Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Ligninolytic enzyme potential of *Trametes* spp. associated with leaf litter in riparian forest of the Amazônia region

Potencial enzimático ligninolítico de *Trametes* spp. associado à serapilheira em áreas de mata ciliar da região amazônica

I. A. L. De Sousa[a]*, A. J. Boari[b] and A. S. Santos[c]

[a] Universidade Federal do Pará - UFPA, Programa de Pós-graduação em Biodiversidade e Biotecnologia - PPG-REDE BIONORTE, Instituto de Ciências Biológicas, Belém, PA, Brasil

[b] Embrapa Amazônia Oriental - EMBRAPA, Laboratório de Fitopatologia, Belém, PA, Brasil

[c] Universidade Federal do Pará - UFPA, Instituto de Ciências Biológicas, Belém, PA, Brasil

Abstract

The present study explored the potential of leaf litter as a source of fungi able to produce ligninolytic enzymes for the biodegradation of anthraquinone dyes. Within the colonies isolated from the leaf litter, only three colonies of two species *Trametes* were selected based on the detection of oxidation and decolorization halos in Petri dishes with PDA (potato-dextrose-agar) + Guaicol and PDA + RBBR (Remazol Brilliant Blue R). The identification of the colonies was done through sequencing of the ITS region. The enzymatic activity of Lac (lacase), MnP (manganês peroxidase) and LiP (lignina peroxidase) was analyzed by spectrophotometry during fermentation in PD+RBBR imedium. Isolates A1SSI01 and A1SSI02 were identified as *Trametes flavida*, while A5SS01 was identified as *Trametes* sp. Laccase showed the highest enzymatic activity, reaching 452.13 IU.L^{-1} (A1SSI01, 0.05% RBBR) after 96h. Isolate A1SSI02 reached the highest percentage of decolorization, achieving 89.28% in seven days. The results imply that these *Trametes* isolates can be highly effective in waste treatment systems containing toxic anthraquinone dyes.
Keywords: laccase, peroxidases, basidiomycete, litter and biodecolorization.

Resumo

Este estudo investigou o potencial da serapilheira como fonte de fungos capazes de produzir enzimas ligninolíticas para a biodegaradação de corante antraquinônico. Entre as colônias isoladas do material de serapilheira, apenas três colônias de duas espécies de *Trametes*. foram selecionados com base na detecção de halos de oxidação e descoloração em placas de Petri com PDA (Batata-Dextrose-Ágar) + Guaicol e PDA + RBBR (Azul Brilhante de Remazol R). A identificação das colônias foi através do sequenciamento da região ITS. A atividade enzimática de Lac (lacase), MnP (manganês peroxidase) e LiP (lignina peroxidase) foi avaliada por espectrofotometria durante a fermentação em meio BD+RBBR. Os isolados A1SSI01 e A1SSI02 foram identificados como *Trametes flavida*, enquanto A5SS01 foi identificado como *Trametes* sp. A lacase mostrou a maior atividade enzimática, atingindo 452,13 UI.L^{-1} (A1SSI01, 0,05% RBBR) após 96h. O isolado A1SSI02 alcançou o maior percentual de descoloração, atingindo 89,28% em sete dias. Os resultados sugerem que esses isolados de *Trametes* podem ser eficazes em sistemas de tratamento de resíduos contendo corantes antraquinônicos tóxicos.

Palavras-chaves: lacase, peroxidases, basidiomiceto, serapilheira e biodescoloração.

1. Introduction

In forest ecosystems, fungi have an active role in the leaf litter decomposition process, being responsible for most of the cycling of nutrients and carbon trapped in organic matter (Yilmaz et al., 2016). The main role of these microorganisms in the decomposition of this material is due to their ability to produce a wide range of extracellular enzymes, which transform the lignocellulosic matrix into energy and nutrients for microbial and plant growth (Hernández and Hobbie, 2010).

This process is mainly mediated by fungi through production of extracellular ligninolytic enzymes, including laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP). These enzymes are non-specific in their substrate preference, and their ability to debase lignin suggests significant potential for the degradation of synthetic polymers such as textile dyes (Ahsan et al., 2021), such as Remazol Brilliant Blue Reactive (RBBR). This reactive anthraquinonic dye is composed by anthracene derivatives, a polycyclic aromatic

*e-mail: ialanaleites@gmail.com; ieda.sousa@icb.ufpa.br
Received: January 9, 2024 – Accepted: April 24, 2024

hydrocarbon (Eichlerová et al., 2007), being widely used in the textile and dyeing industry (Ahmad and Alrozi, 2011).

RBBR is considered toxic, carcinogenic and mutagenic and, for these reasons, it must be removed before wastewater from these industries disposed in the environment (Rodríguez-Couto, 2011). Studies show that due to their similar structure to lignin, the use of ligninolytic fungi, such as *Ganoderma lucidum*, *Trametes hirsuta* and *Phanerochaete velutina*, represents a biosustainable alternative for the degradation of reactive compounds, including anthraquinonic dyes (Rainert et al., 2021; Alam et al., 2021; Zafiu et al., 2021).

At the present time, the interest in studying new fungal sources of laccases, lignin peroxidase and manganese peroxidase has grown, with the expectation of finding enzymes with new or robust properties for application in the decolorization of dyes (Mishra and Maiti, 2019). In addition, the use of RBBR on a laboratory scale is commonly examined as a substrate for screening assays of certain polycyclic aromatic compounds, which are the target substances for biological remediation, as this dye enables simple, rapid and quantitative observations in spectrophotometric methods in agar and liquid media (Machado et al., 2005; Sari et al., 2012).

Considering the potential of fungi to act in the decolorization of toxic synthetic dyes, the purpose of this study was to evaluate the enzymatic activity of laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP), as well as the decolorization potential of the RBBR dye in liquid fermentation of three isolates of *Trametes* spp. that were obtained from leaf litter collected in a riparian forest in the municipality of Salvaterra, Pará.

2. Material and Methods

2.1. Sample collection

The leaf litter samples used to isolate the fungi were collected in February 2022 in areas of riparian forest in the Amazon region, the island of Marajó in the municipality of Salvaterra (53°19'00" S and 48°35'39" W), in the state of Pará (Figure 1), in accordance with the project's registration in SISGEN, under registration number A5E8BAD. Five sampling points were delimited in two collection areas, in 50m stretches of each water stream present in the areas. A single leaf litter collection was made at these points, which were approximately 3 m apart. A 0.25 m^2 (0.5 × 0.5 m) plot made from PVC pipe was used to delimit the collecting area, and all the litter present in this area was collected. In the end, a total of 10 random samples of leaf litter were gathered. The material was transferred to trays, dried in the open air for 12 hours, packed in paper bags and kept in the fridge until the samples were transported to the Laboratório de Investigação Sistemática em Biotecnologia e Biodiversidade Molecular – Universidade Federal do Pará.

