazonas, in the Amazon region, and to compare the enzymatic production obtained in specific and general culture media. We selected ten strains of fungi associated, which were subjected to molecular identification and subsequently cultivated in general culture medium (potato, dextrose and yeast extract) and in specific culture medium (Manachini solution with appropriate substrate for each enzyme). From the isolation of the basidiomes of *Fomitiporia bambusarum*, *F. neotropica*, *Fuscoporia gilvus*, *Hymenochaete damicornis* and *Phylloporia spathulata*, we obtained fungicolous fungi capable of producing

amylases, cellulases and proteases, with the exception of oxidoreductases, for which only five strains produced this enzyme. Both of the culture media were effective; however, the Manachini solution with specific substrates produced between 3 to 60% more enzymes. Fungi isolated from Hymenochaetaceae species have great potential for the production of enzymes of biotechnological interest.

Keywords: Agaricomycetes; Oxidoreductases; Hydrolytics; Microorganisms.

INTRODUCTION

Hymenochaetaceae is a family of basidiomycete macrofungi that is commonly found on dead wood and acts on the degradation of lignocellulolytic polymers [1], some of the species of this family are opportunistic and infect living trees and cause damage, while others live as ectomycorrhizae, in association with live tree roots [2]. These organisms occur in forests in different ecosystems [3,4] and are conducive to hosting parasitic or are symbiotic beings such as bacteria, insects, microfungi and viruses [5,6,7,8].

Hymenochaetaceae fungi are described as excellent producers of primary and/or secondary metabolites, which can have antitumor, immunomodulation, antioxidant, neuroprotection, antimicrobial properties, and also help in the prevention of diabetes and its complications [9,10,11]. Interestingly, some of these species are found in the Amazon [12]. In addition, the fungal bodies (basidiomes) of Hymenochaetaceae are inhabited by several fungicolous fungi (also called associates), in which the primary and secondary metabolites, and the community structure of those that inhabit the basidiomes are rarely explored and reported [13]. However, studies in the Brazilian Amazon have demonstrated that fungicolous fungi isolated from the basidiomes of Hymenochaetaceae are sources of primary and secondary metabolites [14].

Among the primary metabolites, enzymes are proteins that are originally formed from a long chain of amino acids linked together through peptide bonding of living beings (animals, plants and microorganisms, among others) and act as catalysts for biochemical processes [15], as well as being able to decompose complex molecules into smaller units [16]. From this process, they can generate new products [17] or obtain energy for their survival.

Species and genera of microorganisms isolated from different habitats can produce enzymes with distinct properties [18], and these products (enzymes) arouse interest in different industries, such as in the food industry [19], the detergents manufacturing [20], pharmaceuticals [21], diagnostic kits [22], biofuels [23], textile processing [24], and pulp and paper, leather and chemicals manufacturing, among others [25,26]. The market for industrial enzymes currently amounts to US$ 7.4 billion and, by 2028, it is expected to grow to US$ 10.2 billion – an estimated growth rate of 6.6% per annum [27].

Fungicolous fungi are a promising source for obtaining hydrolytic enzymes and oxidases [28]. Among the hydrolytic enzymes, amylases form the main group of enzymes used in the food industry and are exploited in baking for improving the physicochemical and sensory quality of products [29]. These are enzymes that hydrolyze starches and they are classified into different forms according to the starch or glycogen molecule [30]. Cellulases are exploited in the fabric and stationery industry [31] for the breakdown of the bonds of cellulose molecules with release of disaccharide cellobiose [32]. Proteases are more commonly used in the treatment of leather, in the development of new drugs, different detergents and in the food industry [33]. These groups of enzymes are currently being investigated for use in biorefineries for the production of second-generation bioethanol, and oxidases are highlighted for bioremediation of areas contaminated by oil and its derivatives.

Laccases, which are part of the group of oxidoreductases, are enzymes of low specificity to their reducing substrates, becoming generalist in relation to their substrates, with the power to catalyze a variety of phenolic and aromatic compounds via oxidation [34,35]. They are extracellular enzymes, which facilitates their action and activity [36], and are mainly used in bioremediation processes of contaminated water and soils or in paper bleaching [37,38].

Currently, fungal enzymes account for more than half of the market for enzymes used in different industrial sectors [39]. In this context, this study aimed to investigate the production of enzymes (amylases, cellulases, proteases and oxidoreductases) from fungicolous fungi isolated from the basidiomes of Hymenochaetaceae species collected in the Brazilian Amazon and compare the efficiency of two cultivation media, a generalist and a specific medium, in order to aid sites where research resources are scarce.

MATERIAL AND METHODS

Collection and identification of Hymenochaetaceae macrofungi

The Hymenochaetaceae macrofungi were obtained from the Catuaba Experimental Farm at UFAC (10°03'25.8" S, 67°36'00.9" W), the Humaitá Forest Reserve (9°45'49.36" S, 67°38'25.54" W), both located in the state of Acre, and the UFAM Experimental Farm (2°39'1.12" S, 60°3'12.30" W), located in the state of Amazonas.

At the time of collection, the basidiomes of the Hymenochaetaceae found were georeferenced and photographed with the aid of a camera, a scale and given a collector's number. Then, the specimens were manually removed with the help of a chisel and packed in a paper bag duly identified with the date, place of collection, substrate and geographical coordinates [40]. Other basidiomes from the same specimen were placed in a Petri dish for isolation, as described in the section below. The collected macrofungi were taken to the Laboratory of Bioassays and Microorganisms of the Amazon, at the Analytical Center of the Federal University of Amazonas (LaBMicrA/UFAM), where they were kept in an oven with air circulation at 35 °C (±2 °C), for 48 to 96 h for complete drying. Then, they were stored in envelopes with all their information until the taxonomic identification.

