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POTENCIAL ENZIMÁTICO LIGNINOLÍTICO DE FUNGOS FILAMENTOSOS ISOLADOS DE SERAPILHEIRA DE FLORESTA AMAZÔNICA NA BIODEGRADAÇÃO DE RESÍDUOS

IÊDA ALANA LEITE DE SOUSA

Belém-PA
2025

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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia - Rede BIONORTE, na Universidade Federal do Pará, como requisito parcial para a obtenção do Título de Doutora em Biodiversidade e Biotecnologia.

Orientador (a): Prof. Dr. Alberdan Silva Santos

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SOUSA, Iêda Alana Leite de. **Potencial biotecnológico de fungos ligninolíticos isolados de serapilheira de floresta amazônica na biodegradação de resíduos**. 2025. 127 f. Tese (Doutorado em Biotecnologia) - Universidade Federal do Pará, Belém, PA - Brasil, 2025.

RESUMO

A decomposição da serapilheira amazônica é conduzida por invertebrados e microrganismos, com destaque para fungos dos filos Basidiomycota e Ascomycota, produtores de enzimas ligninolíticas como lacase (Lac), manganês peroxidase (MnP) e lignina peroxidase (LiP). Esses fungos têm potencial para degradar resíduos agroindustriais e atuar no tratamento de efluentes contaminados. Esta pesquisa teve como objetivo investigar a produção de enzimas ligninolíticas por fungos isolados de serapilheira de floresta ciliar, coletadas em Salvaterra – PA, visando sua aplicação na biodegradação de resíduos industriais como corante antraquinônico e caroço de açaí. Foram selecionadas quatro colônias fúngicas com base na formação de halos de oxidação e descoloração em meio batata-dextrose-ágar suplementado com guaiacol ou Remazol Brilliant Blue R (RBBR). A identificação molecular por sequenciamento da região ITS revelou dois isolados como *Trametes flavida*, um como *Trametes lactinea* e um como *Syncephalastrum sympodiale*. Apenas os isolados de *Trametes* spp. promoveram descoloração do corante RBBR, sendo o maior percentual (89,28%) alcançado pelo isolado A1SSI02 após sete dias em comparação aos demais isolados. A Lac apresentou a maior atividade entre as enzimas testadas, atingindo 452,13 $U \cdot mL^{-1}$ em meio líquido com RBBR (A1SSI01) e 797,73 $U \cdot mL^{-1}$ em fermentação sólida com caroço de açaí (A1SSI02). Ensaios de fermentação sólida com caroço de açaí também evidenciaram modificações estruturais no substrato degradado, confirmadas por microscopia eletrônica de varredura (MEV) e espectroscopia no infravermelho por transformada de Fourier (FTIR), com observação de hifas, esporos e alterações na integridade da superfície, como rachaduras e quebras da parade celular, além de alterações na quantificação do teor de lignina do caroço. Os resultados revelam o potencial dos isolados de *Trametes* spp. e *S. sympodiale* na produção de enzimas ligninolíticas e sua aplicação em processos biotecnológicos sustentáveis, como a biodescoloração de efluentes e o aproveitamento de resíduos lignocelulósicos. O estudo também representa o primeiro registro de *S. sympodiale* em serapilheira da Amazônia e contribui para o conhecimento taxonômico de *T. flavida* no Brasil.

Palavras-Chave: Lacase; Peroxidases; *Trametes* spp.; *Syncephalastrum sympodiale*; Biotransformação.

SOUSA, Iêda Alana Leite de. **Biotechnological potential of ligninolytic fungi isolated from amazonian forest litter for waste biodegradation**. 2025. 127 f. Thesis (Doctorate in Biotechnology) - Federal University of Pará, Belém, PA - Brazil, 2025.

ABSTRACT

The decomposition of Amazonian litter is driven by invertebrates and microorganisms, with particular emphasis on fungi from the phyla Basidiomycota and Ascomycota, which produce ligninolytic enzymes such as laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP). These fungi have potential for degrading agro-industrial waste and treating contaminated effluents. This study aimed to investigate the production of ligninolytic enzymes by fungi isolated from riparian forest litter collected in Salvaterra – PA, with a focus on their application in the biodegradation of industrial residues such as anthraquinone dye and açaí seeds. Four fungal colonies were selected based on the formation of oxidation and discoloration halos on potato-dextrose-agar medium supplemented with guaiacol or Remazol Brilliant Blue R (RBBR). Molecular identification through ITS region sequencing revealed two isolates as *Trametes flavida*, one as *Trametes lactinea*, and one as *Syncephalastrum sympodiale*. Only the Trametes spp. isolates promoted RBBR dye discoloration, with the highest percentage (89.28%) achieved by isolate A1SSI02 after seven days compared to the other isolates. Lac showed the highest activity among the tested enzymes, reaching 452.13 $U \cdot mL^{-1}$ in liquid medium with RBBR (A1SSI01) and 797.73 $U \cdot mL^{-1}$ in solid-state fermentation with açaí seeds (A1SSI02). Solid-state fermentation assays with açaí seeds also revealed structural modifications in the degraded substrate, confirmed by scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR), with observations of hyphae, spores, and changes in surface integrity such as cracks and cell wall breakage, as well as alterations in lignin content quantification of the seeds. The results demonstrate the potential of *Trametes* spp. and *S. sympodiale* isolates in producing ligninolytic enzymes and their application in sustainable biotechnological processes, such as effluent biobleaching and the utilization of lignocellulosic waste. The study also represents the first record of *S. sympodiale* in Amazonian litter and contributes to the taxonomic knowledge of *T. flavida* in Brazil.

Keywords: Lacase; Peroxidases; *Trametes* spp.; *Syncephalastrum sympodiale*; Biotransformation.

LISTA DE FIGURAS

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

ANEXOS

LISTA DE TABELAS

CAPÍTULO II

CAPÍTULO III

LISTA DE SIGLAS

BD	**Meio de cultura batata-dextrose**
BDA	**Meio de cultura batata-dextrose-ágar**
CTAB	**Cationic Hexadecyl Trimethyl Ammonium Bromide**
DB38	**Corante Direct Black 22**
DMP	**2,6-dimetoxifenol**
DNS	**Ácido dinitrosalicílico**
DR5B	**Corante Direct red 5B**
EML	**Enzimas modificadoras de lignina**
SFm	**Fermentação em estado líquido**
SSF	**Fermentação em estado sólido**
FTIR	**Espectroscopia de infravermelho por transformada de Fourier**
G	**Guaiacil**
H	**p-cumaril**
HAP	**Hidrocarbonetos aromáticos policíclicos**
HCl	**Ácido Clorídrico**
Lac	**Lacase**
LiP	**Lignina peroxidase**
MCMC	**Monte Carlo em cadeia de Markov**
MEV	**Microscopia Eletrônica de Varredura**
MnP	**Manganês peroxidase**
MP	**Máxima Parcimônia**
NCBI	**National Center for Biotechnology Information**
PCR	**Reação em cadeia de polimerase**
PP	**Probabilidade posterior**
PV	**Peroxidase versátil**
RBBR	**Remazol brilliant blue reactive**
Rpm	**Rotação por minuto**
S	**Siringil**
SY	**Corante amarelo pôr do sol**
TMCS	**Trimetilclorossilano**

SUMÁRIO

1. INTRODUÇÃO

O processo de decomposição da serapilheira é mediado por diferentes invertebrados detritívoros e decompositores microbianos (Gessner et al., 2010). Entre os microrganismos decompositores as bactérias e fungos são os principais responsáveis pela degradação desse material na natureza. Durante esse processo, distintas enzimas produzidas por esses microrganismos atuam na conversão do material orgânico bruto em compostos mais simples (Bai et al., 2021), permitindo a ciclagem de nutrientes essenciais para a sustentação da produtividade dos ecossistemas Amazônicos (Cotrufo et al., 2013).

Entre os fungos, os saprófitos dos filos Basidiomycota e Ascomycota são frequentemente isolados da serapilheira e de madeira em decomposição, sendo os agentes da degradação da matriz lignocelulósica (Canto-Canché et al., 2020). Esses fungos são classificados em três grupos: fungos de podridão branca, de podridão parda e de podridão mole (Kuuskeri et al., 2016). A decomposição da matéria orgânica tem ação das seguintes principais enzimas: manganês peroxidase (MnP), lignina peroxidase (LiP) e lacase (Lac), também conhecida como fenol-oxidase (Januz et al., 2017).

A matriz lignocelulósica está presente em grande quantidade de resíduos agroindustriais (Xu et al., 2019), e estudos recentes têm demonstrado a biodegradação desses resíduos por fungos (Díaz et al., 2022; Lei et al., 2022; Zang et al., 2022). Por exemplo, Díaz et al. (2022) investigaram a biodegradação de licor negro, um resíduo da indústria de papel, e observaram que os fungos Aspergillus uvarum e Phanerochaete chrysosporium foram capazes de reduzir significativamente a quantidade desse resíduo industrial. Já Zang et al. (2022), ao estudar a degradação da palha de milho, revelaram que o uso sinérgico das enzimas Lac, LiP e MnP foi eficaz na quebra das principais ligações químicas da lignina, fornecendo uma base teórica para a biodegradação dessa macromolécula.

Esses resultados evidenciam o potencial dos fungos ligninolíticos na bioconversão de resíduos ricos em lignina, como os gerados a partir do processamento do açaí — uma atividade econômica relevante na região metropolitana de Belém. Apesar da importância comercial do fruto, apenas 15 a 20% é convertido em polpa, enquanto cerca de 80 a 85% correspondem a caroços e fibras descartados como resíduo (Zavarize, 2021). O descarte inadequado desse material acarreta sérios impactos ambientais, como poluição do solo e da água, emissão de odores e gases de efeito estufa, além de sobrecarga em aterros urbanos (Melo et al., 2021; Martins et al., 2021). Diante disso, torna-se urgente a busca por alternativas sustentáveis para o

reaproveitamento desses resíduos, especialmente em regiões de alta produção e consumo do fruto.

Nesse contexto, os fungos ligninolíticos se destacam por seu elevado potencial biotecnológico, devido à capacidade de secretar enzimas oxidativas — como lacases (Lac), lignina peroxidase (LiP) e manganês peroxidase (MnP) — que degradam compostos aromáticos complexos, como a lignina. Além disso, essas enzimas apresentam baixa especificidade de substrato, sendo também eficazes na biodegradação de corantes sintéticos, especialmente os do tipo antraquinônico, como o Remazol Brilliant Blue R (RBBR), amplamente utilizado na indústria têxtil (Eichlerová et al., 2007; Ahmad & Alrozi, 2011; Ahsan et al., 2021). A semelhança estrutural entre a lignina e esses corantes permite que sua degradação seja utilizada como modelo experimental para avaliar o desempenho e o perfil catalítico das enzimas ligninolíticas, por meio da análise dos padrões de clivagem e formação de compostos fenólicos (Herath et al., 2024; Saratale et al., 2020).

Complementarmente, os fungos produtores dessas enzimas têm sido amplamente reconhecidos por sua eficácia no tratamento de águas residuais contaminadas com corantes industriais. A inespecificidade das enzimas ligninolíticas em relação aos substratos permite a degradação de polímeros sintéticos, como os corantes têxteis (Ahsan et al., 2021), incluindo o RBBR. Este corante antraquinônico reativo é composto por derivados de antraceno, um hidrocarboneto aromático policíclico (Eichlerová et al., 2007), amplamente empregado na indústria de tingimento (Ahmad & Alrozi, 2011). Os fungos produtores de Lac, LiP e MnP são considerados organismos promissores na biorremediação desse corante em águas residuais, utilizando sua biomassa como adsorvente e fonte de enzimas extracelulares responsáveis pela biodegradação (Saratale et al., 2020; Herath et al., 2024).

Além do tratamento de efluentes, o reaproveitamento de frações residuais ricas em lignina é estratégico para promover a sustentabilidade e a economia circular nas indústrias (Buratto et al., 2021). A biodegradação desses materiais por meio da ação enzimática de fungos tem sido proposta como uma abordagem eficiente para sua valoração (Hu et al., 2018). O processo resulta na conversão da macromolécula fenólica em moléculas de baixo peso molecular, frações hidrofílicas e compostos fenólicos (Ueno et al., 2016; Fedoseeva et al., 2019), os quais possuem diversas aplicações industriais.