2.2. Isolation of fungi

The leaf litter obtained was fragmented and 1g of each sample was macerated in 100 mL of water using a crucible and a porcelain pistil. This solution was dissolved to concentrations of 10^{-2}, 10^{-3} and 10^{-4}. An aliquot of 0.2mL of the last two dilutions was sown on PDA (potato-dextrose-agar infusion) culture medium, supplemented with 100 $\mu g.mL^{-1}$ chloramphenicol in Petri dishes (90 mm diameter), in duplicate, with a total of four dishes per

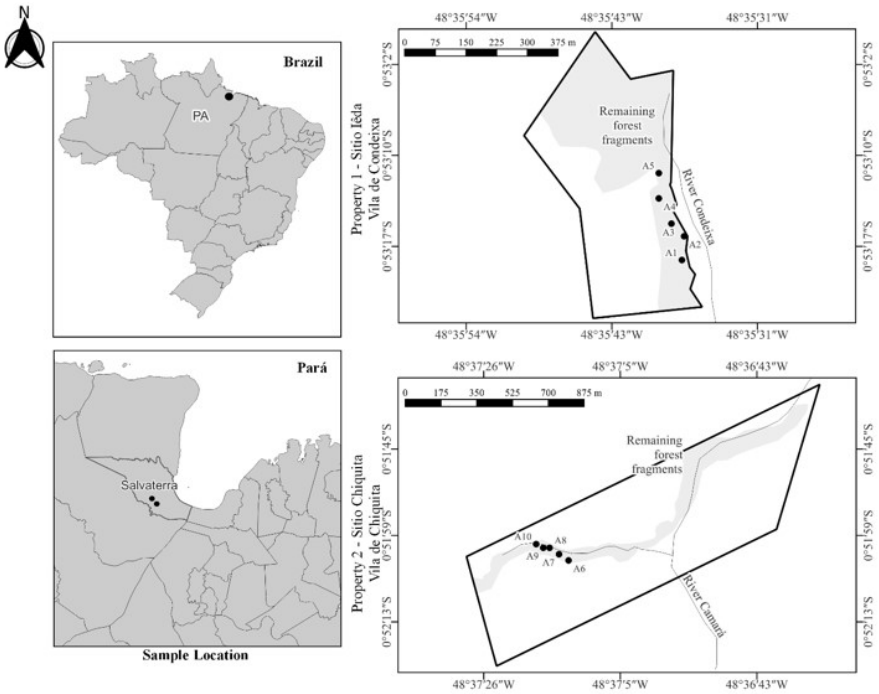


Figure 1. Geographic location of the litter sampling sites in riparian forest in the municipality of Salvaterra, state of Pará, Brazil.

sample. The plates were incubated at 28ºC for up to 48h. The fungal isolates obtained were transferred individually to Petri dishes containing PDA and incubated at 28 ± 2ºC, photoperiod of 12h for seven days for purification. Once pure fungal cultures had been obtained, they were stored in sterile microtubes with distilled water (Castellani, 1939).

2.3. Selection and identification of fungal colonies

Each isolate was chosen through the qualitative determination of enzymatic activities based on the oxidation of guaiacol and the decolorization of Remazol Brilliant Blue R dye (RBBR). This enzymatic detection was made by growing the fungal colonies in Petri dishes containing PDA (potato-dextrose-agar) medium plus 0.05% (v/v) guaiacol and PDA plus 0.01% (w/v) RBBR dye for five days at 28ºC. Once this period had finished, the presence of a brown halo of oxidation for guaiacol and a halo of discoloration for RBBR were visually examined. The colonies capable of producing these halos were considered positive for the presence of these enzymes and were selected to the next step.

In the identification phase, the colonies with positive enzymatic activity were previously grown in Petri dishes containing PDA for 7 days at 28ºC and a 12-hour photoperiod. The mycelial mass was then removed from each plate to extract total genomic DNA using the CTAB method (Cationic Hexadecyl Trimethyl Ammonium Bromide®, Sigma, USA) (Doyle and Doyle, 1987). The obtained DNA was analyzed by electrophoresis on a 0.8% (w/v) agarose gel containing GelRed™ (Biotium Inc., USA) and analyzed under ultraviolet light in a photodocumentation system (Loccus Biotechnology, Brazil).

The primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTA TTGATATGC-3') (White et al., 1990) were used to amplify the ITS (Internal Transcribed Spacer) region of the ribosomal DNA conserved 5.8S gene, following these conditions: initial denaturing at 94°C for 1 minute, 34 cycles of 94°C for 30 seconds for denaturing , 55°C for 30 seconds for annealing and 72°C for 30 seconds for extending, with final extending at 72°C for 3 minutes. The amplified ITS fragments were submitted to agarose gel electrophoresis, dyed with GelRed® and visualized under ultraviolet light. The 1 Kb DNA Ladder (Promega, USA) was used as a molecular weight indicator.

The amplified products were then sequenced in both directions using the same PCR primers. The purification and the sequencing of the PCRs were conducted by ACTgene, Brazil. All the sequences were adjusted, when necessary, in the SeqAssem software (Hepperle, 2004) and compared with the reference sequences deposited in the NCBI GenBank database (Supplementary Material 1). The MAFFT online platform (Katoh et al., 2019) was used to align the sequences obtained with those of phylogenetically close species. The sequences of *Dentocorticium sulphurellum* (FP11801) and *Lopharia cinerascens* (FP105043sp) were used as an out-group (Olou et al., 2020).

The maximum parsimony (MP) analysis was conducted using the PAUP* 4.0 software (Swofford, 2002), with a bootstrap value of 1000 replicates to determine the confidence levels of the branches. The number of informative parsimony sites and the retention index (RI) were also calculated during this analysis. Bayesian inference was used to produce posterior probability (PP) values for a consensus using the software MRBAYES v 3.1 (Huelsenbeck and Ronquist, 2001). The analyses were done using the Markov chain Monte Carlo (MCMC) algorithm, using the best-fit model selected by the Akaike information criteria in MrModelTest 2.3 (Larget and Simon, 1999). The trees were visualized and adjusted using FigTree software.

2.4. Enzymatic test

The selected isolates (A1SSI01, A1SSI02 and A5SSI01) were reactivated on PDA for 7 days at 28ºC and then transferred to PD (potato-dextrose) liquid medium in triplicates, containing different concentrations of RBBR as a carbon source. Each 200mL flask was filled with 125mL of PD liquid with concentrations of 0, 0.01, 0.05 and 0.1% (w/v) RBBR, and the pH was adjusted to 5.5. The flasks were incubated on a rotary shaker (140 rpm) for seven days at 30 ± 2ºC in the dark. Samples of 1.5mL were taken from each flask every 24h for enzyme analysis. The mycelium was collected, filtered and the rest centrifuged at 4000 rpm for 10 minutes, using the supernatant as the enzyme source.