The macroscopic analyses were performed based on observations of the basidiomes with the naked eye, verifying: shape of the basidiome (sessile/resupinate/effusive-reflex/pileate/stipitate), dimension of the basidiome (length, width and thickness) with a ruler in millimeter, and insertion into the substrate, consistency, and with the aid of a stereoscopic magnifying glass, the characteristics of the surfaces of the pores, tubes, context and margin of the basidiome, with a stereo microscope [41].

For the microscopic analysis, free-hand sections of different parts of the basidiomes (cap, context and tubes) were obtained with the aid of a steel blade, under a stereoscopic magnifying glass. Then, the sections were arranged between slides and coverslips with different aqueous solutions: distilled water, potassium hydroxide 3% (moisturizer), phloxin 1% (dye), cotton blue (dye) and Melzer (reagent) to show the reactions of the walls of the microstructures, which can be positive or negative according to each species – amyloid (blue to purple/violet tone), dextrinoid (brown to reddish tone) and kinophilia reaction (lighter tone) [41,42]. The hyphalic system (monomitic, dimitic, di-trimitic), reproductive structures (basidia and basidiospores) and sterile structures (cystidia, cystidioles and setae) were also analyzed [41].

After the specimens were analyzed, the characteristics were compared with the specialized literature of Hymenochaetaceae [41,43,44]. The nomenclature for the species has been continuously updated according to the Index Fungorum (http://www.indexfungorum.org). All fungal species in this study are registered in SisGen under code A91107E.

Isolation of fungicolous fungi with the basidiomes of Hymenochaetaceae

The basidiomes of the Hymenochaetaceae fungi were subjected to asepsis for isolation adapted from [45]. In a biological safety booth, the basidiomes were fragmented and immersed in 70% alcohol for 1 min, in 4% hypochlorite for 4 min, and again in 70% alcohol for 30 s. To remove the excess hypochlorite, they were placed in autoclaved distilled water for 2 min or more for the removal of the excess hypochlorite and to control asepsis.

After the asepsis process, fragments of the fungi (0.5 × 0.5 cm) were removed and inoculated in a 2 × 2 matrix in another Petri dish containing oat agar culture medium (10 g/L of oats, 10 g/L of malt extract, 4 g/L of yeast extract, 4 g/L of dextrose, 15 g/L of microbiological agar) plus ampicillin and tetracycline (100 µg/mL) to inhibit bacterial growth.

The inoculated Petri dishes were incubated in a BOD chamber at 28 ºC for seven days and, according to the emergence of the mycelia, cuts were performed for dishes containing only oat agar culture medium, which were kept at the same isolation temperature for 10 days recording the obverse and reverse of the dishes.

Morphological and molecular identification of fungicolous fungi

For the morphological and molecular characterization, 10 out of 203 strains of fungicolous fungi with the basidiomes of the Hymenochaetaceae were selected using the random system available in Microsoft Excel (formula: =RANDOMBETWEEN(1;203)). We used this method to ensure randomness and impartiality in the selection of strains, avoiding bias and ensuring that the sampling more fairly represented the entire data set. For the macrophological characterization, the isolates were cultured for 8 days on oat agar, at 28 °C in a BOD chamber, after which, the strains were grouped into morphogroups which were recorded the front and

back of the Petri dishes by photographs. For the micromorphological analysis, the isolates, which were grouped according to vegetative characteristics, were submitted to microcultures, and slides were made for the observation of their vegetative (hyphae) and reproductive (spores) structures under an optical microscope with 40x magnification. These were then compared with the specific literature for the taxonomy of each group [46,47,48].

The selected strains were subjected to genomic DNA extraction and cultured in 250 mL Erlenmeyer flasks containing 100 mL of potato dextrose yeast medium (PDY: 200 g/L potato, 20 g/L dextrose and 4 g/L yeast extract), which were incubated in a shaker for 72 h at 28 (±2 °C) and 120 rpm. After this period, the culture was vacuum filtered with filter paper in a Büchner flask with a Büchner funnel to obtain approximately 200 mg of mycelial mass, and the surplus was discarded. Total DNA was extracted with a ZR Fungal/Bacterial DNA MicroPrep™ kit, according to the manufacturer's protocol with some modifications [49] to improve yield and adapt it to the laboratory conditions.

The quantification of genomic DNA was performed in a spectrophotometer (NanoDrop, Thermo Fisher) to standardize the DNA concentrations of each sample. An aliquot of this was diluted to the final concentration of 10 ηg/µL for PCR with specific primers for molecular identification of the species. The concentrated and diluted DNAs were stored at -20 °C. After obtaining the total DNA, an internal rDNA fragment of approximately 700 bp from the *ITS-1* and *ITS-2* regions was amplified using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 primers (5'-TCCTCCGCTTATTGATATGC-3') [50].

Amplification was performed using an initial cycle of 94 °C for 4 min, 35 cycles at 94 °C for 2 min, 55 °C for 2 min and 72 °C for 2 min. At the end of the cycles, another cycle was performed at 72 °C for 10 min and the final PCR product was kept at 4 °C until it was removed from the thermocycler [44]. PCR product verification was performed using GelRed™ stained 1% agarose gel electrophoresis using Invitrogen® 1 Kb Ladder plus as DNA marker. The gels were photographed using the L-Pix Touch Loccus® system and the amplicons were then sequenced.