Entre esses produtos, destacam-se os ácidos fenólicos, que apresentam potencial de uso nas indústrias farmacêutica, cosmética e alimentícia (Kumar & Goel, 2019). Assim, considerando a oportunidade de transformar resíduos lignocelulósicos em moléculas menos

tóxicas e com valor agregado, este estudo propõe investigar fungos degradadores de serapilheira capazes de produzir enzimas oxirredutoras com potencial para descoloração do corante RBBR e desconstrução do caroço de açaí. O objetivo é valorizar esse resíduo como alternativa econômica para a produção de enzimas e subprodutos de interesse biológico.

1.1 HIPÓTESES

- A serapilheira é um material potencial para o isolamento de fungos ligninolíticos;
- As enzimas produzidas por fungos isolados de serapilheira são capazes de degradar resíduos ricos em lignina e corantes de tingimento;
- A degradação do resíduo de açaí por fungos ligninolíticos isolados da serapilheira produz moléculas fenólicas.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Avaliar o potencial de fungos decompositores de serapilheira na produção de enzimas ligninolíticas, com foco na eficiência dessas enzimas na descoloração do corante antraquinônico Remazol Brilliant Blue R (RBBR) e na biodegradação da matriz lignocelulósica presente no caroço de açaí.

1.2.2 Objetivos Específicos

- Investigar a diversidade de fungos filamentosos produtores de enzimas ligninolíticas presentes na serapilheira de floresta ripária de Salvaterra-PA;
- Selecionar linhagens de fungos filamentosos que produzem concentrados enzimáticos capazes de desconstruir corante têxtil e resíduos gerados no processamento do açaí;
- Verificar a capacidade de descoloração do corante têxtil Remazol Brilliant Blue R (RBBR) pelas linhagens selecionadas.
- Verificar a capacidade de desconstrução da lignina do resíduo de açaí pelas linhagens selecionadas.
- Identificar compostos fenólicos gerados a partir do processo de desconstrução do caroço de açaí.

2. REVISÃO DE LITERATURA

2.1 Fungos de serapilheira na Amazônia

A serapilheira é definida como uma camada formada por folhas, galhos, cascas e outros resíduos orgânicos que se acumulam no solo das florestas. Essa camada desempenha um papel crucial no funcionamento dos ecossistemas florestais, contribuindo para a retenção de umidade, proteção contra a erosão e servindo como substrato para uma variedade de formas de vida (Golley et al., 1978), incluindo fungos. Os fungos têm um papel fundamental na decomposição da matéria orgânica, liberando nutrientes essenciais para outras plantas e microrganismos (Krishna e Mohan, 2017).

Na serapilheira de ecossistemas florestais amazônicos é observada uma alta diversidade de espécies de fungos, incluindo representantes de táxons como *Ascomycota*, *Basidiomycota* e *Mucoromycota*. Estudos relacionados à diversidade desses fungos em ambientes florestais na Amazônia relataram cerca de 409 espécies até o momento. Os filos *Ascomycota* e *Basidiomycota* possuem um número maior de espécies entre os filos de fungos identificados no material (Braga-Neto et al., 2008; Castro et al., 2012; Santos et al., 2018; Monteiro et al., 2019).

Estudos mostram que a distribuição dos fungos na camada de serapilheira está associada à composição deste material, o que refletirá diretamente nas atividades enzimáticas desses detectadas nesses microrganismos. Assim, fungos com atividades celulolíticas são frequentemente encontrados em folhas, enquanto aqueles com capacidade ligninolítica são mais comuns em substratos lenhosos, como os galhos (Castro et al., 2012; De Melo et al., 2018).

2.2 Enzimas ligninolíticas

Uma das grandes dificuldades de realizar o reaproveitamento de resíduos de biomassa lignocelulósica deve-se a presença de lignina, que é um polímero aromático de ficil degradação, devido as fortes ligações entre seus monômeros. A lignina é composta principalmente de monômeros p-cumaril (H), guaiacil (G) e siringil (S) e é conectada por ligações β-O-4, β-5 e β-β (Kamimura et al., 2019). No entanto, essas ligações podem ser rompidas através das enzimas produzidas por fungos, devido sua alta adaptabilidade à matéria orgânica em ambientes naturais, em que desenvolveram a capacidade de produzir compostos degradantes, como as enzimas ligninolíticas, também conhecidas como enzimas modificadoras de lignina (EML). As lacases e peroxidases compõem este grupo de enzimas e apresentam matrizes de oxidorredutases com potencial redox intenso (Asemoloye et al., 2021).

As EMLs estão divididas em dois grupos: heme-peroxidase e fenoloxidase. No primeiro grupo, as principais representantes são a lignina peroxidase (LiP) e a manganês peroxidase

(MnP), enquanto, no segundo grupo tem-se as lacases (Lac). Conforme o banco de dados de enzimas ativas em carboidratos (CAZy), as lacases são classificadas na superfamília multicobre oxidase (AA1), e as lignina e manganês peroxidases estão incluídas na família de atividade auxiliar (AA2) (CAZy; http://www.cazy.org/). As três enzimas compõem um sistema que podem degradar a lignina de forma eficaz (Rodríguez-Couto, 2016; Suryadi et al., 2022), como também demonstraram capacidade de degradação de vários xenobióticos, incluindo corantes, clorofenóis, hidrocarbonetos aromáticos policíclicos (HAP), compostos organofosforados e fenóis (Wesenberg et al., 2003).

2.2.1 Lignina Peroxidase (LiP)

A lignina peroxidase (LiP EC1.11.1.14) é uma peroxidase da superfamília peroxidase-catalase da Classe II, assim como a manganês peroxidase (MnP) e a peroxidase versátil (PV), que são peroxidases de natureza fúngica ou bacteriana, relacionadas à degradação de lignina na presença de peróxido de hidrogênio como aceptor de elétrons (Falade et al., 2017).

Conforme Abdel-Hamid et al. (2013), o ciclo catalítico da enzima envolve três etapas: i. oxidação da enzima férrica em repouso (Fe^{+3}) por peróxido de hidrogênio (H_2O_2) como um aceptor de elétrons, resultando na formação do composto intermediário oxo-ferril; ii. redução do intermediário oxo-ferril (deficiente em 2e-) por uma molécula de substrato, como o substrato aromático não fenólico que doa um elétron (1e-) ao composto intermediário para formar o segundo intermediário, composto II (deficiente de 1e-); iii. doação subsequente de um segundo elétron ao composto II pelo substrato reduzido, retornando assim a LiP ao estado de oxidação férrica em repouso (figura 1A). As reações catalisadas por LiP resultam principalmente na quebra de ligações Cα-Cβ, abertura de anéis aromáticos, oxidação de álcoois benzílicos nos aldeídos ou cetonas correspondentes, oxidação do fenol para produzir radicais livres, hidroxilação de metileno em grupos específicos e clivagem do fenilglicol (Singh et al., 2021).

Entre as enzimas ligninolíticas, a LiP foi a primeira a ser descoberta no meio extracelular do fungo da podridão branca *Phanerochaete chrysosporium* (Tien e Kirk, 1983; Glenn e Gold, 1985). Desde então, tem sido relatada nos gêneros de basidiomicetos como *Trametes*, *Phanerochaete*, *Pleurotus*, *Phlebia* e *Bjerkandera* (Peralta et al., 2017), e em ascomicetos como *Aspergillus*, *Trichoderma* e *Rhizopus* (Ikubar et al., 2018; Onianwah et al., 2019; Fan et al., 2019).

O método de detecção desta enzima em grande parte dos ensaios realizados nas publicações científicas baseia-se na oxidação do álcool veratrílico (Tien e Kirk, 1983), ou consiste em adaptações dessa metodologia, como a realizada por Takamiya et al. (2008). A

adição de peróxido de hidrogênio resulta na oxidação do álcool veratrílico em aldeído veratrílico, permitindo uma mudança na absorbância, que é lida em aproximadamente 310 nm. Outra metodologia de detecção alternativa ao uso do álcool veratrílico para LiP consiste no uso de Azure B (Archibald, 1992; Arora e Gill, 2001), na qual se avalia a oxidação do corante a 651 nm. No entanto, é uma metodologia menos usual entre os artigos catalogados e, em alguns casos, não permite detectar a presença da enzima, conforme mostrado por Sergentani et al. (2016).

2.2.2 Manganês Peroxidase (MnP)

A enzima manganês peroxidase (MnP - EC 1.11.1.13) é uma glicoproteína extracelular dependente de H_2O_2 e requer Mn^{2+} para oxidar fenóis monoaromáticos e corantes aromáticos. Essa proteína apresenta uma massa molecular que varia entre 38 e 62,5 kDa (Hofrichter, 2002). O ciclo catalítico da MnP é semelhante ao da LiP, sendo a única diferença o uso de Mn^{2+} como doador de elétrons.

Na primeira fase da reação, ocorre a ligação do H_2O_2 à enzima férrica, formando um complexo radical de Fe^{4+} + oxo-porfirina (Composto I MnP). Nesta etapa, um íon de Mn^{2+} monoquelado doa um elétron ao intermediário porfirina. Imediatamente ocorre o segundo ciclo da reação, em que o íon Mn^{2+} monoquelado atua como doador de um elétron para o intermediário porfirina e é oxidado a Mn^{3+}, formando o complexo Fe^{4+}oxo-porfirina (Composto II MnP). Posteriormente, o composto MnP II se combina com Mn^{2+} de maneira semelhante para gerar Mn^{3+}, liberando uma molécula de H_2O, e é reduzido ao estado original de MnP férrico, completando o ciclo catalítico (figura 1B). A oxidação por MnP ocorre através da formação de Mn^{3+} quelado, que atua como mediador em pequenas moléculas capazes de atingir estruturas fenólicas, como fenóis simples, aminas, corantes e compostos modelo de lignina fenólica (Rodríguez-Couto, 2016; Mondal et al., 2019; Periasamy et al., 2019).

A atividade de MnP já foi descrita para diversos fungos do filo Basidiomicota, como os pertencentes as ordens Agaricales, Cortiales, Polyporales e Hymenochaetales da classe Agaricomycetes (Gold e Alic, 1993; Tello et al., 2000; Morgenstern et al., 2010). Algumas espécies notáveis incluem *Irpex lacteus* F17 (Ying et al., 2016), *Trametes* sp. 48424 (Zhang et al., 2017), *Echinodontium taxodii* 2538 (Kong et al., 2016), *Ganoderma lucidum* IBL-05 (Bilal e Asgher et al., 2016), e *Trametes versicolor* (Shi et al., 2021). Além disso, há registros de atividade de MnP em fungos do filo Ascomicota, como *Fusarium* sp. HUIB01 (Huy et al., 2017), *Mucor irregularis* B-Yorla10 e *Aspergillus oryzae* C-Effurun (Asemoloye et al., 2020).

Com relação aos principais métodos de detecção da atividade MnP, alguns substratos específicos são utilizados, como o vermelho de fenol, 2,6-dimetoxifenol (DMP) e guaiacol, com absorbâncias lidas em 610 nm, 469 nm e 465 nm, respectivamente, para cada substrato (Paszczyński et al., 1988; Stasolla e Yeung, 2007; Silva et al., 2014). Em todos os métodos de detecção, as reações são suplementadas com solução contendo Mn^{2+} ou solução de $MnSO_4$ para determinar a atividade desta enzima.

2.2.3 Lacase (Lac)

Entre as enzimas modificadoras de lignina, a lacase (Lac – EC 1.10.3.2) destaca-se por sua ampla aplicabilidade e elevada estabilidade. Trata-se de uma enzima oxidorredutase multicobre pertencente à família das glicoproteínas monoméricas extracelulares. Seu sítio ativo é composto por quatro átomos de cobre distribuídos em três centros distintos: o centro tipo 1 (Cu T1), que é mononuclear e responsável pela oxidação direta dos substratos fenólicos; o centro tipo 2 (Cu T2); e o centro tipo 3 (Cu T3), que juntos formam um aglomerado trinuclear envolvido na redução do oxigênio molecular a água. Essa organização estrutural confere à lacase sua capacidade catalítica única. O peso molecular da enzima varia entre 50 e 130 kDa, sendo superior ao das enzimas LiP e MnP (Jaiswal et al., 2015; Debnath e Saha, 2020).