All enzymatic activities were measured by spectrophotometry (GENESYS 10uv Scanning). MnP (Manganese Peroxidase) activity was tested using a mixture of 800µL phenol red (0.01% w/v), 100µL sodium lactate (0.25M), 200 µL bovine albumin (0.5% w/v), 50 µL $MnSO_4$ (2mM), 50µL hydrogen peroxide (H_2O_2) + Sodium Succinate buffer (20mM, pH 4.5) (adapted from Bonugli-Santos et al. (2010)). LiP (Ligin Peroxidase) activity was measured using the method of Tien and Kirk (1988) with the reaction mixture consisting of 1mL of sodium tartarate buffer (125mM and pH 3.0), 500µL of veratryl alcohol (4mM), 500 µL of H_2O_2 (2mM). Lac (Laccase) was tested by mixing 500µL of guaiacol solution (50mM), 1000µL of sodium acetate buffer (0.1 M and pH 5.0) (adapted from Monssef et al., 2016). For each of these reactions, 500µL of enzyme source was added. All the blends were incubated at 30ºC for 10min, and the absorbance was read at 610, 310 and 450nm, respectively, for MnP, LiP and Lac. As a blank, the specific volume of each medium, without inoculum, was used instead of the enzyme solution. The enzymatic activities were expressed in units per liter (UI.L^{-1}), with one unit of enzymatic activity determined as the amount of enzyme that catalyzed the formation of 1µmol of the corresponding products in one minute under the given test conditions. The obtained values were calculated according to the Equation 1 below (Monssef et al., 2016).

$$\frac{UI}{L} = \frac{Abs * 10^6}{\epsilon * V * t} \quad (1)$$

Abs is the absorbance, ε is the molar absorption coefficient, which determines the capability of one mole of the substance to absorb the light at a given wave length, V is the volume of the extract (mL), and t is the time of reaction in minutes.

2.5. Decolorization test

The decolorization of the RBBR dye was measured seven days after fermentation by taking a spectrophotometer reading

of the supernatant at the wavelength of the dye (590nm). The decolorization percentage (%) was thus calculated according to the Equation 2 below (Khan et al., 2023).

$$Decolorization(\%) = \frac{A0 - A1}{A0} * 100 \quad (2)$$

A0 is the original absorbance of the dye and A1 is the absorbance at the end of the test.

The data obtained in triplicate for enzymatic activity and decolorization were submitted to ANOVA using the Rstudio program version 4.2.2. The results of the analytical determinations are the average of the results in triplicate and the significant differences between the treatments were compared using the Tukey test at a 5% probability level.

3. Results

3.1. Identification of ligninolytic fungi

Twenty-eight fungal colonies were isolated from the leaf litter collected. After screening on PDA (potato-dextrose-agar) + RBBR (Remazol Brilliant Blue R) (0.01% w/v) and Guaiacol (0.05% v/v) media, it was verified that only isolates A1SSI01, A1SSI02 and A5SSI01 showed a halo of discoloration and oxidation, respectively, in the media tested, showing the isolates' ability to produce ligninolytic enzymes (Figure 2). These colonies grown on PDA medium had a slightly whitish color and a velvety mycelium. In liquid, the colonies' growth promoted the formation of pellets. Microscopically, the hyphae were hyaline, thin walled, branched, with long hyphal segments and simple septate with dispersed single staple connections.

The ITS region was sequenced in both directions and phylogenetic trees generated by the maximum parsimony (MP) and Bayesian inference methods indicated the three selected isolates belonged to the genus *Trametes*. The sequences were compared to 68 reference sequences available in GenBank for the genus, totaling a dataset of 73 sequences (Supplementary Material 1).

Similar topology phylogenetic trees were obtained. The MP statistics obtained for the ITS region tree were the following: RI 0.852, with a total of 581 sites, 342 constant sites, 72 non-informative variable sites and 167 informative variable sites. In the tree, 35 species formed thirteen major clusters. The sequences of isolates A1SSI01 and A1SSI02 grouped into monophyletic clades corresponding to *Trametes flavida* (Lév.) Zmitr., which was supported by a bootstrap value of 100% and pp of 1.0, being the first reported occurrence of this species in Brazil. While isolate A5SSI01 was grouped in a paraphyletic clade, as the *Trametes lactinea* (Berk.) Sacc. clade was also composed of *Trametes cubensis* (Mont.) Sacc., supported by high bootstrap values (100%) and pp (1.0), both species were previously reported in the country (Figure 3).

The phylogenetic analysis also showed that there is no formation of subclades within *T. flavida*, given the absence of polymorphisms at specific nucleotide positions for the ITS region when compared to the reference sequences. However, in the *T. cubensis* and *T. lactinea* clade, the isolates *T. cubensis* TJU93_213sp., UZ526_17 and *T. lactinea* LIP:GUY09_110, DMC346 and OAB0232 form a sub-clade due to the presence of a polymorphic nucleotide absent in the other isolates at positions 155, 156, 174 and 291 (Supplementary Material 2).

3.2. Enzymatic activity

The results indicated that using RBBR in PDA culture medium induces an increase in the production of ligninolytic enzymes. In the absence of the dye (0% treatment), it was not quite possible to identify the enzymes MnP and LiP. Only Lac was detectable, but to a lower extent when compared to the other treatments with RBBR (Figure 3).

Enzymatic activities of MnP, LiP and Lac were found in fermentations with different concentrations of RBBR, with maximum activity peaks 120h (day 5) after the start of fermentation for MnP and 96h (day 4) for LiP, while Lac varied between 96h and 144h for the isolates tested in this study. Among the ligninolytic enzymes, the peaks in enzyme activity were highest for laccase, with enzyme activities above 400 UI.L^{-1} being detected for the isolates tested using RBBR.