The PCR products were purified with the enzyme ExoSAP-IT (GE Healthcare) and quantified and adjusted to the concentration of 10 ng/µL. Sequencing was performed using the Sanger method and the BigDye Terminator kit (Applied Biosystems). The sequences obtained from the strains were compared with the sequences deposited in the GenBank database (NCBI) using the BLASTn alignment tool (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The alignment of the sequenced products was performed using the CLUSTAL W program (MEGA 11, Molecular Evolutionary Genetics Analysis).

Acquisition of the enzyme extract

The enzymatic assay was performed in triplicate in two ways: (i) two 0.5 × 0.5 cm fragments of fungicolous fungi were inoculated in 100 mL of PDY liquid culture medium without the inducer in 250 mL Erlenmeyer flasks. In this experiment, we did not test for oxidoreductases [51]; (ii) two 0.5 × 0.5 cm fragments of fungicolous fungi were inoculated in 100 mL of Manachini solution [52], (2 g/L potassium phosphate monobasic (KH_2PO_4), 1 g/L ammonium sulfate $(NH_4)2SO_4$, 0.1 g/L magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 0.9 g/L sodium phosphate monobasic ($NaHPO_4{\cdot}2H_2O$), 1 g/L yeast extract) in 250 mL Erlermeyer flasks and we added 5% amylase-inducing substrate (corn starch), cellulases (carboxymethylcellulase), proteases (colorless and tasteless gelatin) and oxidoreductases (wheat bran), which had been previously sterilized in an autoclave. After inoculation, the Erleymeyer flasks were placed in a shaker incubator at 28 ºC for 198 h and 120 rpm.

After the cultivation period, the enzyme extracts were separated from the fungal mycelia by vacuum filtration with a Büchner flask, Büchner funnel and autoclaved filter paper. Then, the enzyme extracts were transferred to 50 mL Falcon tubes where they were subjected to centrifugation at 4,000 rpm, 4 ºC for 20 min. Subsequently, 10 mL of the enzyme extract was filtered with a sterile 0.22 µm polyethersulfone (PES) membrane syringe filter and maintained at -20 ºC in a 15 mL Falcon tube for the qualitative assay.

Qualitative assay

Aliquots of 100 µL of the enzyme extract (in triplicate) were placed in wells with a 6 mm diameter (cup plate) made in specific culture media for each enzyme: (i) amylases, starch agar (18 g/L of agar, 10 g/L of starch; 0.1 M citrate-phosphate buffer [0.1865 g/L of citric acid + 0.3665 g/L of dibasic sodium phosphate, pH 5.0]); (ii)) cellulases, agar-CMC (18 g/L Agar, 10 g/L carboxymethylcellulase, 0.1 m acetate buffer [8.203 g/L sodium acetate, pH 5.0]); (iii) proteases, agar-milk (36 g/L agar-milk); (iv) oxidoreductases, agar-gallic acid (18 g/L agar, 5 g/L gallic acid; 0.1 M citrate-phosphate buffer [0.1865 g/L citric acid + 0.3665 g/L sodium phosphate dibasic, pH 5.0]). For this culture medium, it is necessary that the gallic acid is autoclaved separately from the other reagents since heating the homogenized reagents together causes hydrolysis in

the agar, making it liquid. After autoclaving, the gallic acid solution can be homogenized with the other reagents [53]. The control for this experiment consisted of PDY and Manachini solution with addition of the substrate in the absence of fungicolous fungi.

The qualitative assays were incubated at 28 ºC for 24 h and, after the reaction time, the plates were stained with 10 mL of halo visualization reagent for amylases (Iugol 5%), cellulases (Congo red 1%), proteases (acetic acid 5%) and, for the oxidoreductases, there was no need for developer solution [54,55,-56].

Data analysis

After the application of the visualization reagents and a wait of 30 min, the diameters of the halos were measured (mm) with the aid of a ruler on the back of the Petri dish. The mean values obtained among the samples were compared using a one-way analysis of variance (ANOVA), and two-way analysis was used between the two experiments, with the Tukey test being performed a posteriori in the R 4.2.2 software [57] using the vegan package [58]. The determination of the enzymatic index (*EI*) was calculated using the formula: $EI = dh/ dc$, where dh is the diameter of the halo formed by the liquid extract and dc is the diameter of the well in the cup plate [59].

RESULTS

Altogether, 34 specimens of hymenochaetaceae macrofungi were collected. These belong to five genera and 13 species, namely, *Fomitiporia apiahyna* (2 specimens), *Fo. bambusarum* (5), *Fo. neotropica* (1), *Fo. punctata* (3), *Fuscoporia* aff. *gilva* sp. (1), *Fu. gilva* (4), *Hymenochaete iodina* (1), *H. damicornis* (6), *H. luteobadia* (2), *H. rubiginosa* (1), *Phylloporia elegans* (1), *P. spathulata* (5) and *Sclerotus extensus* (2). From these specimens, after isolation, 203 pure cultures of fungicolous fungi were obtained and cluster in 14 morphological groups, from which 10 random strains were selected to obtain the enzyme extract (Figure 1).

The 10 selected strains were identified through the *ITS-1* and *ITS-2* regions of the rDNA and compared on the NCBI platform (Table 1). The strain LaBMicrA 1345, identified 100% as *Chaetomium globosum*, while the data for LaBMicrA 1346 and LaBMicrA 1348 are *Xylaria* sp. 1 and 2, obtained from different hosts comprising 76.84%, and *X. mellisis* over 98%. From two specimens of *Hymenochaete damicornis*, two strains of *Colletotrichum* sp. were identified with 94.05% similarity. In this research, we used the 98% nucleotide identity cut-off because it is more robust than the other cut-off that is adopted (96%). In other words, strains with identification below 98% require more molecular markers and possibly sequencing of the entire genome (Table 1).