O ciclo catalítico desta enzima ocorre basicamente em três etapas: i. redução do cobre tipo 1 através da redução do substrato; ii. transporte interno de elétrons do tipo 1 para o cobre tipo 2 e tipo 3; e iii. redução de oxigênio na água no local de cobre tipo 2 e tipo 3. Ou seja, os elétrons extraídos do substrato são transferidos do sítio T1 para o sítio T2/T3 através do tri-peptídeo conservado His-Cys-His e o oxigênio molecular é reduzido a água, como mostrado na figura 1C (Messerschmidt, 1997; Singh e Gupta, 2020). Na primeira fase, o sítio de cobre T1 é responsável pela cor azul da Lac com grande capacidade de oxirredução, possibilitando a absorção eletrônica substancial em quase 600 nm e sua detecção por meio da leitura de absorbância. Essa característica da enzima permite sua aplicabilidade em processos biotecnológicos de biobranqueamento e biorremediação, como em águas residuais das indústrias têxteis e celulósicas. Já o sítio de cobre T2 é incolor e não pode ser identificado por espectrofotometria (Solomon et al., 2008). No entanto, o sítio binuclear de cobre T3 é possível de se identificar, devido a possuir um espectro de fluorescência característico, com uma absorção espectral na zona de 330 nm (Shin e Lee 2000).

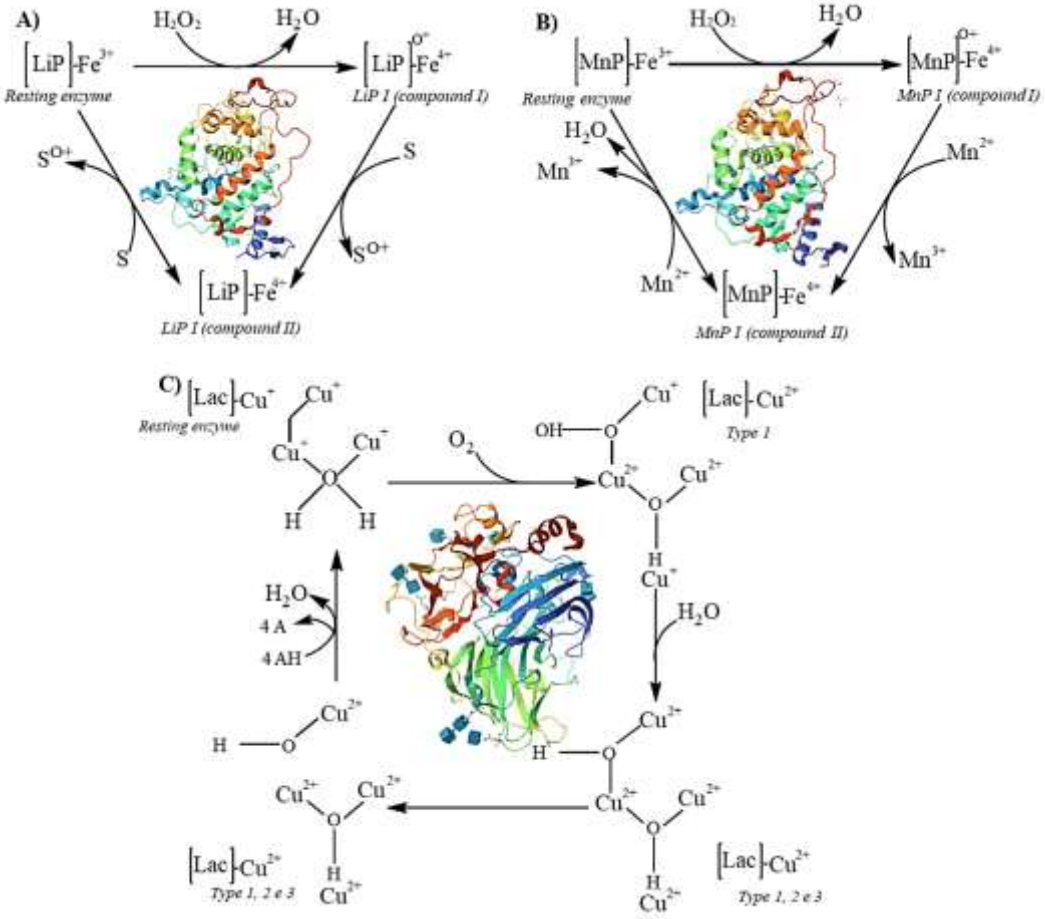

Figura 1 - A) Representação esquemática da catálise de substrato pela lignina peroxidase, adaptado de Singh et al. (2021), sendo S igual a substrato. A estrutura molecular tridimensional de LiP: referência PDB ID: 1B85. B) Representação esquemática da catálise de substrato por manganês peroxidase (MnP), adaptado de Wesenberg et al. (2003). A estrutura molecular tridimensional de MnP: referência PDB ID: 4BM2. C) Representação esquemática da catálise de substrato por lacase, adaptado de Wesenberg et al. (2003). A estrutura molecular tridimensional de Lac na sua forma oxidada: referência PDB ID: 1GYC.

A produção em microrganismos é identificada principalmente em fungos de podridão de madeira, como os de podridão branca. Podem ser citadas como exemplos as espécies *Phanerochaete chrysosporium*, *Theiophora terrestris* e *Lenzites true* (Suryadi et al., 2022), além de *Pleurotus ostreatus* e *Trametes versicolor* (Mallak et al., 2020). O gênero *Trichoderma*, como a espécie *T. harzianum*, também foi identificado como fonte produtora de Lac (Al Farraj et al., 2020; Ruan et al., 2023).

Diferentes métodos são relatados na literatura para a detecção da atividade de Lac, mas todos incluem a oxidação de fenóis como DMP e guaiacol, e o não fenólico ABTS. A oxidação desses substratos permite a determinação da atividade por espectrofotometria, sendo lida em 469 nm para DMP, 450 nm para guaiacol e 420 nm para ABTS (Arora e Sandhu, 1985; Jiang et al., 2011; Younes e Sayadi, 2011; Litwińska et al., 2019).

2.3 Biodegradação de Resíduos

Diversos resíduos gerados por processos industriais têm sido amplamente mencionados na literatura como fontes de carbono para a produção e/ou aplicação de enzimas modificadoras de lignina (EML), visando à degradação sustentável desses materiais. Entre os mais utilizados destacam-se os corantes industriais de tingimento e os resíduos agroindustriais, ambos frequentemente empregados em processos de biodegradação mediados por fungos ligninolíticos (Cagide e Castro-Sowinski, 2020).

Os corantes, como o Remazol Brilliant Blue R (RBBR), apresentam elevada resistência à degradação química devido à presença de estruturas antraquinonas altamente estabilizadas por ressonância, o que dificulta sua oxidação (Mahdy e Suttinu, 2023). Por outro lado, os resíduos agroindustriais são ricos em lignina, um dos principais obstáculos ao reaproveitamento da biomassa lignocelulósica. Esses resíduos são gerados em grandes volumes, especialmente pela indústria alimentícia, e incluem bagaço de cana-de-açúcar, casca de arroz, palha de milho, casca de laranja, resíduos de café, casca de soja e farelo de trigo (Cordeiro et al., 2020).

Em ambos os casos, a utilização de fungos ligninolíticos, como os do gênero *Pleurotus*, *Phanerochaete* e *Trametes*, tem se mostrado uma alternativa promissora para a biotransformação desses resíduos, contribuindo para a redução da carga poluente e mitigação de impactos ambientais (Kumar e Chandra, 2020; Qian et al., 2020).

2.3.1 Corantes residuais

No processo de tingimento na indústria têxtil e de papel, ocorre a geração de águas residuais tóxicas devido ao uso de corantes, os quais geralmente são resistentes a degradação e podem produzir resíduo mutagênicos e/ou carcinogênicos. As etapas de tratamento desses efluentes dependem principalmente de procedimentos físicos e químicos, aumentando os custos de produção e gerando resíduos perigosos (Shindhal et al., 2021).

Como método alternativo, de baixo custo e sustentável, o uso de fungos produtores de enzimas ligninolíticas tem sido investigado, pois são microrganismos que possuem sistemas oxidativos extracelulares caracterizados por baixa especificidade de substrato, o que permite a transformação ou degradação de uma ampla gama de contaminantes ambientais (Chen et al., 2023; Gao et al., 2024). Entre os principais responsáveis por essa biodegradação estão as EML que oxidam eficazmente os corantes sintéticos (Bilal et al., 2017; Kumar e Chandra, 2020).

Em um estudo realizado para avaliar o desempenho de uma nova Lac de *T. polyzona* MUCL 38443 na descoloração de cinco corantes diferentes, denominados como amido preto 10B, laranja G, sal sódico roxo de bromocresol, eritrosina B e oxalato verde malaquita,

verificou-se que a enzima isolada e purificada TP-Lac2 descolora de forma eficiente (> 75%) os corantes testados, com exceção de eritrosina, na presença de indutores como acetosiringona e ABTS, podendo ser indicada como um sistema mediador potencial e sustentável para o tratamento de águas residuais na indústria têxtil (Bucchieri et al., 2024).

Physisporinus vitreus (PHYS-L) é um fungo de podridão branca que também tem se mostrado promissor na degradação e descoloração de corante, sendo responsável por 90% da descoloração de corantes azos (corantes orgânicos aromáticos com um ou mais grupo azo em sua cadeia) em fermentações líquidas, como demonstrados nos resultados do estudo de Alhujaily et al. (2024). Além disso, foi observado que a suplementações dos meios fermentativos com siringaldeído como a gente mediador, em concentrações de 0,1 ou 0,2 mM, promovera o aumento do percentual de descoloração dos corantes azos como SY (amarelo pôr do sol), DB38 (Direct Black 22) e DR5B (Direct red 5B). Esses resultados são explicados devido o siringaldeído favorecer no meio o aumento da atividade enzimática da Lac (Alhujaily et al., 2024).

Aspergillus flavus também é uma espécie de fungo promissora, que produz diversos metabólitos ao longo do crescimento de sua colônia, como as EML. A cepa A5P1 deste fungo Ascomycota possui potencial para degradação do corante azo devido à produção de Lac extracelular e LiP intracelular por esta colônia. Verificou-se que houve a descoloração do corante RO16, nas concentrações de 50 a 600 mg/L, com fermentação em pH = 3, favorecendo o processo de remoção do RO16, assim como a capacidade de eliminação de radicais livres (Qin et al., 2024).

O tratamento de efluentes originados da coloração de produtos em fábricas de palma de óleo com fungos de podridão mostrou-se efetivo em um estudo realizado por Ridtibud et al. (2024). Neste estudo, os autores ao analisarem isolados *Trametes elegans* (PP17-06) e *Ganoderma* sp.2 (PW17-06 e PW17-177) verificaram a eficiências das colônias na presentaram descoloração do efluente após 5 dias de incubação. Na comparação dos resultados, o isolado de *T. elegans* foi o mais promissor, com taxa de descoloração de 47,4% do efluente. Para um aumento no percentual dessas taxas a adição de indutores, como mostrado por Bucchieri et al. (2024) e Alhujaily et al. (2024) poderia melhorar os resultados desta investigação, uma vez que a atividade de Lac foi a maior registradas para os isolados estudados por Ridtibud et al. (2024).

2.3.2 Resíduos agroindustriais

O uso de resíduos agrícolas como substrato de cultivo para fungos produtores de EML não apenas reduz a dependência de fontes de carbono convencionais, mas também contribui

para a minimização do impacto ambiental associado ao descarte inadequado desses resíduos. Desta maneira, o tratamento biológico de materiais de biomassa lignocelulósica tem recebido diversas atenções de pesquisas porque oferece muitas vantagens, como custo-benefício, ecologia e alta propensão na degradação de materiais lignocelulósicos (Qian et al., 2020).

Estudo realizado por Ying et al. (2024), ao testar três resíduos (colmo de milho, palhas de arroz e trigo) verificou que o isolado *Cerrena unicolor* GC.u01 foi capaz de produzir Lac nos três substratos, com atividade máxima registrada para o colmo de milho, sendo igual a 8,396 $U.mL^{-1}$ sete dias após o início da fermentação. A produção de MnP e LiP foi ausente no uso de palhas de trigo, e teve seus maiores registros de atividade no colmo de milho, respectivamente iguais a 15,22 e 24,31 $U.mL^{-1}$. Os resultados da taxa de degradação dos componentes dos resíduos revelam este isolado como promissor na degradação de resíduos agrícolas, principalmente em palha de arroz, onde foi responsável pela degradação de 34,3% da lignina do material após 15 dias de fermentação.

A faixa de degradação apresentada pelo isolado acima está dentro da faixa já demonstrada por fungos de podridão branca, que podem degradar entre 30 e 70% da lignina de diferentes biomassas (Li et al., 2022). Outro ponto relevante que auxilia nessa degradação é o percentual de lignina no material residual, podendo estar diretamente relacionado à produção enzimática dos fungos, como para três isolados de *Trametes hirsuta* empregados na degradação de diferentes acessos de sorgo com alto teor de lignina (19,7–22,9%), lignina moderada (17,9–18,4%) e baixo teor de lignina (14,4–16,7%). Os resultados da fermentação revelaram que o resíduo com alto teor de lignina favoreceu a produção de Lac e MnP, não havendo uma relação direta na produção de LiP, que por sua vez apresentou menor atividade ao ser comparada com as demais enzimas em todos os acessos.