An increase in enzymatic activity was noticed for MnP in relation to fermentation time and the concentration of

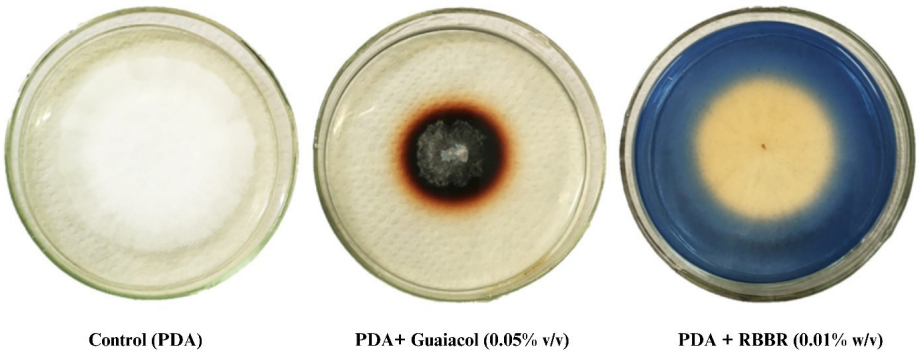


Figure 2. Zones of reddish-brown color in culture medium PDA + Guaiacol (0.05% v/v) and a halo of discoloration in PDA + Remazol Brilliant Blue R (0.05% v/v) as a positive result to produce oxy-reducing enzymes by leaf isolates, compared to the control test (PDA).

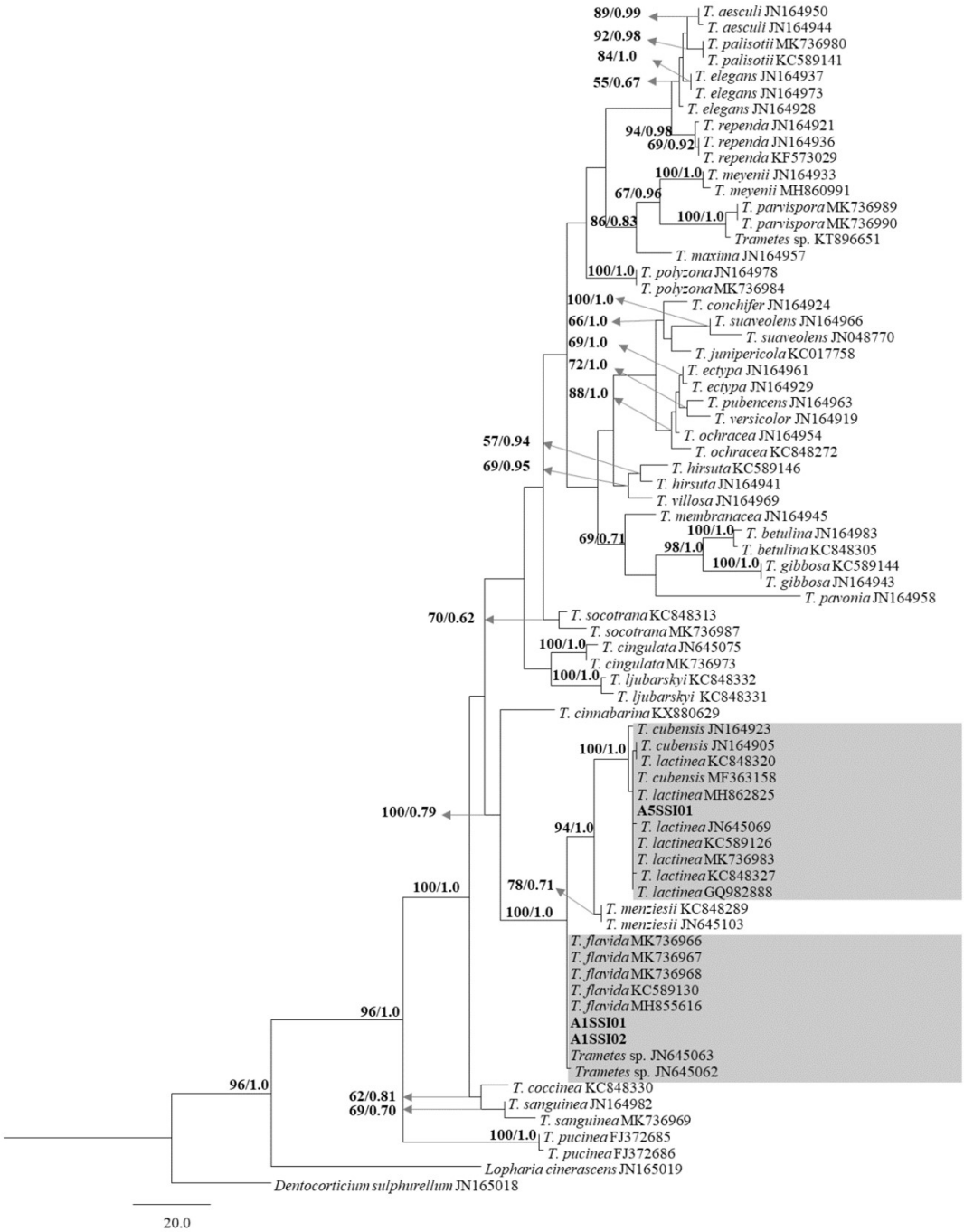


Figure 3. Phylogenetic tree based on MP and Bayesian analysis of ITS data. Bootstrap values (>55%) for PM analysis and PP values (>0.67) for Bayesian inference are indicated near branches (MP/PP). The bold isolates were sequenced in this study. *Dentocorticium sulphurellum* and *Lopharia cinerascens* were selected as the outrgroup. The toopology of the tree shown refers to the MB analysis.

the RBBR dye. Concentrations of 0.0 to 0.05% increased the expression of this enzyme in the environment, with a decrease in activity at the highest concentration of the dye (0.1%). Apart from isolate A1SSI01, which maintained the increase in MnP activity proportional to the concentration of RBBR. Nevertheless, all the isolates showed a maximum at 120h and a drop-in activity after this period. The highest MnP activity was registered for isolate A1SSI01 in the treatment with 0.1% RBBR, equal to 59.03 UI.L^{-1}, followed by A1SSI02 and A5SSI01 at 0.05% (51.89 and 48.29 IU.L^{-1}, respectively) at 120h (day 5) after fermentation (Figure 4).

For LiP, the enzyme activity was highest at 96h after the experiment had started and in the highest concentration of RBBR (0.1%), and was the same for all the examined