The strains obtained, such as *C. globosum*, *Nemania abortiva*, *N. feicuensis*, *N. fusoidisporia* and *Trichoderma inhamatum* are new records for the Amazon region, and we expanded the distribution of *X. mellissii* isolated from the basidiomes of Hymenochaetaceae found in the city of Manaus.

Irrespective of the experiment employed (PDY or Manachini solution), the fungicolous fungi isolated from the basidiomes of Hymenochaetaceae macrofungi were positive for the production of amylases, cellulases, proteases and 50% of the strains were positive for oxidoreductases with Manachini solution (Tables 1 and 2).

In regard to the amylases, in the experiment with PDY, the largest halos were obtained by *N. fusoidispora* (LaBMicrA 1356) and *T. inhamatum* (LaBMicrA 1352), both with an average of 13.33 mm and, in the Manachini solution, *Xylaria* sp. 1 (LaBMicrA 1346) had an average of 17.67 mm (Tables 1 and 2; Figures 2 and 3). Among the experiments, there was a significant difference (ANOVA $F= 105.9$, $p<0.001$), as well as between the strains in the Manachini solution (ANOVA $F= 8.28$, $p<0.001$). It can be noted that the isolates presented enzymatic indices ranging from 2.0 to ≤3.0, and the formation of halos exceeding the control value (Tables 1 and 2).

In the cellulases, in the PDY experiment, the largest halos produced were by Colletotrichum sp. 1 (LaBMicrA 1350), with an average of 15.67 mm, and *N. abortiva* (LaBMicrA 1351) and *T. inhmatum* (LaBMicrA 1352) with halos of 18.00 mm in the Manachini solution (Tables 1 and 2; Figures 2 and 3). The enzymatic indices ranged from 1.9 to 3.0, being higher in Manachini solution and, in the data, there was a statistical difference between the experiment with PDY and the Manachini solution (ANOVA $F= 19.49$, $p<0.001$) and between the samples in PDY (ANOVA $F= 3.11$, $p<0.01$). However, eight strains produced cellulases below the control value, demonstrating that the experiment with PDY is more efficient. However, eight strains showed cellulase production below the control value, indicating that the experiment with the PDY medium is effective for screening. However, the Manachini solution and the substrate demonstrated better performance, as the medium is specific for cellulase induction, resulting in a 17% increase in enzymatic production.

For the proteases produced by the fungicolous fungi, in the experiment with PDY, *Xylaria* sp. 2 (LaBMicrA 1348) had the largest halo with an average of 12.67 mm, while in the Manachini solution, *Xylaria* sp. 2 (LaBMicrA 1348) and *X. mellissii* (LaBMicrA 1349) produced halos of 16.00 mm (Tables 1 and 2; Figures 2 and 3). With the statistical data obtained for the proteases, it is observed that in this case there was also a difference between PDY and the Manachini solution (ANOVA F= 260.1, p<0.001), and between the samples (ANOVA F= 22.09, p<0.001 - PDY; ANOVA F= 39.15, p<0.001 – Manachini solution). Five strains in the Manachini solution produced proteases lower than the control, while the others were relatively high for the enzymatic index.

For oxidoreductase activity, the halo is characterized by a dark brown color, and only five strains showed positive results. Among the species, *X. mellissii* (LaBMicraA 1349) was the one that produced the largest halo with 18.67 mm (Table 2; Figure 3). According to the statistical analyses, there was a significant difference between the samples (ANOVA F= 320.7, p<0.001).

Figure 1. Hymenochaetaceae macrofungi and the fungicolous fungi obtained. A1) *Fuscoporia gilvus*, A2-A3) *Chaetomium globosum* (LaBMicrA 1345); B1) *Hymenochaete damicornis*, B2-B3) *Xylaria* sp. 1 (LaBMicrA 1346); C1) *F. gilvus*, C2-C3) *Xylaria* sp. 2 (LaBMicrA 1348); D1) *H. damicornis*; D2-D3) *X. mellissii* (LaBMicrA 1349); E1) *H. damicornis*; E2-E3) *Colletotrichum* sp. 1 (LaBMicrA 1350); F1) *Phylloporia spathulata*; F2-F3) *Nemania abortiva* (LaBMicrA 1351); G1) *P. chrysites*; G2-G3) *Trichoderma inhamatum* (LaBMicrA 1352); H1) *Fomitiporia bambusarum*; H2-H3) *N. feicuiensis* (LaBMicrA 1353); I1) *H. damicornis*; I2-I3) Colletotrichum sp. 2 (LaBMicrA 1354); J1) *Fo. neotropica*; J2-J3) *N. fusoidispora* (LaBMicrA 1356). Scale: A1-J1 – 1 cm; Petri dish – 90 mm.

Table 1. Mean (mm) ± standard deviation of three replicates of enzymatic activity in potato dextrose yeast (PDY) medium produced by fungicolous fungi isolated from Hymenochaetaceae macrofungi. The means followed by the same letter do not differ significantly by the Tukey test (5%). Values in bold were the most produced. EI – enzymatic index.