Além dos fungos de podridão branca, alguns isolados de ascomicetos são capazes de colonizar resíduos agrícolas e produzir esse grupo de enzimas, como mostrado por Sijinamanoj et al. (2021) para *Aspergillus nomius* e *Trichoderma harzianum*. No estudo realizado para estes isolados, foi demonstrado que as enzimas Lac, MnP e LiP produzidas por esses fungos, sobretudo na presença de glicose para Lac e LiP, e na presença de maltose para MnP em fermentação em estado sólido, foram capazes de reduzir o percentual de celulose, hemicelulose e lignina dos resíduos de palha de arroz, bagaço de cana e casca de coco, com maior percentual de redução da lignina atribuído à palha de arroz, igual a 65% para fermentação com *A. nomius*, sendo indicados pelos autores como alternativa de valorização e biorrefinaria de biomassa lignocelulósica.

O aproveitamento de resíduos agrícolas na produção de enzimas ligninolíticas por fungos é uma abordagem promissora para a gestão sustentável de resíduos orgânicos e a produção de biocatalisadores de alto valor. Assim, no caso do estado do Pará pode ser uma alternativa para o reaproveitamento residual de indústrias despolpadoras de açaí, em que em grande parte é descartada no ambiente de forma inadequada (figura 2).

2.3.2.1 Caroço de Açaí

O açaizeiro é uma palmeira tropical nativa da região Amazônica, com aproximadamente 28 espécies, incluindo *Euterpe edulis* e *Eeuterpe oleracea*, amplamente disseminadas na região amazônica e com a produção comercial de seu fruto centralizada na região Norte. O estado do Pará é o principal produtor da cultura no Brasil, sendo a polpa do açaí, que é separada dos caroços e processada, gerando o suco, a forma de consumo mais difundida na região (Tavares et al., 2022; IBGE, 2022). Em termos de produtividade dados para o ano de 2021 demonstram que o estado alcançou uma produção igual a 154 mil toneladas de frutos, equivalente a 68% da produção nacional, com rendimento de R$ 6.200 milhões para economia paraense (IBGE, 2022).

No estado do Pará, devido ao consumo do açaí se configurar como uma característica cultural, 60% da produção total é consumido pela população local, enquanto 40% apenas é exportado para outros estados e países (Tavares et al., 2022). Entretanto, os elevados índices de produção e consumo de açaí traz consigo a alta taxa de geração de resíduos, pois apenas 15 a 20% do fruto é transformado em polpa, os demais 80 a 85% constituem em material residual de sementes e fibras (Zavarize, 2021). A alta quantidade desses resíduos representa um ônus ambiental as processadoras, pois grande parte do material vegetal é convertido em resíduo. Além disso, o caroço residual é em grande parte descartado de forma irregular, provocando impactos ambientais (Melo et al., 2021; Zavarize, 2021), principalmente em áreas urbanas da região metropolitana de Belém, como observado na figura 2.

Figura 2 - Descarte em áreas urbanas a céu aberto de resíduo de açaí após o despolpamento do fruto para produção da polpa em ruas da região metropolitana de Belém – PA, 2024.

A busca de alternativas para o aproveitamento de resíduos agroindustriais tem aumentado consideravelmente dada a necessidade de reduzir os impactos ambientais de seu descarte incorreto, como poluição dos lençóis freáticos, emissão de odores, aterros lotados e produção descontrolada de gases de efeito estufa (Martins et al., 2021). Nesse contexto, alguns processos como a produção de carvões ativados, extração de antioxidantes, produção de briquetes energéticos, produção de manose via hidrólise catalisada, biocombustíveis e a inclusão na dieta dos animais, foram desenvolvidos a fim de amenizar este problema, entretanto as alternativas para o aproveitamento desse resíduo ainda são insuficientes (Zavarize et al., 2021).

O reaproveitamento dessa fração residual através da biodegradação de fungos ligninolíticos pode ser uma alternativa sustentável e rentável para o tratamento dos caroços residuais de açaí. Pois, uso desse resíduo pode baratear o processo de produção enzimática da EMLs por fungos (Tišma et al., 2021). Além disso, nos últimos dez anos as pesquisas têm focado em alternativas biotecnológicas envolvendo fungos ligninolíticos para explorar e

produzir produto de valor agregado a partir desses resíduos agroindustriais, principalmente os alimentícios, visto que são fontes biodegradáveis e gerada em grande volume (Buratto et al., 2021; Zavarize et al., 2021), como o caso do resíduo do açaí no estado do Pará.

2.5 Rotas de biodegradação de resíduos por fungos ligninolíticos e geração de moléculas de valor agregado

No processo de degradação as enzimas catalisam reações que resultam na quebra das ligações químicas das moléculas orgânicas, permitindo a sua conversão em produtos degradados. Os produtos da degradação podem apresentar uma variedade de propriedades e potenciais aplicações, como exemplo, a produção de substratos em processos industriais, ou até mesmo o desenvolvimento de novos materiais e produtos com valor agregado. O conhecimento de moléculas resultantes desse processo pode otimizar estratégias de biodegradação e valorização de resíduos, contribuindo para a sustentabilidade ambiental e a economia circular.

A previsão da via de degradação dos corantes azo RO16 e Remazol Brilliant Blue R (RBBR) são similares através do uso de EML produzidas por fungos (Pype et al., 2019; Qin et al., 2024). Esses corantes apresentam grupos retiradores de elétrons (como grupos de ácido sulfônico), que contribuem para taxas mais rápidas de redução química e biológica. Duas vias de degradação são propostas para esses corantes na literatura envolvendo o uso das enzimas Lac e LiP produzidas por fungos que realizam a clivagem do grupo N=N das substâncias. Na primeira via, ocorre reações de dessulfonação e desmetoxilação do corante, levando à formação de dimetilnaftaleno (3), que após sofrer uma reação de abertura é transformado em 1,2 fenilenodibutirato (5), que sofrerá hidrólise, descarboxilação e acetilação e resultará no final desta rota na molécula 1,2-acetilbenzeno (6), como mostra a figura 7. A rota descrita acima, é o primeiro caminho proposto na segunda via de degradação, enquanto, o segundo caminho revela a ocorrência de reações como deetilação e dessulfonilação na presença das oxiredutases, resultando na produção de ácido p-aminobenzeno sulfônico (1), que sofrerá dessulfonação e hidrólise para produzir fenilhidroxilamina (4) (figura 3) (Pype et al., 2019; Qin et al., 2024).

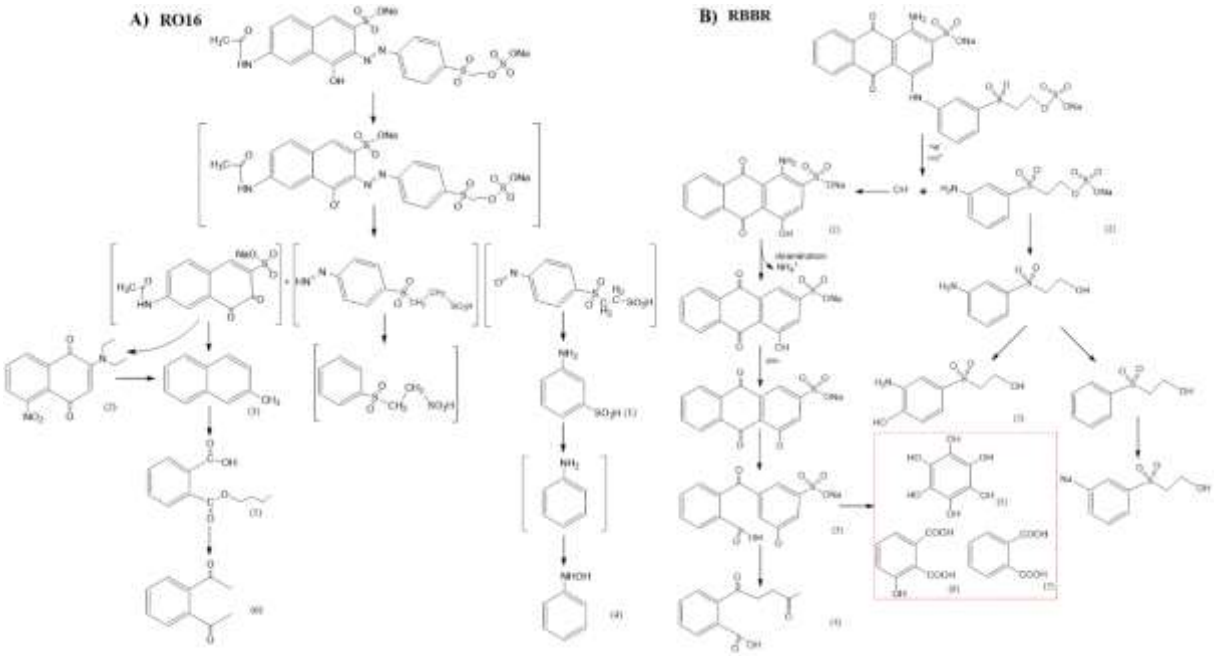

Figura 3 - Vias proposta para biodegradação de corantes azo, como RO16 e Remazol Brilliant Blue R (RBBR), através de lacase (Lac) e lignina peroxidase (LiP) produzidas por fungos.
Fonte: Qin et al., 2024.

Em resíduos agroindustriais, o modelo proposto por Kumar e Chandra (2019) em sua revisão sobre os mecanismos de degradação de resíduos lignocelulósicos por essas enzimas, mostra que as enzimas ligninolíticas extracelulares atuam primeiramente na quebra de diversas ligações na estrutura da lignina, como a ligação éter β-O-4 (éter β-arílico) e a ligação bifenil (β-β), para geração de radicais catiônicos livres. Esses radicais passam por reações químicas espontâneas, como hidroxilação e clivagem da ligação C–C, resultando em produtos hidrofílicos.

A partir da fragmentação da lignina, moléculas classificadas como monômeros de lignina são geradas, como os álcoois ρ-cumarílicos, coniferílicos e sinapilicos, a estrutura desses compostos pode ser visualizada na figura 4a. Esses álcoois são polimerizados e transformados nos respectivos polímeros: fenilpropanóides 4-hidroxifenil (p-hidroxifenil), guaiacil e siringil (figura 4b). A partir desse processo ocorre maior liberação da celulose e hemicelulose do material, que são biotransformados por outras enzimas específicas, em monômeros de celulose e carboidratos, como xylose, manose, galactose, rhaminose e arabinose, respectivamente.

Com relação a especificidade de quebra das ligações a LiP, por exemplo, é mais eficaz na quebra das ligações Cα-Cβ presente na estrutura da lignina, sendo capaz de oxidar compostos modelos de lignina fenólica mais facilmente do que os não fenólicos, enquanto a ação desta enzima em ligações β-O-4 é 25 vezes menor, em relação as demais enzimas (Pollegioni et al.,

2015). A enzima MnP apresenta capacidade de catalisar também a ligação Cα-Cβ, como Cα-oxidação e as ligação das subestruturas fenólicas β-1 (1,2-diarilpropano) e β-O-4 (Higuchi, 2004). Por fim, a Lac é enzima capaz de catalisar as ligações β-1 e β-O-4 da lignina na presença do oxigênio e de outros compostos contendo fenol (Atiwesh et al., 2022). Na figura 4c é possível visualizar a molécula de lignina, suas principais ligações onde ocorrem as ações enzimáticas.

A quebra controlada das ligações carbono-carbono e carbono-oxigênio na lignina, através da despolimerização enzimática pode produzir diferentes moléculas monoméricas e aromáticas, tornando este material uma fonte renovável de produtos químicos aromáticos finos (Pollegioni et al., 2015; Cui et al., 2022). Entre essas moléculas encontram-se como exemplos ácido gálico e seu derivado ácido 2-pirona-4,6-dicarboxílico, originados da clivagem do álcool sinapílico; ácido ferulico e seus derivados ácido vanílico e 4-hidroxibenzoato, originados da clivagem do álcool coniferilíco; e ácido protocatecuico é um dos principais produtos da clivagem do álcool ρ-cumarílicos (Zhao et al., 2022).