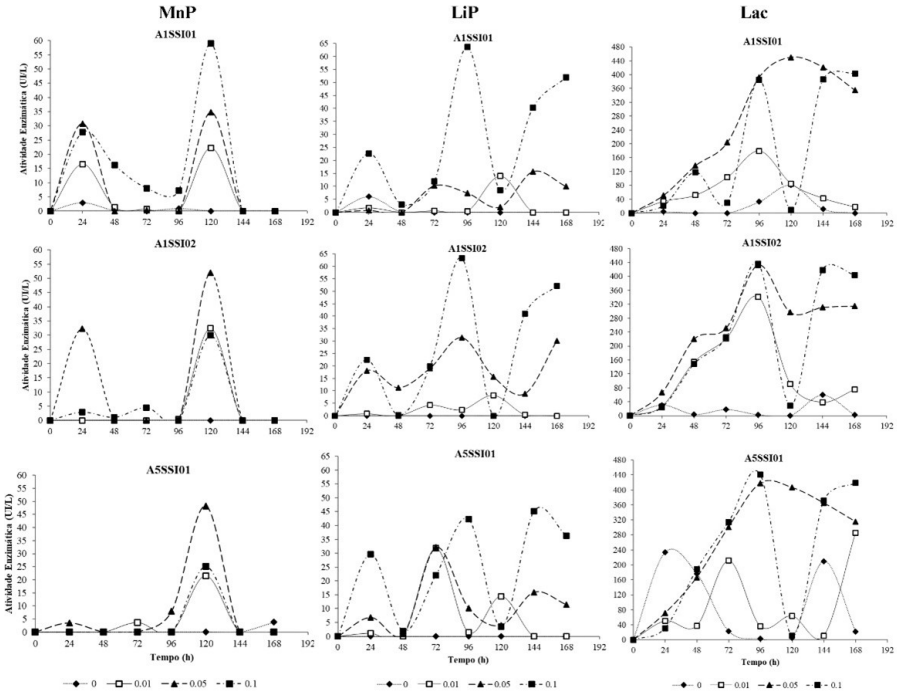


Figure 4. Daily enzymatic activity of manganese peroxidase (MnP), lignin peroxidase (Lip) and laccase (Lac) of isolates A1SSI01, A1SSI02 and A5SSI01 of *Trametes* spp. in BD (Potato-Dextrose) medium with different concentrations of Remazol Brilliant Blue Reactive (RBBR): 0, 0.01, 0.05 and 0.1% (v/v). NOTE: The isolates presented in bold were sequenced in this study and are part of the isolates of the Microbiology collection of the Laboratory of Systematic Research in Biotechnology and Biodiversity from the Federal University of Pará, Brazil.

colonies in this study, with activities oscillating between 42.25 and 63.64 UI.L^{-1}. Remarkably, the activity recorded for the fourth day of fermentation of A5SSI01 was similar to the activity found for the sixth day of fermentation of this colony (45.10 UI.L^{-1}), after a decrease in the determination of the enzyme's activity at 120h.

Lac activity was proportional to the increase in RBBR concentrations up to 0.05%. At the highest RBBR concentration (0.1%), there was a slight decline in enzyme activity when compared to the 0.05% treated. Colony A1SSI01 had the highest Lac activity at 0.05% RBBR after 120 hours (day 5) from the start of fermentation (452.13 UI.L^{-1}), which was statistically equal to the activity of the A5SSI01 colony at 0.1% RBBR concentration (4th day; 440.63 UI.L^{-1}) and A1SSI02 at 0.05 and 0.1% concentrations (432.43 and 435.60 IU.L^{-1}), in both cases 96h (4th day) after the start of fermentation.

3.3. RBBR decolorization

The highest percentage of decolorization was obtained by colony A1SSI02 at the highest concentration of RBBR (0.1%), equal to 89.28% (Table 1). The second highest percentages of decolorization were 87.03% and 86.15%, the result of growing A1SSI01 and A1SSI02 at 0.01% and 0.05%, respectively. The A5SSI01 isolate presented the lowest percentage of decolorization at the lowest concentration of the dye registered at the end of this study (24.77%), but at the 0.05 and 0.1% concentrations, it achieved decolorizations equal to 81.27% and 78.87% (Table 1).

Thus, according to the obtained results, the *T. flavida* colonies were more effective at decolorizing RBBR through the amounts of ligninolytic enzymes produced, when compared to the *Trametes* sp. colony (A5SSI0) at the dye concentrations tested. Considering that for the 0.01% concentration of the dye in medium liquid, colony A1SSI01 was the most effective in decolorization compared to the other colonies, but at concentrations of 0.05 and 0.1%, colony A1SSI02 was the most effective in decolorization after 7 days of fermentation in medium liquid (Table 1). Therefore, isolate A1SSI01, through its enzymatic production, is able to oxidize 0.09 mg.mL^{-1} of RBBR up to seven days of cultivation, while isolate A1SSI02 is able to oxidize 0.43 mg.mL^{-1} to 0.86 mg.mL^{-1} in the same amount of time.

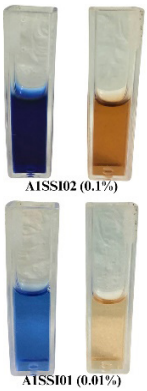
4. Discussion

4.1. Isolates of Trametes spp. obtained from leaf litter

The litter of the riparian forest in the Amazon is home to several fungal species that can be isolated and cultivated *in vitro* for further investigations, such as the 28 colonies isolated in this study. The high diversity of microorganisms associated with this material was reported in previous research that involved only the morphological identification of fungi from palm litter and cedar plantations in the Amazon, revealing the presence of around 100 species in the analyzed materials (Santos et al., 2018; Monteiro et al., 2019).

Table 1. Dye discoloration (%) of fungal isolates grown in flasks stirred with PD medium (potato-dextrose infusion) and 0, 0.01, 0.05 and 0.1% w/v of RBBR stirred after 168 hours of fermentation (7 days).

ISOLATES	RBBR CONCENTRATION	DISCOLORATION (%) *
A1SSI01	0.01	87.03 ± 0.33 ab
	0.05	82.76 ± 0.08 cd
	0.1	77.48 ± 0.35 f
A1SSI02	0.01	84.56 ± 1.30 bc
	0.05	86.15 ± 0.42 b
	0.1	89.28 ± 0.22 a
A5SSI01	0.01	24.77 ± 2.40 g
	0.05	81.27 ± 0.14 de
	0.1	78.87 ± 0.21 ef



*Note: Treatments with the same letter do not differ statistically.

These results indicate the presence of several species acting in the litter decomposition process in tropical forests (Monteiro et al., 2019), including microorganisms that produce extracellular ligninolytic enzymes (Janusz et al., 2017). The lower number detected in the present study is due to the fact that a portion of the fungi present in the soil and litter are not cultivable (Silvani et al., 2017). Litter can be considered a natural source of ligninolytic microorganisms with sufficient enzymatic activity to biodegrade systems involving synthetic anthraquinone dyes, as in this study, and agro-industrial residues, which are rich in lignocellulosic matrix.

Only isolated *Trametes* colonies were able to oxidize guaiacol and decolorize RBBR in culture medium. The morphological characteristics presented in PDA by these colonies resemble the morphology of species of the genus described in other studies (Yang et al., 2009; Sari et al., 2012). These results contribute to affirming that the genus does not present morphological markers capable of differentiating its species (Welti et al., 2012; Olou et al., 2020), making it necessary to complement it with molecular analyses.