Voucher	Hymenochaetaceae isolated	Sequenced fungicolousspecies	Blast (%)	Enzyme extract (PDY)					
				Amylases	EI	Cellulases	EI	Proteases	EI
1345	*Fuscoporia gilva*	*Chaetomium globosum*	100	12.67 ± 0.94^{a}	2.11	13.67 ± 0.47^{ab}	2.28	10.00 ± 0.82^{ab}	1.67
1346	*Hymenochaete damicornis*	*Xylaria* sp. 1	76.84	12.67 ± 0.94^{a}	2.11	11.67 ± 1.70^{ab}	1.94	10.67 ± 0.47^{ab}	1.78
1348	*Fu. gilva*	*Xylaria* sp. 2	76.84	12.33 ± 0.47^{a}	2.06	15.33 ± 0.47^{a}	2.56	**12.67 ± 0.47^{a}**	**2.11**
1349	*H. damicornis*	*X. mellissii*	98.38	12.00 ± 0.82^{a}	2.00	13.67 ± 1.89^{ab}	2.28	10.33 ± 2.62^{ab}	1.72
1350	*H. damicornis*	*Colletotrichum* sp. 1	94.05	12.00 ± 0.82^{a}	2.00	**15.67 ± 0.94^{a}**	**2.61**	7.67 ± 0.47^{b}	1.28
1351	*Phylloporia spathulata*	*Nemania abortiva*	93.03	13.00 ± 0.00^{a}	2.17	13.00 ± 0.00^{ab}	2.17	8.33 ± 0.47^{b}	1.39
1352	*P. chrysites*	*Trichoderma inhamatum*	98.20	**13.33 ± 0.47^{a}**	**2.22**	13.33 ± 0.47^{ab}	2.22	8.33 ± 0.47^{b}	1.39
1353	*Fomitiporia bambusarum*	*N. feicuiensis*	94.41	12.67 ± 0.47^{a}	2.11	12.67 ± 0.47^{ab}	2.11	10.67 ± 0.47^{ab}	1.78
1354	*H. damicornis*	*Colletotrichum* sp. 2	94.05	12.00 ± 0.82^{a}	2.00	13.67 ± 0.47^{ab}	2.28	7.33 ± 0.47^{ab}	1.22
1356	*Fo. neotropica*	*N. fusoidispora*	92.77	**13.33 ± 047^{a}**	**2.22**	13.00 ± 0.82^{ab}	2.17	9.33 ± 1.25^{b}	1.56
			Control	11.17 ± 0.47^{a}		14.33 ± 0.47^{ab}		0.00 ± 0.00^{c}	

Table 2. Mean (mm) ± standard deviation of three replicates of enzymatic activity in Manachini solution with substrate produced by fungicolous fungi isolated from Hymenochaetaceae macrofungi. The means followed by the same letter do not differ significantly by the Tukey test (5%). Values in bold were the most produced. EI – enzymatic index.

Voucher	Sequenced fungicolous species	Enzyme extract (Manachini solution with substrate)							
		Amylases	EI	Cellulases	EI	Proteases	EI	Oxidoreductases	EI
1345	*Chaetomium globosum*	16.33 ± 1.25^{ab}	2.72	14.33 ± 1.70^{a}	2.39	15.67 ± 0.16^{ab}	2.61	0.00 ± 0.00^{c}	0.00
1346	*Xylaria* sp. 1	**17.67 ± 0.94^{a}**	**2.94**	16.00 ± 3.27^{a}	2.67	15.67 ± 0.42^{ab}	2.61	13.67 ± 0.83^{b}	2.28
1348	*Xylaria* sp. 2	14.33 ± 0.47^{bcd}	2.39	14.67 ± 1.25^{a}	2.44	**16.00 ± 0.00^{a}**	**2.67**	0.00 ± 0.00^{c}	0.00
1349	*X. mellissii*	14.33 ± 0.47^{bcd}	2.39	17.67 ± 2.49^{a}	2.94	**16.00 ± 0.00^{a}**	**2.67**	**18.67 ± 0.68^{a}**	**3.11**
1350	*Colletotrichum* sp. 1	14.67 ± 0.47^{bcd}	2.44	14.67 ± 0.94^{a}	2.44	13.67 ± 0.31^{bc}	2.28	17.33 ± 0.42^{a}	2.89
1351	*Nemania abortiva*	15.33 ± 0.47^{bc}	2.56	**18.00 ± 4.24^{a}**	**3.00**	12.00 ± 0.47^{cd}	2.00	12.00 ± 0.47^{b}	2.00
1352	*Trichoderma inhamatum*	13.67 ± 0.47^{cd}	2.28	**18.00 ± 1.41^{a}**	**3.00**	8.67 ± 0.16^{f}	1.44	17.33 ± 0.42^{a}	2.89
1353	*N. feicuiensis*	14.33 ± 0.47^{bcd}	2.39	16.00 ± 2.16^{a}	2.67	11.33 ± 0.42^{de}	1.89	0.00 ± 0.00^{c}	0.00
1354	*Colletotrichum* sp. 2	14.00 ± 0.00^{cd}	2.33	16.00 ± 1.41^{a}	2.67	9.67 ± 0.42^{ef}	1.61	0.00 ± 0.00^{c}	0.00
1356	*N. fusoidispora*	15.00 ± 0.82^{bcd}	2.50	15.67 ± 0.94^{a}	2.61	10.67 ± 0.00^{def}	1.78	0.00 ± 0.00^{c}	0.00
	Control	13.00 ± 0.00^{d}		13.00 ± 0.00^{a}		13.00 ± 0.00^{a}		0.00 ± 0.00^{c}	