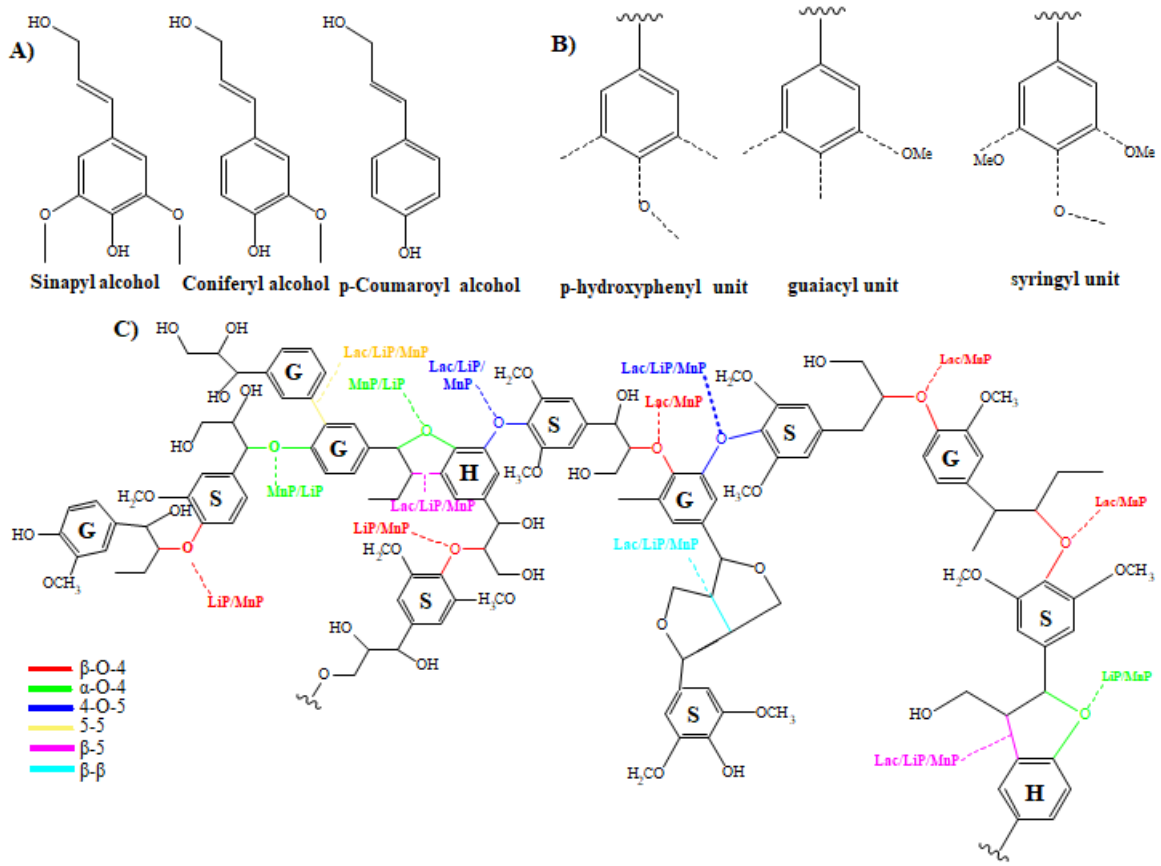

Figura 4 - Representação da estrutura química da molécula de lignina: a) os três monômeros que compõem a lignina; b) três unidades de álcoois oriundos da polimerização dos respectivos monômeros de lignina; e c) diferentes tipos de ligações moleculares da lignina e as principais enzimas envolvidas em suas quebras.

Diferentes moléculas são geradas no processo de degradação por fungos ligninolíticos sejam eles de origem sintética, como os corantes, ou de origem orgânica, como os resíduos agrícolas. A partir das quebras de corantes moléculas menos tóxicas geradas no processo favorecem o descarte do resíduo no ambiente sem provocar poluições em córregos d'água, tornando a produção industrial mais limpa e sustentável, sendo um ótimo catalizador de produtos químicos (Machado et al., 2005). Enquanto, em resíduos agroindustriais moléculas fenólicas são geradas e essa tem inúmeras aplicações em diversos setores industriais, como alimentícios, farmacêuticos e cosméticos (Singh et al., 2021).

A vanilina é um composto aromático originado na degradação da lignina (clivagem do álcool coniferilíco) amplamente utilizado nas indústrias alimentícia e cosmética, e que podem ser acumulados no final da degradação da lignina (Beckham et al., 2016). O ácido gálico (origem em álcool sinapílico) é outro componente interessante para aplicação em alimentos, podendo ser utilizado na indústria através da sua incorporação em biofilmes, melhorando a atividade antioxidante das embalagens e aumentando o tempo de prateleira dos produtos (Goudar et al., 2020). E outro exemplo para valorização das moléculas geradas pós-degradação da lignina é ácido protocatecuico (origem em ρ-cumarílico), com aplicações farmacológicas na regulação do estresse oxidativo e as respostas inflamatórias através de múltiplas vias de sinalização. Essa funcionalidade da molécula pode auxiliar em repostas neuroprotetoras, atividades antitumorais, atividades antiosteoporóticas, efeitos protetores contra atividades hepatotóxicas e nefrotóxicas (Song et al., 2020).

A análise dos dados ao longo desta revisão indica que a serrapilheira de ecossistemas florestais amazônicos apresenta uma diversidade significativa de fungos com potencial para a produção de enzimas ligninolíticas. Estes fungos desempenham um papel vital na biorremediação de águas residuais contendo corantes tóxicos, promovendo a limpeza e preservação ambiental. E a utilização de resíduos agroindustriais no cultivo de fungos de interesse e na produção de enzimas lignocelulolíticas surge como uma estratégia promissora para enfrentar desafios globais na gestão de resíduos sintéticos e orgânicos. Enzimas como LiP, MnP e Lac não apenas reduzem o impacto ambiental, mas também geram moléculas de valor agregado, com aplicações na indústria farmacêutica e alimentícia.

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3. CAPÍTULOS

3.1 CAPÍTULO I

Título: Fungi from litter in Amazonian forest ecosystems and biocatalytic aspects in the biotransformation of residues: a review

Fungos da serapilheira de ecossistemas florestais amazônicos e aspectos biocatalíticos na biotransformação de resíduos: uma revisão

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ABSTRACT
Decomposition of Amazonian litter is driven by microorganisms, particularly fungi from the Basidiomycota and Ascomycota, which produce ligninolytic enzymes such as manganese peroxidases, lignin peroxidase, and laccase. These fungi exhibit remarkable capabilities in degrading agro-industrial waste and treating contaminated wastewater, offering a sustainable approach to pollution mitigation. Research has unveiled substantial fungal diversity, notably within the Chaetosphaeriaceae and Russulaceae families. Ligninolytic enzymes demonstrate adaptability and catalytic efficiency, enabling the breakdown of complex lignin bonds into simpler molecules. This process generates compounds, including phenolic acids, methyl gallate, and vanillin, which have significant applications in the food, cosmetics, and pharmaceutical industries. Utilization of agro-industrial waste as substrates for enzyme production not only reduces costs but also fosters sustainable technological solutions. Furthermore, Amazonian fungi possess immense potential for biotechnological applications, facilitating the transformation of organic and synthetic waste into less toxic by-products. This approach enhances the accessibility and sustainability of these technologies. However, advancing the scalability, as well as the economic and environmental feasibility of these biotechnologies, remains crucial for their sustainable industrial adoption. This review underscores the pressing need for continued research focusing on these aspects to achieve environmentally effective and sustainable industrial applications. Consequently, fungi associated with Amazonian leaf litter emerge as highly promising biological resources, paving the way for innovations in biotechnology, improved waste management, and the production of high-value-added enzymes and products.

Keywords: Ligninolytic fungi. Enzymes. Bioremediation. Laccase. Peroxidases.

RESUMO

A decomposição da serapilheira na Amazônia é conduzida por microrganismos, especialmente fungos de Basidiomycota e Ascomycota, que produzem enzimas ligninolíticas, como manganês peroxidases, lignina peroxidase e lacase. Esses fungos são capazes de degradar resíduos agroindustriais e úteis no tratamento de águas residuais contaminadas, oferecendo uma abordagem sustentável para a mitigação da poluição. As pesquisas revelaram uma diversidade substancial de fungos, principalmente nas famílias Chaetosphaeriaceae e Russulaceae. As enzimas ligninolíticas demonstram adaptabilidade e eficiência catalítica, permitindo a quebra de ligações complexas de lignina em moléculas mais simples. Esse processo gera compostos, incluindo ácidos fenólicos, galato de metila e vanilina, que têm aplicações significativas nos setores de alimentos, cosméticos e farmacêutico. A utilização de resíduos agroindustriais como substratos para a produção de enzimas não apenas reduz os custos, mas também promove soluções tecnológicas sustentáveis. Além disso, os fungos amazônicos possuem um imenso potencial para aplicações biotecnológicas, facilitando a transformação de resíduos orgânicos e sintéticos em subprodutos menos tóxicos. No entanto, o avanço da escalabilidade, bem como a viabilidade econômica e ambiental dessas biotecnologias, continua sendo crucial para sua adoção industrial sustentável. Esta revisão ressalta a necessidade premente de pesquisas contínuas com foco nesses aspectos para obter aplicações industriais ambientalmente eficazes e sustentáveis. Consequentemente, os fungos associados à serapilheira amazônica surgem como recursos biológicos altamente promissores, abrindo caminho para inovações em biotecnologia, melhor gerenciamento de resíduos e produção de enzimas e produtos de alto valor agregado.
Palavras-chave: Fungos ligninolíticos. Enzimas. Biorremediação. Lacase. Peroxidases.

RESUMEN

La descomposición de la hojarasca amazónica corre a cargo de microorganismos, en particular hongos de los géneros Basidiomycota y Ascomycota, que producen enzimas ligninolíticas como el manganeso peroxidasa, la lignina peroxidasa y la lacasa. Estos hongos exhiben notables capacidades para degradar residuos agroindustriales y tratar aguas residuales contaminadas, ofreciendo un enfoque sostenible a la mitigación de la contaminación. La investigación ha revelado una diversidad sustancial de hongos, sobre todo dentro de las familias Chaetosphaeriaceae y Russulaceae. Las enzimas ligninolíticas demuestran adaptabilidad y eficiencia catalítica, permitiendo la descomposición de enlaces complejos de lignina en moléculas más simples. Este proceso genera compuestos, como ácidos fenólicos, galato de metilo y vainillina, que tienen importantes aplicaciones en las industrias alimentaria, cosmética y farmacéutica. La utilización de residuos agroindustriales como sustratos para la producción de enzimas no sólo reduce costes, sino que también fomenta soluciones tecnológicas sostenibles. Además, los hongos amazónicos poseen un inmenso potencial para aplicaciones biotecnológicas, facilitando la transformación de residuos orgánicos y sintéticos en subproductos menos tóxicos. Este enfoque mejora la accesibilidad y la sostenibilidad de estas tecnologías. Sin embargo, el avance en la escalabilidad, así como en la viabilidad económica y medioambiental de estas biotecnologías, sigue siendo crucial para su adopción industrial sostenible. Esta revisión subraya la acuciante necesidad de seguir investigando estos aspectos para conseguir aplicaciones industriales sostenibles y eficaces desde el punto de vista medioambiental. En consecuencia, los hongos asociados a la hojarasca amazónica emergen como recursos biológicos muy prometedores, que allanan el camino para las innovaciones en biotecnología, la mejora de la gestión de residuos y la producción de enzimas y productos de alto valor añadido.
Palabras clave: Hongos ligninolíticos. Enzimas. Biorremediación. Lacasa. Peroxidasas.

1. Introduction

In the litter decomposition process, various enzymes are produced by microorganisms, playing a critical role in the chemical transformation of the material. These include fungal enzymes such as laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP). The production of these enzymes by fungi has been investigated in recent studies, demonstrating their potential in the biodegradation of waste, especially agro-industrial waste, which, like litter, is mainly composed of a lignocellulosic matrix (Shing et al., 2019; Diaz et al., 2022).

As an example of these investigations, an analysis of the biodegradation of black liquor, a waste product from the paper industry, by isolates of the fungi *Aspergillus uvarum* and *Phanerochaete chrysosporium* revealed that these fungi were able to significantly reduce the amount of this waste (Diaz et al., 2022). Meanwhile, another study carried out with the aim of evaluating the degradation of corn straw showed that the synergistic use of the enzymes Lac, LiP and MnP broke the main chemical bonds of lignin, providing a theoretical basis for the biodegradation of this macromolecule (Zhang et al., 2022).