The results obtained with the sequencing of the ITS region (internal transcribed spacer) of rDNA (ribosomal DNA) were able to separate most of the clades of species identified in the genus, including the specimens in this study, with this gene region being efficient in identifying a large part of fungal species (Al-Fadhal et al., 2018). The clades formed with high support values (≥ 57% for MP and ≥ 0.62 for MB) were similar to those found in other studies based on these sequences (Justo and Hibbett, 2011; Welti et al., 2012; Olou et al., 2020).

The molecular analysis also showed that, for *T. flavida* species, such as isolates A1SSI01 and A1SSI02, there is no presence of polymorphism in the ITS region analyzed. However, the species *T. lactinea* and *T. cubensis*, despite being grouped in the same clade, present among their isolate's single nucleotide polymorphisms in the amplified gene region. This result allows us to infer that the polymorphism found may be associated with the geographic origins and adaptations of crops, resulting in variations in their secondary structures, as shown for the species *Ganoderma lucidum* by Zhang et al. (2017) or may be associated with high genetic variability and gene flow between isolates of the species, as shown for *Chrysoporthe puriensis* by Oliveira et al. (2022).

4.2. The role of ligninolytic enzymes in RBBR decolorization

As RBBR's structure resembles the chemical characteristics of lignin, the dye can function as an inducer of Lac, LiP and MnP enzymatic activity, as demonstrated in this investigation, when comparing the enzymatic activity of *Trametes* spp. isolates in liquid fermentation with and without RBBR. Moreover, the use of dyes in laboratory experiments provides a number of advantages over conventional substrates, as they are stable, soluble and have high molar extinction rates and low toxicity to microorganisms (Machado et al., 2005). It also enables primary qualitative tests to be carried out which help to screen a large number of isolates, as it is less time-consuming and easier to be interpreted (Rao et al., 2019).

The results obtained reveal that the significant decolorization of RBBR may be mainly related to the enzymatic production of laccase by the selected colonies, since the highest activities were found for this enzyme, as had already been demonstrated in other studies involving fungal isolates (Sing et al., 2017; Rao et al., 2019; Rainert et al., 2021). Meanwhile, the highest decolorization rate (A1SSI02, 0.1% = 89.28%) was not associated with the treatment with the highest peak activity of this enzyme (A1SSI01, 0.05% = 450.17 UI.L^{-1}), which is probably due to the difference in laccase isoenzymes produced by the different colonies (Nyanhongo et al., 2002).

Osma et al. (2007) demonstrated that the same concentration of laccases obtained from different colonies of *T. pubescens* contributed to a difference in the decolorization efficiency of the anthraquinone dye RBBR and the triphenylmethane dye Methyl Green, suggesting that there is a difference in the redox potential of laccases

from different microorganisms (Osma et al., 2007). Despite the important role of laccase in the decolorization of RBBR, the action of each enzyme (MnP, Lac and LiP) occurs in an additive manner, since each one can attack different chemical structures of the substance (Champagne and Ramsay, 2005).

According to Anita et al. (2020), the rate of decolorization of azo dyes is linearly proportional to the increase in ligninolytic enzyme activity during fermentation. During the experiment, from the 4th day of fermentation onwards it was possible to observe changes in the color of the media, turning brownish, which corresponds to an increase in the activity of the enzymes studied, a period in which the peaks of enzymatic activity were also identified.

Nonetheless, the discoloration rate can decrease as a result of the toxicity of the dye to the enzymes at higher concentrations (Chaudhari et al., 2017), which may possibly have occurred for isolate A1SSI01. High concentrations of the dye can inhibit the oxidation process of ligninolytic enzymes, especially processes catalyzed by laccases, since a higher concentration of RBBR can cover the active site and saturate the enzyme (Navada et al., 2018).

As has been proposed for the fungus *Marasmius cladophyllus*, the use of *Trametes flavida* and *Trametes* sp. isolates may be feasible by using wastewater containing the dye as a substrate to produce decolorizing enzyme, which could simultaneously cause the decolorization of the wastewater before disposal into nature (Sing et al., 2017). If this technology is successfully applied, it will not only help to reduce enzyme production costs but will also reduce wastewater treatment costs.

5. Conclusion

The present study has shown that the saprophytic basidiomycete colonies A1SSI01, A1SSI02 and A5SSI01 of *Trametes*, isolated from leaf litter, have the potential to produce ligninolytic enzymes and can be considered outstanding candidates for the biodecolorization of wastewater containing anthraquinone reactive textile dye, without the need to add mediators. This study is the first report of the identification of the species *Trametes flavida* in Brazil. The production of ligninolytic enzymes by these colonies also indicates the need for further studies to explore their potential biotechnological applications.

Acknowledgements

To the Laboratório de Fitopatologia of EMBRAPA Amazônia Oriental for the partnership developed. To Fundação Amazônia de Amparo a Estudos e Pesquisas - FAPESPA for providing the PhD scholarship.

References

AHMAD, M.A. and ALROZI, R., 2011. Optimization of rambutan peel based activated carbon preparation conditions for Remazol Brilliant Blue R removal. *Chemical Engineering Journal*, vol. 168, no. 1, pp. 280-285. http://doi.org/10.1016/j.cej.2011.01.005.

AHSAN, Z., KALSOOM, U., BHATTI, H.N., AFTAB, K., KHALID, N. and BILAL, M., 2021. Enzyme-assisted bioremediation approach for synthetic dyes and polycyclic aromatic hydrocarbons degradation. *Journal of Basic Microbiology*, vol. 61, no. 11, pp. 960-981. http://doi.org/10.1002/jobm.202100218. PMid:34608659.

ALAM, R., ARDIATI, F.C., SOLIHAT, N.N., ALAM, M.B., LEE, S.H., YANTO, D.H.Y., WATANABE, T. and KIM, S., 2021. Biodegradation and metabolic pathway of anthraquinone dyes by *Trametes hirsuta* D7 immobilized in light expanded clay aggregate and cytotoxicity assessment. *Journal of Hazardous Materials*, vol. 405, pp. 124176. http://doi.org/10.1016/j.jhazmat.2020.124176. PMid:33131941.

AL-FADHAL, F.A., AL-ABEDY, A.N. and AL-JANABI, M.M., 2018. Molecular identification of novel isolates of *Rhizoctonia solani* Kühn and *Fusarium* spp. (Matsushima) isolated from petúnia plants (*Petunia hybrida* L.). *Plant Archives*, vol. 18, no. 1, pp. 703-711.

ANITA, S.H., ARDIATI, F.C., OKTAVIANI, M., SARI, F.P., NURHAYAT, O.D., RAMADHAN, K.P. and YANTO, D.H.Y., 2020. Immobilization of laccase from *Trametes hirsuta* EDN 082 in light expanded clay aggregate for decolorization of Remazol Brilliant Blue R dye. *Bioresource Technology Reports*, vol. 12, pp. 100602. http://doi.org/10.1016/j.biteb.2020.100602.