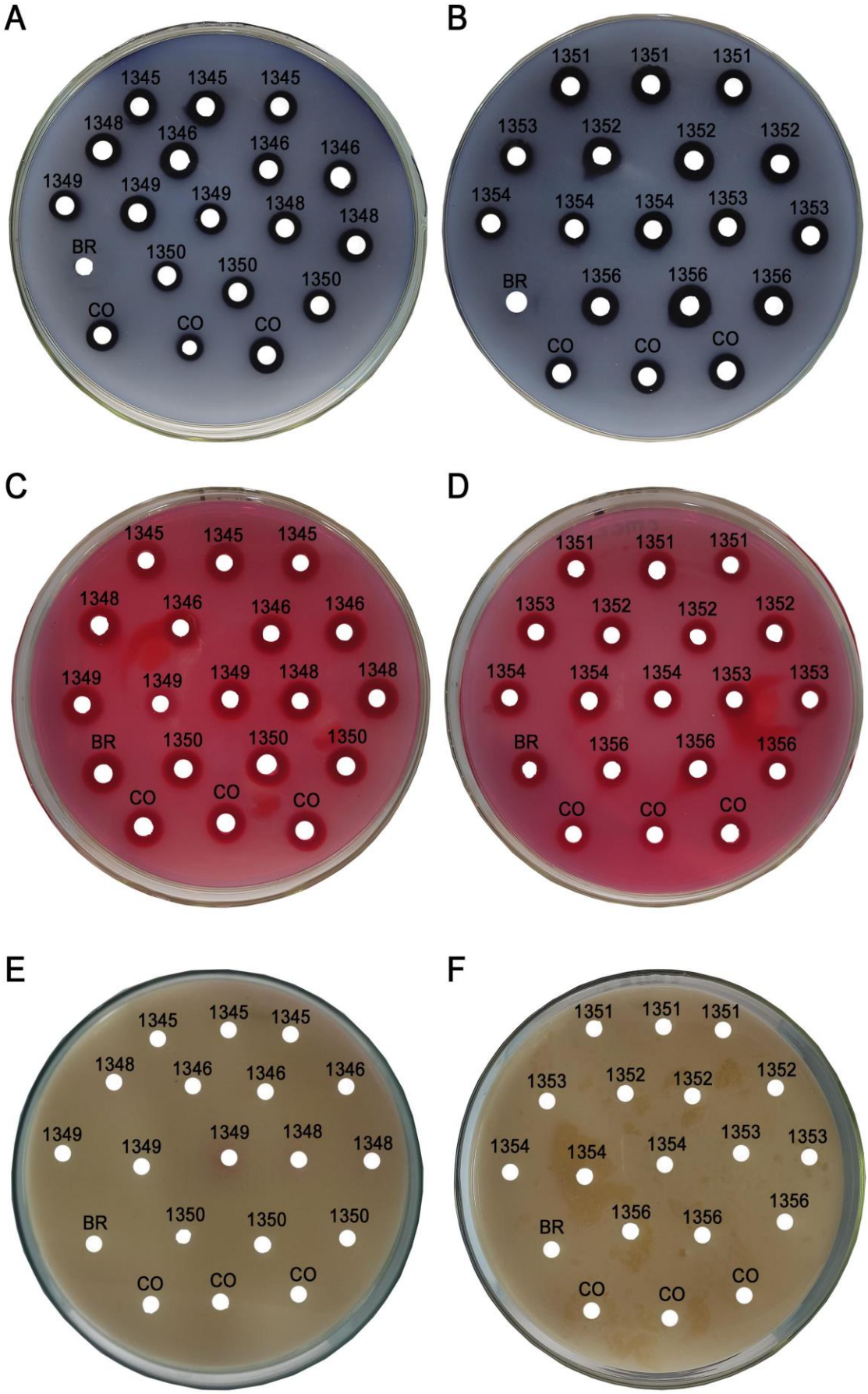

Figure 2. Detection of amylases (A-B), cellulases (C-D) and proteases (E-F) enzymes produced by fungicolous fungi with the basidiomes of Hymenochaetaceae specimens in PDY with inducing substrates. CO – Control, BR – White, Petri dishes – 200 mm.