Another biotechnological application using fungi that produces ligninolytic enzymes is their potential for treating wastewater contaminated with industrial dyes. These enzymes are nonspecific in relation to the substrates and can degrade synthetic polymers, such as textile dyes (Ashan et al., 2021). The fungi that produce Lac, LiP and MnP are considered promising organisms for the bioremediation of dyes in wastewater (Saratale et al., 2020), and their biomass is used as an adsorbent and/or producer of extracellular enzymes responsible for the biodegradation of dyes (Herath et al., 2024).

Filamentous fungi isolated from leaf litter are a promising and renewable source to produce oxidative-reducing enzymes, such as lacases and peroxidases, with potential application in the biotransformation of synthetic and organic waste. The careful selection of strains is essential for the development of biotechnological processes aimed at making better use of these organisms and promoting the production of lignolytic enzymes on a large scale. This review sought to evaluate the diversity of fungi associated with litter from forest environments in the Amazon and the role of fungal species in the process of biodegradation of waste, highlighting the main enzymes involved and their catalytic mechanisms, as well as the possible value-added products generated in this process of enzymatic biotransformation of waste. It also addresses possible waste matrices that can act as substrates to produce these enzymes, offering a perspective on this conversion into possible new biotechnological products and a sustainable technological solution for biomass from industrial activity.

2. Methodology

For this scientific production, different academic publications from various recognized databases, including Scopus, Google Scholar, ACS, Scielo and Web of Science, were analyzed. The keywords used in the literature search were fungal diversity, litter, Amazon rainforest, biotransformation, lignin, oxidizing enzymes, lacase, dyes, textile dyes, manganese peroxidase, lignin peroxidase, residues, degradation routes, phenolic compounds, antioxidants, anti-inflammatories and antimicrobials. The selection criteria for these studies were limited to fungi identified in litter material related to publications with a study area in the Amazon rainforest, in addition to reporting on the use of these fungi in the biodegradation of agro-industrial waste and studies related to the potential use in biological activities of the new molecules generated after biodegradation. Also, for data analysis of the diversity of fungi found in the published articles, the phylum, class and family of fungi identified in the litter material were corrected and confirmed according to the Mycobank Database (2024).

3. Results and discussions

3.1 Diversity of fungi identified in leaf litter in the Amazon

Saprophytic fungi of the phyla Basidiomycota and Ascomycota are frequently identified in the substrate of leaf litter and decaying wood and are the main agents of lignocellulosic matrix degradation in nature (Canto et al., 2020). These fungi are classified into three groups: white rot, brown rot and soft rot fungi (Hakkinen et al., 2014). They are microorganisms known for producing extracellular ligninolytic enzymes released during the decomposition of organic matter, including manganese peroxidases (MnP), lignin peroxidase (LiP) and laccase (Lac), also known as phenol-oxidase (Jasnuz et al., 2017). These fungi play a fundamental role in the decomposition of organic matter, releasing essential nutrients for other plants and micro-organisms (Song et al., 2023). The use of these microorganisms in the biotransformation of waste can bring significant environmental benefits, such as reducing the amount of solid waste and producing compounds with high added value (Kuthiala et al, 2023).

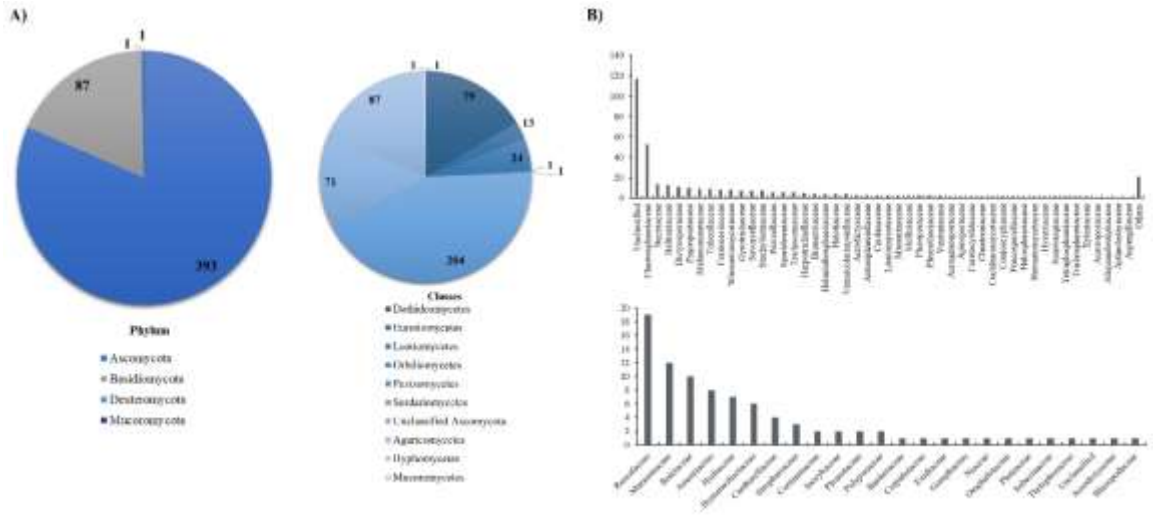
In the litter of Amazon forest ecosystems that have already been studied, a high diversity of fungal species is observed, including representatives of taxa such as *Ascomycota*, *Basidiomycota* and *Mucoromycota*. Studies related to the diversity of these fungi in forest environments in the Amazon have reported around 409 species to date. The phyla Ascomycota and Basidiomycota showed the highest abundances and richness of species among those identified in the material (Singer e Araújo, 1979; Braga-Neto et al., 2008; Castro et al., 2012; Yilmaz et al., 2016; Santos et al., 2018; Vasco-Palacios et al., 2018; Monteiro et al., 2019; De Queiroz et al., 2021; Gates et al., 202; De Sousa et al., 2024). Most of the species identified are present in the classes Dothideomycetes and Sordariomycetes, among the ascomycetes, and the class Agaricomycetes is the only one that appears for the phylum Basidiomycota among the species identified, especially abundant in the leaf litter and with the ability to degrade lignocellulosic materials (Figure 1A).

According to data obtained from, the most abundant and diverse family in the Ascomycota phylum is the Chaetosphaeriaceae family with 53 individuals and 42 species, followed by Nectriaceae (14 and 12), Beltraniaceae (13 and 9) and Dictyosporaceae (11 and 8). In the Basidiomycota, the Russulaceae family has 19 species identified in litter from forest ecosystems, followed by Marasmiaceae (12), Boletaceae (10) and Amanitaceae (7). Many representatives of these families play a crucial role as environmental biodegraders. In addition, around 71 species did not have families and/or classes defined (Figure 1B).

The most representative genres found in this survey of the fungal community present in the leaf litter were *Dictyosporium*, *Ellisembia*, *Sporidesmium* and *Stachybotrys*, with 7, 6, 6 and

6 species, followed by *Zygosporium*, *Chloridium* and *Helicosporium*, with 5 species each. In relation to the species, some were common in the leaf litter of different studies in native and planted forest areas in the Amazon, such as *Beltraniella portoricensis*, *Beltrania rhombica*, *Beltraniella portoricensis*, *Brachysporiella gayana* and *Circinotrichum olivaceum* (Braga-Neto et al., 2008; Castro et al., 2012; Yilmaz et al., 2016; Santos et al., 2018; Vasco-Palacios et al., 2018; Monteiro et al., 2019). The distribution of fungi in the litter may be associated with the composition of this material, which will have a direct impact on the enzymatic activities of these microorganisms. Fungi with cellulolytic activities are often found in leaves, while those with ligninolytic capacity are more common in woodier substrates (Castro et al., 2012; De Melo et al., 2018).

Figure 1. Distribution of fungal species identified in litter material in forest ecosystems in the Amazon, according to - a) phylum e taxonomic classes; b) phylum Ascomycota Basidiomycota, Deuteromycota and Mucoromycota.



Source: Elaborated by the authors, 2025.

3.2 Ligninolytic enzymes produced by fungi from Amazonian litter

The ligninolytic enzymes (LEs) produced by fungi represent a promising biotechnological alternative to overcoming the primary challenge in reusing lignocellulosic biomass residues: the deconstruction of lignin, one of the most recalcitrant components of the organic matrix that has great potential to produce valuable metabolites, turns in this way a biopharm. Lignin is predominantly composed of p-coumaryl (H), guaiacyl (G), and syringyl (S) monomers, linked by chemical bonds such as β-O-4, β-5, and β-β, which confer high structural resistance (Kmimura et al., 2019). These bonds can be efficiently broken by the action of LEs produced by fungi, which, due to their high adaptability to organic matter in natural environments, have evolved the capacity to synthesize these enzymes. Among the LEs, laccases and peroxidases stand out, possessing oxidoreductase matrices with high redox potential, making them highly effective in lignin deconstruction (Asemoloye et al., 2021).

One of the great difficulties in reusing lignocellulosic biomass waste is due to the lignin component, an aromatic polymer and one of the main components of plant biomass. Lignin is mainly composed of p-coumaryl (H), guaiacyl (G) and syringyl (S) monomers and is connected by β-O-4, β-5 and β-β bonds (Asemoloye et al., 2021). However, these strong bonds can be broken through enzymes produced by fungi, which due to their high adaptability to organic matter in natural environments have developed the ability to produce degrading biocatalysts, such as ligninolytic enzymes, also known as lignin-modifying enzymes (LME). Lacases and peroxidases make up this group of enzymes and present oxidoreductase matrices with intense redox potential (Suryadi et al., 2022).

LMEs can be associated with both structural modifications and the degradation of lignin into less complex compounds. These enzymes are divided into two groups: heme-peroxidase and phenoloxidase. In the first group, the main representatives are lignin peroxidase (LiP) and manganese peroxidase (MnP), while the second group includes laccases (Lac). According to the Carbohydrate Active Enzyme Database (CAZy), laccases are classified in the multicopper oxidase superfamily (AA1), and lignin and manganese peroxidases are included in the auxiliary activity family (AA2) (CAZy; http://www.cazy.org/). The three enzymes make up a system that can deconstruct a lignin effectively[23]. In addition, they are enzymes capable of degrading various xenobiotics, including dyes, chlorophenols, polycyclic aromatic hydrocarbons (PAHs), organophosphorus compounds and phenols (Wesenberg et al., 2003).

Fungi associated with leaf litter stands out for producing this complex ligninolytic enzyme system, which catalyzes non-specific reactions that lead to the depolymerization of lignin. The genes for these enzymes are constantly reported in white and brown rot fungi present in dead plant material (Lombard et al., 2014), such as litter and tree trunks. Among the 30 genera of fungi with the highest abundance of species identified in this review in leaf litter, only 10 have records in the literature that prove ligninolytic enzyme activity. The other genera have been reported to occur in this environment, but without confirmation of the production of these LMEs. The genera *Dictyosporium* and *Stachybotrys* (Castro et al., 2012; Yilmaz et al., 2016; Santos et al., 2018; Vasco-Palacios et al., 2018; Monteiro et al., 2019; De Queiroz et al., 2021) have already been described as potential producers of ligninolytic enzymes (Sin et al., 2002; Singh et al., 2014; Duong et al., 2022). On the other hand, for the genera *Ellisembia*, *Sporidesmium*, *Zygosporium*, *Chloridium* and *Helicosporium*, there are no studies that correlate their species with the production of these enzymes. Specifically, the genus *Chloridium* is described as a soft rot fungus, characterized by secondary colonization of the substrate after the degradation of lignin (Gams et al., 1976).

3.3 General principles of LMES functioning

3.3.1 Lignin peroxidase (LiP)

Lignin peroxidase (LiP EC1.11.1.14) of the Class II peroxidase-catalase superfamily, as well as manganese peroxidase (MnP) and versatile peroxidase (PV), which are peroxidases of fungal or bacterial origin, may be related to the degradation of lignin in the presence of hydrogen peroxide as an electron acceptor. In general, during lignin degradation, LiP targets non-phenolic components, while PV and MnP act to oxidize phenolic structures. These enzymes are H_2O_2-dependent, have high redox potential and acidic pH optima, and can oxidize a wide variety of aromatic compounds and non-phenolic structures that make up 90% of lignin (Riley et al., 2014).

LiP has the distinction of being able to oxidize methoxylated aromatic rings without a free phenolic group, which results in cation radicals capable of reacting via a variety of pathways, including ring opening, demethylation and phenol dimerization. In contrast to Lac, LiP does not need mediators to degrade compounds with high redox potential, but it does need H_2O_2 to initiate catalysis (Falade et al., 2017). The reactions catalyzed by LiP mainly result in the breaking of Cα-Cβ bonds, opening of aromatic rings, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, oxidation of phenol to produce free radicals, hydroxylation of methylene to specific groups and cleavage of phenylglycol (Vandana et al., 2019).