BONUGLI-SANTOS, R.C., DURRANT, L.R., SILVA, M. and SETTEC, L.D., 2010. Production of laccase, manganese peroxidase and lignin peroxidase by Brazilian marine-derived fungi. *Enzyme and Microbial Technology*, vol. 46, no. 1, pp. 32-37. http://doi.org/10.1016/j.enzmictec.2009.07.014.

CASTELLANI, A., 1939. Viability of some pathogenic fungi in distilled water. *The Journal of Tropical Medicine and Hygiene*, vol. 42, pp. 225-226.

CHAMPAGNE, P.-P. and RAMSAY, J.A., 2005. Contribution of manganese peroxidase and laccase to dye decoloration by *Trametes versicolor*. *Applied Microbiology and Biotechnology*, vol. 69, no. 3, pp. 276-285. http://doi.org/10.1007/s00253-005-1964-8. PMid:15834615.

CHAUDHARI, A.U., PAUL, D., DHOTRE, D. and KODAM, K.M., 2017. Effective biotransformation and detoxification of anthraquinone dye reactive blue 4 by using aerobic bacterial granules. *Water Research*, vol. 122, pp. 603-613. http://doi.org/10.1016/j.watres.2017.06.005. PMid:28628882.

DOYLE, J.J. and DOYLE, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, vol. 19, pp. 11-15.

EICHLEROVÁ, I., HOMOLKA, L., BENADA, O., KOFRONOVÁ, O., HUBÁLEK, T. and NERUD, F., 2007. Decolorization of Orange G and Remazol Brilliant Blue R by the white rot fungus *Dichomitus squalens*: toxicological evaluation and morphological study. *Chemosphere*, vol. 69, no. 5, pp. 795-802. http://doi.org/10.1016/j.chemosphere.2007.04.083. PMid:17604080.

HEPPERLE, D., 2004 [viewed 9 January 2024]. *SeqAssem©: a sequence analysis tool contig as-sembler and trace data visualization tool for molecular sequences. Win32-ver-Sion*. Available from: https://www.sequentix.de/

HERNÁNDEZ, D.L. and HOBBIE, S.E., 2010. The effects of substrate composition, quantity, and diversity on microbial activity. *Plant and Soil*, vol. 335, no. 1-2, pp. 397-411. http://doi.org/10.1007/s11104-010-0428-9.

HUELSENBECK, J.P. and RONQUIST, F., 2001. MRBAYES: bayesian inference of phylogenetic trees. *Bioinformatics (Oxford, England)*, vol. 17, no. 8, pp. 754-755. http://doi.org/10.1093/bioinformatics/17.8.754. PMid:11524383.

JANUSZ, G., PAWLIK, A., SULEJ, J., ŚWIDERSKA-BUREK, U., JAROSZ-WILKOŁAZKA, A. and PASZCZYŃSKI, A., 2017. Lignin degradation: microorganisms, enzymes involved, genomes analysis and

evolution. *FEMS Microbiology Reviews*, vol. 41, no. 6, pp. 941-962. http://doi.org/10.1093/femsre/fux049. PMid:29088355.

JUSTO, A. and HIBBETT, D.S., 2011. Phylogenetic classification of *Trametes* (Basidiomycota, Polyporales) based on a five–marker dataset. *Taxon*, vol. 60, no. 6, pp. 1567-1583. http://doi.org/10.1002/tax.606003.

KATOH, K., ROZEWICKI, J. and YAMADA, K.D., 2019. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, vol. 20, no. 4, pp. 1160-1166. http://doi.org/10.1093/bib/bbx108. PMid:28968734.

KHAN, S.A.N., MEHMOOD, S., SHABNIR, S.B., ALI, S., ALREFAEI, A.F., ALBESHR, M.F. and HAMAYUN, M., 2023. Efficacy of fungi in the decolorization and detoxification of remazol brilliant blue dye in aquatic environments. *Microorganisms*, vol. 11, no. 703, pp. 1-19. http://doi.org/10.3390/microorganisms11030703. PMid:36985276.

LARGET, B. and SIMON, D.L., 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution*, vol. 16, no. 6, pp. 750-759. http://doi.org/10.1093/oxfordjournals.molbev.a026160.

MACHADO, K.M.G., MATHEUS, D.R. and BONONI, V.L.R., 2005. Ligninolytic enzymes production and Remazol Brilliant Blue R decolorization by tropical Brazilian basidiomycetes fungi. *Brazilian Journal of Microbiology*, vol. 36, no. 3, pp. 246-252. http://doi.org/10.1590/S1517-83822005000300008.

MISHRA, S. and MAITI, A., 2019. Applicability of enzymes produced from different biotic species for biodegradation of textile dyes. *Clean Technologies and Environmental Policy*, vol. 21, no. 4, pp. 763-781. http://doi.org/10.1007/s10098-019-01681-5.

MONSSEF, R.A.A.E., HASSAN, E.A. and RAMADAN, E.M., 2016. Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment. *Annals of Agricultural Science*, vol. 61, no. 1, pp. 145-154. http://doi.org/10.1016/j.aoas.2015.11.007.

MONTEIRO, J.S., SARMENTO, P.S.M. and SOTAO, H.M.P., 2019. Saprobic conidial fungi associated with palm leaf litter in eastern Amazon, Brazil. *Anais da Academia Brasileira de Ciências*, vol. 91, no. 3, pp. e20180545. https://doi.org/10.1590/0001-3675201920180545.

NAVADA, K.K., SANJEEV, G. and KULAL, A., 2018. Enhanced biodegradation and kinetics of anthraquinone dye by laccase from an electron beam irradiated endophytic fungus. *International Biodeterioration & Biodegradation*, vol. 132, pp. 241-250. http://doi.org/10.1016/j.ibiod.2018.04.012.

NYANHONGO, G.S., GOMES, J., GÜBITZ, G., ZVAUYA, R., READ, J.S. and STEINER, W., 2002. Production of laccase by a newly isolated strain of *Trametes modesta*. *Bioresource Technology*, vol. 84, no. 3, pp. 259-263. http://doi.org/10.1016/S0960-8524(02)00044-5. PMid:12118703.

OLIVEIRA, M.E.S., KANZI, A.M., VAN DER MERWE, N.A., WINGFIELD, M.J., WINGFIELD, B.D., SILVA, G.A. and FERREIRA, M.A., 2022. Genetic variability in populations of *Chrysoporthe cubensis* and *Chr. puriensis* in Brazil. *Australasian Plant Pathology*, vol. 51, no. 2, pp. 175-191. http://doi.org/10.1007/s13313-021-00847-4.