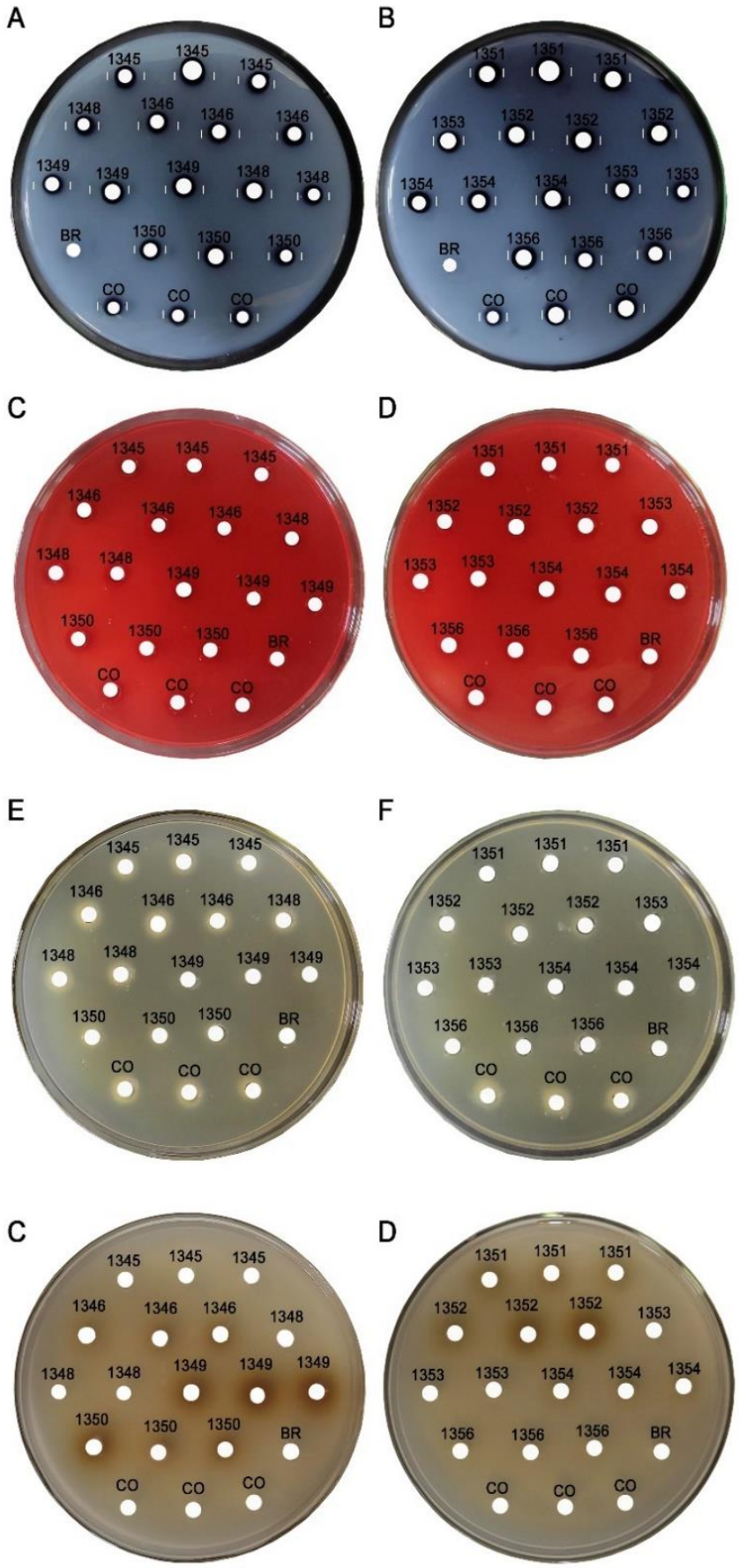

Figure 3. Detection of amylases (A-B), cellulases (C-D), proteases (E-F) and oxidoreductases (G-H) enzymes produced by fungicolous fungi with the basidiomes of Hymenochaetaceae specimens in Manachini solution with inducing substrates. Petri dishes – 200 mm. CO – Control, BR – White, Petri dishes – 200 mm.

DISCUSSION

In this study, we isolated and identified, by morphological aspects and sequencing of the ITSs regions of rDNA, three strains at the species level, and seven others at the genus level, isolated from the basidiome of macrofungi of the Hymenochaetaceae family in the states of Acre and Amazonas, and investigated the production of hydrolytic enzymes and oxidoreductases. Of the 10 specimens, four were from Acre (three from wood and one from litter) and six from the state of Amazonas (four from litter and two from wood). The The sporophores and the substrate where these basidiomycetes grew are important factors that can influence the detected diversity of associated fungi, which is in agreement with the specific literature [60].

The sequenced fungicolous fungi belong to the phylum Ascomycota, which was partially consistent with the results reported by Maurice and coauthors [60] for forest fungus basidiomes and with our morphological analyses. The sequencing results provided us with the fungal diversity of the basidiomes of the Hymenochaetaceae macrofungi. As we expected, the results correspond to the taxonomy of the host fungus found in soils, plants and wood, such as *Chaetomium*, *Xylaria*, *Colletotrichum* and *Trichoderma*, which are good cellulose degradators [61,62]. In addition to having demonstrated good enzymatic production, *Chaetomium* and *Trichoderma* are also used in the biological control of insects and agricultural pests [63,64] and *Xylaria*, *Colletotrichum* and *Trichoderma* produce a diversity of bioactive secondary metabolites that have already been described however, the production of enzymes using the first two is still low according to our surveys [65,66].

For enzymatic studies, it should be remembered that the results of these halos depend on several factors and one of these factors is the reagents that make up the culture medium to be used. The chemical substances present in the culture medium can lead to false-positive results, as they can interfere with the dye, in addition to inhibiting the bonds of these with polysaccharides or even lead the dye to its precipitation [67]. In addition, fungal strains isolated from different habitats may produce enzymes with distinct physicochemical and functional properties [18], which can be observed in the *Xylaria* and *Colletotrichum* strains in this study.

The addition of starch to the substrate generated an increase in the enzymatic production of all the isolates, and it is possible that the substrate was used as a more immediate source of carbon [68]. This indicates that the isolated fungal strains are capable of degrading both α-amylases and glucoamylases substrates [69]; however, we admit that there is a need to execute the production curve to detect the optimal production time of the evaluated enzymes. Souza and coauthors [70] also used corn starch as a substrate and observed that only four of the ten basidiomycete strains were positive; however, when changing the carbon source, the halos of all the strains were positively visible and two strains were larger (>20 mm). It can be noted that different sources of carbon (as the substrate) and of nitrogen can be factors that influence the induction of enzymes, and consequently influence the evaluation.

It was observed that there was an increase in cellulase production of between 5-10% with the addition of the CMC substrate and the salt solution. Surprisingly, cellulase studies on Colletotrichum are scarce [71], with *Trichoderma*, *Aspergillus* and *Penicilliium* being the most explored genera due to them presenting higher cellulase production, thus generating greater industrial interest [72], though not always will these species produce such enzymes [73]. Nonetheless, the knowledge of new fungal species that produce new enzymes can lead to the production of new products by industries.

Found in plant cell walls, cellulose [74] is abundant in forest environments [75,76]. Fungi, including fungicolous ones, have the ability to metabolize this polymer [77], with their versatility being based on abiotic conditions, which can influence the metabolic activities of the fungi [78]. It is believed that these isolated fungi live harmoniously inside the specimens of Hymenochaetaceae until they find their next host, though complementary studies are necessary so that the types of ecological relationships can be identified and verify whether there is mycoparasitism or symbiosis.