In most fungi, LiP is present as a series of isoenzymes encoded by different genes, which have molecular weights ranging from 37 to 47 kDa, ideal enzyme activity temperatures of 35 to 55 °C, with ideal pH values of 3.0 to 4.5 (Singh et al., 2021; Zhao et al., 2023) found that the fungus *Clonostachys compactiuscula*, a species identified in leaf litter from the Amapá Forest by Monteiro et al. 2019, had LME activities, including LiP, with greater stability at acidic pH (equal to 5) and a temperature of 30 °C.

The main inducer to produce this enzyme, which has been widely reported in the literature, is veratryl alcohol, which increases enzyme activity compared to other substrates (Zhao et al., 2023). Meanwhile, compounds such as acetone, dioxane, diethyl ether, acetonitrile, dimethylformamide, cationic surfactant, cetyltrimethylammonium bromide and H_2O_2 in high concentrations act as inhibitors, and this inhibition has been reported in different fungi (Parshetti et al., 2012).

3.3.2 Manganese peroxidase (MnP)

The manganese peroxidase enzyme (MnP - EC 1.11.1.13) is an H_2O_2-dependent extracellular glycoprotein that requires Mn^{2+} to oxidize monoaromatic phenols and aromatic dyes. This protein has a molecular mass of between 38 and 62.5 kDa. The activity of MnP has already been described for various fungi of the phylum Basidiomycota, such as those belonging to the orders Agaricales, Cortiales, Polyporales and Hymenochaetales of the class Agaricomycetes (Manavalan et al., 2015), being species of the order Agaricales. Some genera and species identified in the leaf litter of the phylum show positive activity for this enzyme, such as *Trametes flavida*, *Trametes* sp. (De Sousa et al., 2024), and in some species of *Lactarius* sp. (Morgenstern et al., 2010), *Lactifluus* sp. (Zhao et al., 2021), *Lentinula* sp. (Ematou et al., 2020). In addition, there are records of MnP activity in fungi of the phylum Ascomycota, such as *Clonostachys compactiuscula*, *Dictyosporium* and *Stachybotrys* (Sousa et al., 2019; Shi et al., 2021; Yang et al., 2016).

However, some factors can inhibit the production of this enzyme in microorganisms, such as cultivation methods, temperature, pH, metal ions, among others (Suryadi et al., 2022). Some species show better production in solid cultures, in the presence of minimal humidity to favor the growth of the microorganism, as in the case of the isolate *Echinodontium taxodii* 2538 (Kong et al., 2016). About temperature and pH, the ideal range is 20 to 50 ºC and an acidic pH of 3.5 to 5 (Suryadi et al., 2022).

3.3.3 Laccase (Lac)

Among the lignin-modifying enzymes, laccase (Lac - EC 1.10.3.2) is one of the most studied due to its wide applicability and stability. Lac is an oxidoreductase enzyme characterized by the presence of four copper atoms in its catalytic site and is classified as a multicopper enzyme of the extracellular monomeric glycoprotein family. Lac's active site is composed of: Cu type 1 (T1), Cu type 2 (T2) and Cu type 3 (T3). T1 is a mononuclear site, and types T2 and T3 form a trinuclear cluster. The molecular weight of this enzyme varies between 50 and 130 kDa, being higher than LiP and MnP (Debnath e Saha, 2020).

In fungi, Lac not only plays a role in their metabolism, due to its role in the delignification of lignocellulosic material, but also contributes to the process of sporulation, pigment production, basidiocarp formation and plant pathogenesis (Singh e Gupta, 2020). Laccase is one of the main enzymes identified in litter species, especially in basidiomycete fungi. As an example, among the genera identified in litter from the Amazon rainforest (Singer e Araújo, 1979; Braga-Neto et al., 2008; Vasco-Palacios et al., 2018; Gates et al., 2021), include

Coltricia, *Craterellus*, *Deconica*, *Lactarius*, *Lactifluus* (Ng et al., 2004; Morgenstern et al., 2010; KALYANI, et al., 2012; Martani et al., 2017; Zhao et al., 2021; Gupta et al., 2025).

The ideal conditions reported for this enzymatic activity occur in the temperature range of 25 to 50 °C, in media with a pH varying from 3.5 to 6 (Suryadi et al., 2022; Neto et al., 2022). As well as the LiP and MnP peroxidases, there are agents that induce Lac production. The main inducers are lignin-derived compounds such as 2,5-xylidine, lignin and veratryl alcohol, which are known to increase and induce Lac activity (Litwińska et al., 2019).

The use of synthetic mediators can also favor an increase in Lac activity, because in the presence of acetosyringone and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), an increase in Lac activity has already been observed (Neto et al., 2022). However, some metal ions can decrease the activity of this enzyme, such as Hg^{+2}, which inhibited *Chalara paradoxa* CH32 (Robles et al., 2002). In the same study, reducing agents such as EDTA, potassium cyanide and sodium azide were also shown to inhibit the activity and production of the enzyme. However, in the presence of some organic solvents, such as methanol, ethanol and isopropyl alcohol, enzyme activity was stimulated, with an increase in enzyme production. The species has already been identified in the litter of primary and palm-dominated forests in the Amazon (Monteiro et al., 2019; De Queiroz et al., 2021).

3.4 Biodegradation routes of waste by ligninolytic fungus and value-added molecules generated

In the degradation process of dyes and agro-industrial waste, fungi producing LiP, MnP and Lac transform these complex compounds into simpler, less polluting molecules. Enzymes catalyze oxidation reactions that result in the breaking of the chemical bonds of organic molecules, allowing them to be converted into degradation products. The degradation products can have a variety of properties and potential applications, such as the production of substrates in industrial processes, or even the development of new materials and products with added value. The molecules resulting from this process can be used to optimize biodegradation and waste recovery strategies, contributing to environmental sustainability and the circular economy.

3.4.1 Synthetic dyes as a degradation substrate

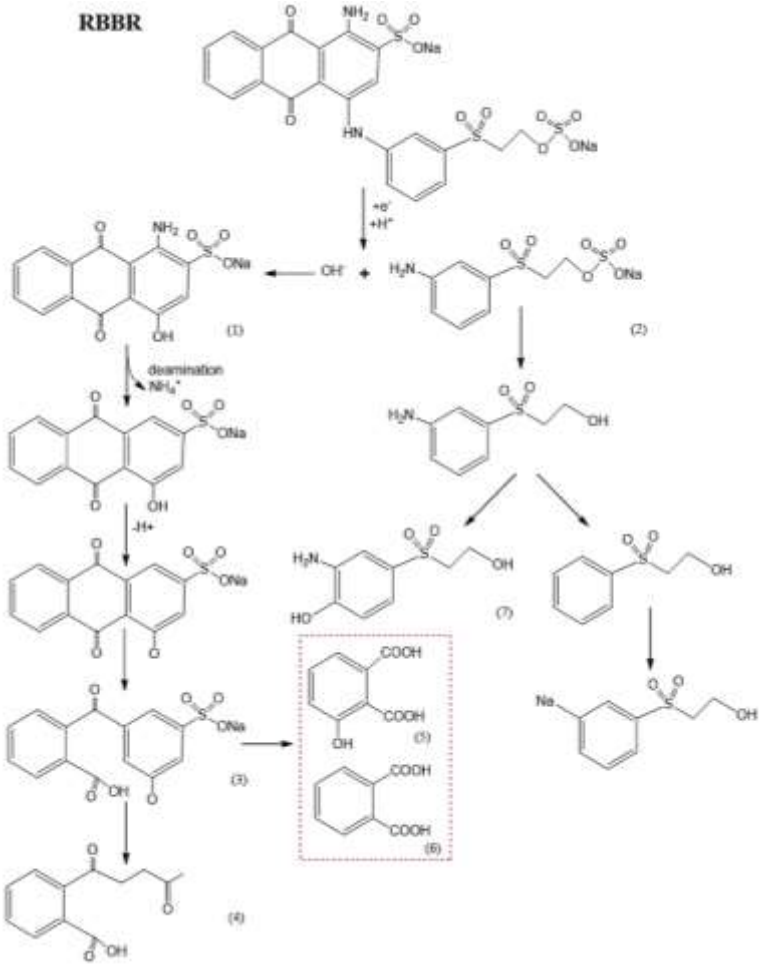
Studies have reported species of genera identified in leaf litter as sustainable and economical alternatives for removing organic dyes from wastewater before they are disposed of. Species of *Crepidotus*, *Dictyosporium* and *Trametes* are described as potential biodegraders of synthetic dyes (Mtui, 2007; Yang et al., 2016; De Sousa et al., 2024) and can be isolated from litter in terra firme forests, native forests with a predominance of palm trees, cedar plantations

and riparian forests (Santos et al., 2018; Monteiro et al., 2019; De Queiroz et al., 2021; Gates et al., 202; De Sousa et al., 2024).

Crepidotus variabilis in a study carried out for the biodegradation of Azure-B, Poly-B and Poly-R dyes and raw wastewater, revealed a decolorization capacity of 84, 86 and 92% for the dyes and 54% for raw wastewater (Mtui, 2007). The *Dictyosporium zhejiangensis* species is also capable of biodegrading dyes through its enzymatic production, being able to decolorize 11 dyes after treatment for 7 days at rates of 44.78 to 77.87% for strain Sy06 and 46 to 77.41% for strain H-6 (Yang et al., 2016). And isolates of Trametes spp. from riparian forest litter were able to decolorize the azo dye RBBR by up to 89.28% (De Sousa et al., 2024).

The proposed enzymatic degradation pathway for RBBR reveals that the biodegradation of dyes by fungal enzymes such as Lac and LiP promotes not only the remotion of the dye in wastewater, but also the detoxification of the waste by converting it into less toxic molecules (Figure 2). The enzymatic action, primarily by laccase, promotes the excision of azo dyes, forming a reaction center deficient in electrons. This action generates highly reactive intermediates that are nucleophilically attacked (-OH, -SO, or halogen ions), leading to an asymmetric cleavage of the azo bond (Ardila-Leal et al. 2021). After this step, successive deamination, hydroxylation, and oxidation occur, causing the opening of the final ring, as shown by the products (3) and (4) in Figure 2 (Bilal et al., 2017). Among the main products accumulated at the end of the degradation of these anthraquinone dyes by fungal enzymes is phthalic acid (molecule (5) in Figure 2), which can be considered a less toxic molecule, reducing the toxicity of residues containing these dyes and providing a sustainable alternative for their disposal (Bucchieri et al., 2024).

Figure 2. Proposed routes for biodegradation of azo dyes Remazol Brilliant Blue R (RBBR), by laccase (Lac) and lignin peroxidase (LiP) produced by fungi.



Source: Adapted of Ardila-Leal et al., 2021.