OLOU, B.A., KRAH, F.-S., PIEPENBRING, M., YOUROU, N.S. and LANGER, E., 2020. Diversity of *Trametes* (Polyporales, Basidiomycota) in tropical Benin and description of new species *Trametes parvispora*. *MycoKeys*, vol. 65, pp. 25-47. http://doi.org/10.3897/mycokeys.65.47574. PMid:32206024.

OSMA, J.F., HERRERA, J.L.T. and COUTO, S.R., 2007. Banana skin: A novel waste for laccase production by *Trametes pubescens* under solid-state conditions. Application to synthetic dye decolouration. *Dyes and Pigments*, vol. 75, no. 1, pp. 32-37. http://doi.org/10.1016/j.dyepig.2006.05.021.

RAINERT, K.T., NUNES, H.C.A., GONÇALVES, M.J., HELEM, C.V. and TAVARES, L.B.B., 2021. Decolorization of the synthetic dye Remazol Brilliant Blue Reactive (RBBR) by *Ganoderma lucidum* on bio-adsorbent of the solid bleached sulfate paperboard coated with polyethylene terephthalate. *Journal of Environmental Chemical Engineering*, vol. 9, no. 2, pp. 104990. http://doi.org/10.1016/j.jece.2020.104990.

RAO, R.G., RAVICHANDRAN, A., KANDALAM, G., KUMAR, S.A., SWARAJ, S. and SRIDHAR, M., 2019. Screening of wild basidiomycetes and evaluation of the biodegradation potential of dyes and lignin by manganese peroxidases. *BioResources*, vol. 14, no. 3, pp. 6558-6576. http://doi.org/10.15376/biores.14.3.6558-6576.

RODRÍGUEZ-COUTO, S., 2011. Production of laccase and decolouration of the textile dye Remazol Brilliant Blue R in temporary immersion bioreactors. *Journal of Hazardous Materials*, vol. 194, pp. 297-302. http://doi.org/10.1016/j.jhazmat.2011.07.098. PMid:21868156.

SANTOS, R.F., SOTÃO, H.M.P., MONTEIRO, J.S., GUSMÃO, L.F.P. and GUITIÉRREZ, A.H., 2018. Conidial fungi associated with leaf litter of red cedar (*Cedrela odorata*) in Belém, Pará (eastern Brazilian Amazon). *Acta Amazonica*, vol. 48, pp. 230-238. http://dx.doi.org/10.1590/1809-4392201704411 .

SARI, A.A., TACHIBANA, S. and MURYANTO., 2012. Correlation of ligninolytic enzymes from the newly-found species *Trametes versicolor* U97 with RBBR decolorization and DDT degradation. *Water, Air, and Soil Pollution*, vol. 223, no. 9, pp. 5781-5792. http://doi.org/10.1007/s11270-012-1314-2.

SILVANI, V.A., COLOMBO, R.P., SCORZA, M.V., FERNÁNDEZ BIDONDO, L., ROTHEN, C.P., SCOTTI, A., FRACCHIA, S. and GODEAS, A., 2017. Arbuscular mycorrhizal fungal diversity in high-altitude hypersaline Andean wetlands studied by 454-sequencing and morphological approaches. *Symbiosis*, vol. 72, no. 2, pp. 143-152. http://doi.org/10.1007/s13199-016-0454-3.

SING, N.N., HUSAINI, A., ZULKHARNAIN, A. and ROSLAN, H.A., 2017. Decolorization capabilities of ligninolytic enzymes produced by Marasmius cladophyllus UMAS MS8 on Remazol Brilliant Blue R and other azo dyes. *BioMed Research International*, vol. 2017, no. 1, pp. 1325754. http://doi.org/10.1155/2017/1325754.

SWOFFORD, D.L., 2002. *PAUP: Phylogenetic analyses using parsimony version 4.0 beta* [software]. Sunderland: Sinauer Associates Inc.

TIEN, M. and KIRK, T.K., 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. *Methods in Enzymology*, vol. 161, pp. 238-249. http://doi.org/10.1016/0076-6879(88)61025-1.

WELTI, S., MOREAU, P.-A., FAVEL, A., COURTECUISSE, R., HAON, M., NAVARRO, D., TAUSSAC, S. and LESAGE-MEESSEN, L., 2012. Molecular phylogeny of *Trametes* and related genera, and description of a new genus *Leiotrametes*. *Fungal Diversity*, vol. 55, no. 1, pp. 47-64. http://doi.org/10.1007/s13225-011-0149-2.

WHITE, T.J., BRUNS, T., LEE, S. and TAYLOR, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, vol. 18, no. 1, pp. 315-322.

YANG, X.Q., ZHAO, X.X., LIU, C.Y., ZHENG, Y. and QIAN, S.J., 2009. Decolorization of azo, triphenylmethane and anthraquinone dyes by a newly isolated *Trametes* sp. SQ01 and its laccase. *Process Biochemistry (Barking, London, England)*, vol. 44, no. 10, pp. 1185-1189. http://doi.org/10.1016/j.procbio.2009.06.015.

YILMAZ, N., LÓPEZ-QUINTERO, C.A., VASCO-PALACIOS, A.M., FRISVAD, J.C., THEELEN, B., BOEKHOUT, R.A.S. and HOUBRAKEN, J., 2016. Four novel *Talaromyces* species isolated from leaf litter from Colombian Amazon rain forests. *Mycological Progress*, vol. 15, no. 10-11, pp. 1041-1056. http://doi.org/10.1007/s11557-016-1227-3.

ZAFIU, C., PART, F., EHMOSER, E.K. and KÄHKÖNEN, M.A., 2021. Investigations on inhibitory effects of nickel and cobalt salts on the decolorization of textile dyes by the white rot fungus *Phanerochaete velutina*. *Ecotoxicology and Environmental Safety*, vol. 215, pp. 112093. http://doi.org/10.1016/j.ecoenv.2021.112093. PMid:33721667.

ZHANG, X., XU, Z., PEI, H., CHEN, Z., TAN, X., HU, J., YANG, B. and SUN, J., 2017. Intraspecific variation and phylogenetic relationships are revealed by ITS1 secondary structure analysis and single-nucleotide polymorphism in *Ganoderma lucidum*. *PLoS One*, vol. 12, no. 1, pp. e0169042. http://doi.org/10.1371/journal.pone.0169042. PMid:28056060.

Supplementary Material

S1. Species, identification of isolates and GenBank access numbers of the reference sequences of the ITS region of the genus Trametes and the outgroups Dentocorticium sulphurellum and Lopharia cinerascens used in this study.

S2. Position of nucleotide polymorphisms (nps) found in the ITS region for Trametes species.

This material is available as part of the online article from https://doi.org/10.1590/1519-6984.282099.