Species of xylariaceous fungi are known for being optimal producers of wood-rotting enzymes such as xylanase and cellulases and these have been reported in different *Xylaria* species [79,80]. Gokhale and coauthors [81] isolated a *Xylaria* sp., an Asian endophyte that is capable of producing amylases and lipase enzymes. In the Amazon, studies on basidiomycete fungi such as *Auricularia* sp. show that they are not amylase producers, while Pleurotus albidus has better performance in this respect [82]. Although this group of ascomycetes has been better described in temperate environments, since the 1990s, when studies on endophytic fungi began in Brazil, the pioneering work of Katia Rodrigues identified their abundance in palm trees and then in several other tropical plants [83,84].

In the case of our study, for proteases, there was an increase in enzyme production with the Manachini and gelatin solution, and the isolates *T. inhamatum* (LaBMicrA 1352) and *Colletotrichum* sp. 2 (LaBMicrA 1354) still obtained the lowest performance. *Trichoderma* was expected to be the most expressive genus in

relation to protease enzymes [85], though these strains are widely used in agriculture to inhibit or kill phytopathogenic fungi, thereby acting as biological control agents [85,86,87].

The genus *Xylaria* is an important source of drug discoveries in scientific fields and in the pharmaceutical industry due to its potential to produce a variety of structured, novel and bioactive secondary metabolites [88]. The study by Indarmawan and coauthors [89] aimed to isolate, purify and test the potential application of the extracellular protease KT30 from *X. psidii* as an antibacterial agent against Gram-positive bacteria, obtaining as a response the inhibition of bacterial growth. In other studies, *Xylaria* is considered a great source of proteases, and is used as an anticoagulant [90]. Another example is the protease produced by *X. hypoxylon*, which is an enzyme with a distinct N-terminal sequence, relatively high thermostability, and the first report of HIV-1 reverse transcriptase inhibitory activity in Ascomycota macrofungi is for this species [91].

The data from this study corroborate with Castaño and coauthors [92], who isolated and identified a *Xylaria* sp. for the production of oxidoreductases and optimized the culture medium in order to enhance the production of oxidoreductases. Different factors were determinants in the production of the enzyme, such as the carbon source and the presence of an inducer. According to Dekker and coauthors [93], the production of oxidoreductases is related to the nutrients found in the substrates, which corroborates the results obtained in this and other studies. It is also suggested that the production of oxidoreductases by Colletotrichum sp. may be linked to phytopathogenicity processes [94].

CONCLUSION

All the isolates from the basidiomes of Hymenochaetaceae specimens studied were able to produce all the enzymes studied, except oxidoreductases, for which only five strains were positive. It was found that enzyme production was enhanced in the Manachini solution with the addition of inducing substrates, this being more expressive for oxidoreductases, amylases and proteases.

Therefore, this study contributes to the investigation of enzymatic production of microfungi isolated from macrofungi and opens up new perspectives on the enzymatic potential of these fungicolous fungi isolated in the Amazon, which are practically unexplored in this field; however, as was found, they have great potential for biotechnological applications particularly in enzyme production. It can be noted that macrofungi of Hymenochaetaceae become reservoir of a fungal richness and are potential producers of enzymes not yet studied.

Author Contributions:

Conceptualization, Couceiro, D.M., Santiago, S.R.S.S., Souza, E.M.M. and Souza, A.Q.L.; methodology, Couceiro, D.M., Santiago, S.R.S.S., Souza, E.M.M. and Souza, A.Q.L.;; software, Couceiro, D.M., Souza, E.M.M. and Souza, A.Q.L.; validation, Couceiro, D.M.; Santiago, S.R.S.S.; Souza, E.M.M.; Silva, G.F.; Souza, A.D.L.; Filho, S.A.; Souza, A.Q.L.; formal analysis, Couceiro, D.M. and Souza, E.M.M.; investigation, Couceiro, D.M., Souza, E.M.M. and Souza, A.Q.L.; resources, Couceiro, D.M., Souza, E.M.M. and Souza, A.Q.L.; data curation, Couceiro, D.M., Souza, E.M.M. and Souza, A.Q.L.; writing—original draft preparation, Couceiro, D.M.; Santiago, S.R.S.S.; Souza, E.M.M.; Silva, G.F.; Souza, A.D.L.; Filho, S.A.; Souza, A.Q.L.; writing—review and editing, Couceiro, D.M.; Santiago, S.R.S.S.; Souza, E.M.M.; Silva, G.F.; Souza, A.D.L.; Filho, S.A.; Souza, A.Q.L.; visualization, Couceiro, D.M.; Santiago, S.R.S.S.; Souza, E.M.M.; Silva, G.F.; Souza, A.D.L.; Filho, S.A.; Souza, A.Q.L.; supervision, Couceiro, D.M.; Santiago, S.R.S.S.; Souza, E.M.M.; Silva and Souza, A.Q.L.; project administration, Souza, A.D.L.; Filho, S.A. and Souza, A.Q.L.; funding acquisition, Souza, A.D.L.; Filho, S.A. and Souza, A.Q.L.

Funding: Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) (resolution N. 008/2021 POSGRAD UEA 2021 edition) via the Graduate Program in Biodiversity and Biotechnology of the Legal Amazon - Rede Bionorte/AM and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

Data Availability Statement: Research data are available in the body of the manuscript.

Acknowledgments: The authors would like to thank the Laboratory of Bioassays and Microorganisms of the Amazon of Central Analítica at the Federal University of Amazonas for the infrastructure made available for this study and Embrapa Western Amazon, where the sequencing of the species was carried out. This study is a partial result of the first author's doctorate degree. A special thanks to my friend Sérgio Dantas for his valuable review of this manuscript before submission.

Conflicts of Interest: The authors declare no conflict of interest.

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