3.4.2 Lignocellulosic waste as degradation substrate

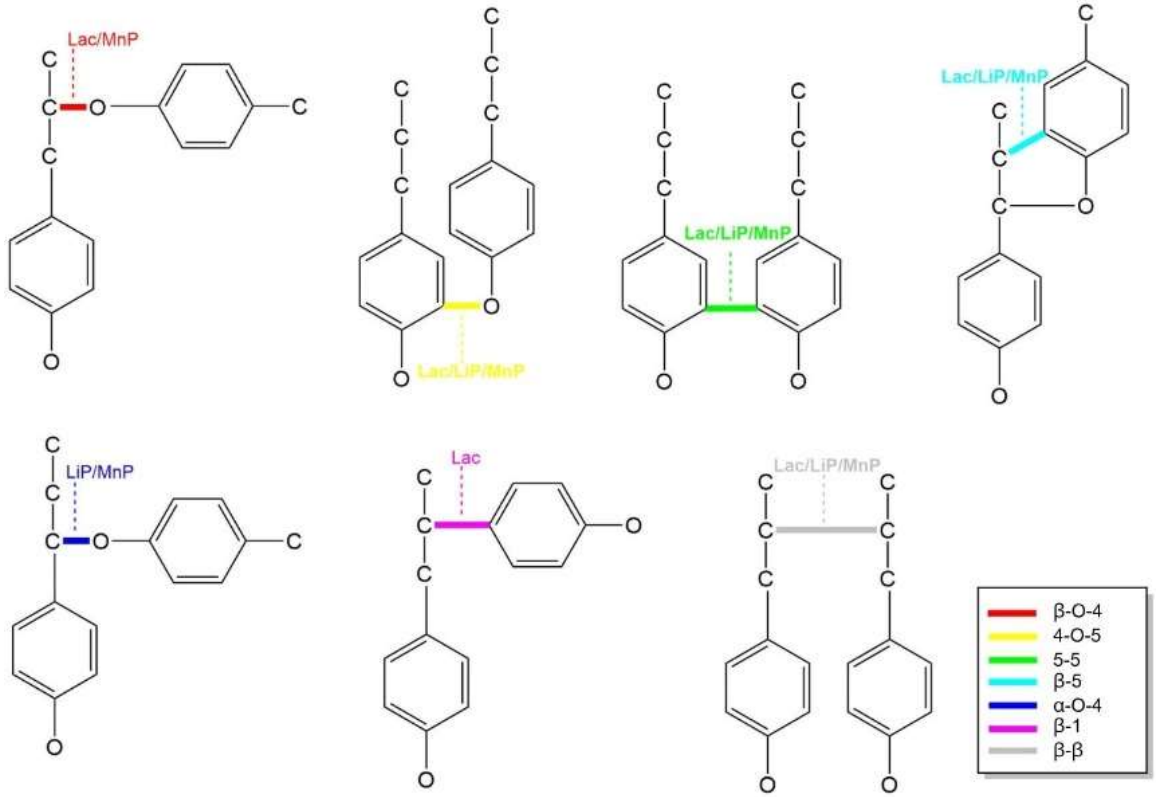
Lignocellulosic waste from agro-industrial residues can be effectively degraded by extracellular ligninolytic enzymes, which initiate the process by breaking key bonds in lignin, such as the β-O-4 ether (β-aryl ether) and β-β (biphenyl) bonds. This enzymatic action generates free cationic radicals that undergo further chemical reactions like hydroxylation and C–C bond cleavage, resulting in hydrophilic degradation products (Asemoloye et al., 2021). As lignin is fragmented, it releases lignin monomers such as ρ-coumaryl, coniferyl, and sinapyl alcohols, which are precursors to the phenylpropanoid polymers p-hydroxyphenyl, guaiacyl, and syringyl. This breakdown facilitates the release of cellulose and hemicellulose, which are further hydrolyzed by specific enzymes into monomeric sugars such as xylose, mannose, galactose, rhamnose, and arabinose.

Different ligninolytic enzymes exhibit varying specificities for bond cleavage. For instance, lignin peroxidase (LiP) is particularly effective at breaking Cα–Cβ bonds in phenolic

structures but has significantly lower activity on β-O-4 bonds (Li et al., 2022). Manganese peroxidase (MnP) also acts on Cα–Cβ bonds and can catalyze reactions involving β-1 and β-O-4 phenolic substructures (Adriani et al., 2020). Laccase (Lac) can degrade β-1 and β-O-4 bonds in the presence of oxygen and phenolic compounds (Pype et al., 2019) (Figure 3).

Enzymatic depolymerization of lignin not only supports biomass deconstruction but also enables the production of valuable aromatic compounds. These include gallic acid and 2-pyrone-4,6-dicarboxylic acid from sinapyl alcohol, ferulic acid and its derivatives vanillic acid and 4-hydroxybenzoate from coniferyl alcohol, and protocatechuic acid from ρ-coumaryl alcohol (Higuchi, 2004; Pype et al., 2019; Li et al., 2022).

Figure 3. Representation of the different types of lignin molecular bonds and the main enzymes involved in breaking them.



Source: Adapted from Datta et al., 2017.

3.5 By-products of biodegradation

Ligninolytic fungi play a significant role in the degradation of both synthetic compounds, such as dyes, and organic materials, like agricultural waste, leading to the generation of various bioactive molecules. Aounallah et al. 2024 demonstrated that enzymatic treatment of the Congo Red azo dye using *Geotrichum candidum* resulted in a decolorization efficiency of up to 85.4 ± 2.6%, along with the production of metabolites that were less phytotoxic to the shoot and root growth of *Lactuca sativa* and *Solanum lycopersicum*. Similar findings from other studies confirm that dye degradation mediated by fungal ligninolytic

enzymes (LMEs) produces metabolites with reduced toxicity to plants and aquatic organisms, including zebrafish larvae and *Daphnia magna* (Przystś et al., 2013; Zouari-Mechichi et al., 2024). These results underscore the environmental benefits of fungal LMEs in reducing pollutant toxicity and promoting sustainable waste management.

Beyond detoxification, lignin degradation also yields valuable phenolic compounds with diverse industrial applications. For instance, vanillin derived from the cleavage of coniferyl alcohol is widely used in the food and cosmetics industries (Przystś et al., 2013). Gallic acid, another byproduct, has applications in food packaging, where it enhances antioxidant activity and extends shelf life (Beckham et al., 2016). Additionally, protocatechuic acid, formed from ρ-coumaryl alcohol, exhibits pharmacological properties including antioxidant, anti-inflammatory, neuroprotective, anti-tumor, and organ-protective effects (Sharma et al., 2022). These multifunctional compounds highlight the potential of ligninolytic processes not only in environmental remediation but also in the development of value-added bioproducts for various industries.

Recent studies have demonstrated the effectiveness of fungal consortia in lignin biodegradation from agricultural residues. For example, the combined use of *Lenizites betulina* and *Trametes versicolor* on corn stalk-derived lignin led to a 50% lignin reduction over 17 days, with increased enzymatic activity of laccase (Lac) and manganese peroxidase (MnP). The degradation process produced several valuable compounds, including substituted aromatics, small molecule acids, and aliphatic acids, such as 4-methylcinnamic acid, p-hydroxybenzoic acid, benzoic acid, oxalic acid, succinic acid, maleic acid, and adipic acid (Song et al., 2020).

Similar degradation products were identified when lignin in bamboo waste was treated with *Phanerochaete chrysosporium* and *Pleurotus ostreatus*, yielding organic acids, esters, and aromatic substances like benzoic acid, propanoic acid, 3,5-dimethylphenol, and ethyl alcohol (Cui et al., 2021). Many of these organic acids have industrial value, particularly in the food and beverage sector, where they are used as acidulants to lower pH, inhibit microbial growth, and extend shelf life (Jin et al., 2021).

Fungal degradation of lignocellulosic waste also enables the recovery of high-value aromatic compounds. Vanillin (4-hydroxy-3-methoxybenzaldehyde), a product of coniferyl alcohol degradation, is widely used in the food and cosmetics industries. A notable yield of vanillin (162 µg/mL) was obtained after 96 hours of fungal transformation of black liquor from *Aleppo pinecones* (Zhao et al., 2022; Chilakamarry et al., 2022). Another valuable compound, gallic acid—derived from sinapyl alcohol—can be extracted from grape pomace treated with fungi like *Rhizopus oryzae*, *Ganoderma* spp., *Phanerochaete chrysosporium*, and *Trametes*

gibbosa. The highest gallic acid yield reached 586.43 ± 12.48 µg/g after three days using *R. oryzae* (Šelo et al., 2022). Gallic acid is used to enhance antioxidant properties in food packaging and shows promise in drug development due to its selective apoptotic effect on cancer cells without harming healthy cells (Messaoudi et al., 2019; AL Zahrani et al., 2020).

Additionally, protocatechuic acid, a product of ρ-coumaryl alcohol degradation, has been recovered in high quantities—up to 699.30 ± 20.78 µg/g—through fermentation of grape pomace with *Humicola grisea* (Messaoudi et al., 2019). This compound has multiple pharmacological benefits, including antioxidant, anti-inflammatory, neuroprotective, anti-tumor, anti-osteoporotic, and organ-protective properties (Šelo et al., 2022).

4. Conclusions

The ability of fungi to degrade leaf litter highlights their potential for the production of ligninolytic enzymes (LMEs), especially in the generation of value-added molecules during the breakdown of lignin. This review shows that leaf litter is an important natural source for isolating fungi that produce biocatalytic enzymes that can be used to reuse and treat chemical and organic waste. However, there is still a need to investigate other promising species isolated from this material. This study has some limitations, such as the limited number of fungal strains evaluated and the lack of in-depth enzymatic characterization under industrial conditions. Future research should prioritize expanding the screening of different taxa of fungi obtained from leaf litter, optimizing the production of enzymes under industrial-scale conditions, and assessing the environmental impact and economic viability of using agro-industrial waste as a substrate. Progress in this field strengthens industrial biotechnology, sustainable alternatives for organic waste management, and the development of greener technologies and practices associated with the circular economy.

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3.2 CAPÍTULO II

Título: 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

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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

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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 biodegradação 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 Hobie, 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 (Ashan et al., 2021), such as Remazol Brilliant Blue Reactive (RBBR). This reactive anthraquinonic dye is composed by anthracene derivatives, a polycyclic aromatic 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).

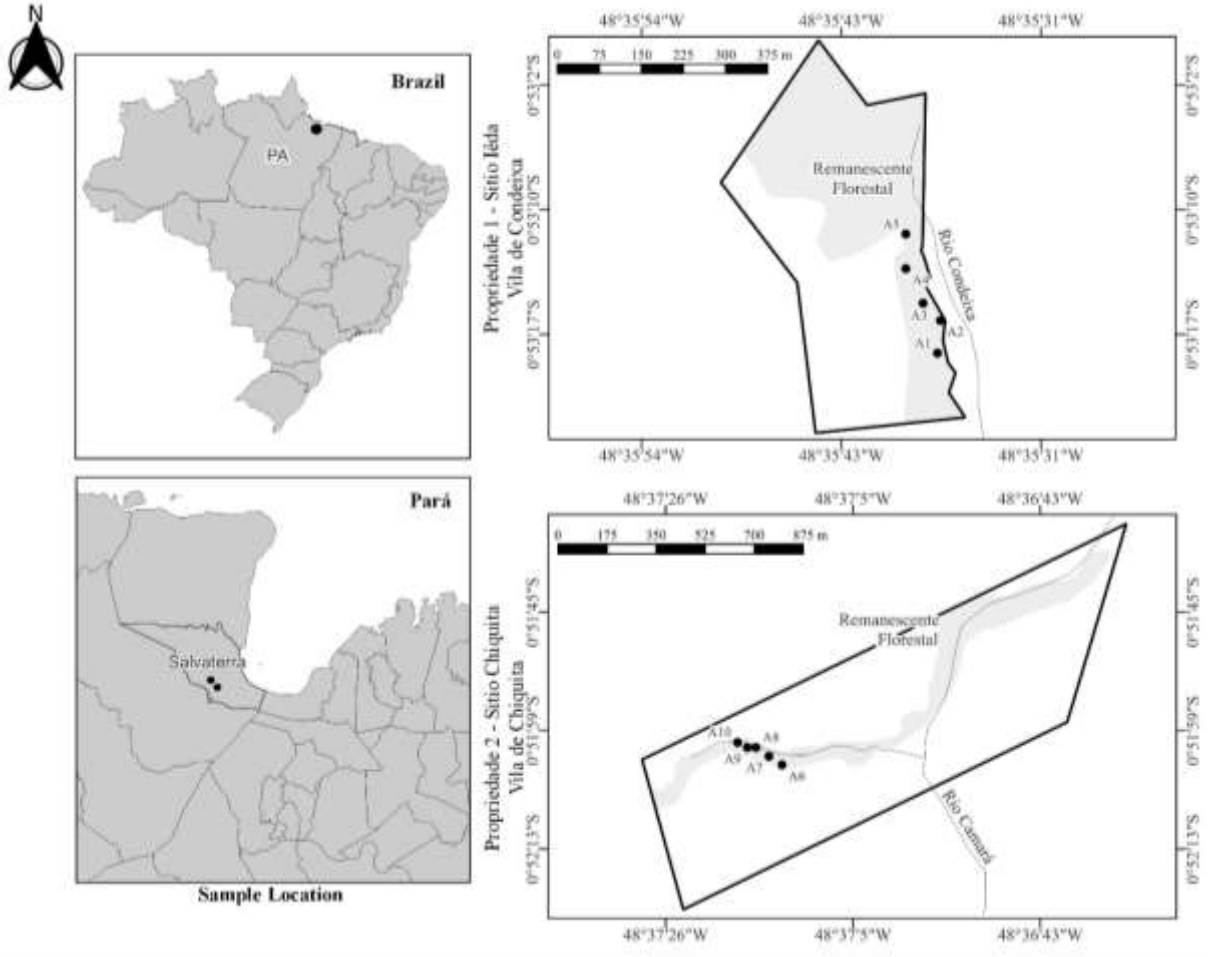
Considering the potential of fungi to act in the decolorization of toxic synthetic dyes, the purpose of this study was to evaluate the enzymatic activity of laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP), as well as the decolorization potential of the RBBR dye in liquid fermentation 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 m² (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 – UFPA.

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



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 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 (Large 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 below (Monssef et al., 2016).

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

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 below (Khan et al., 2023).

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

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% m/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).

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).

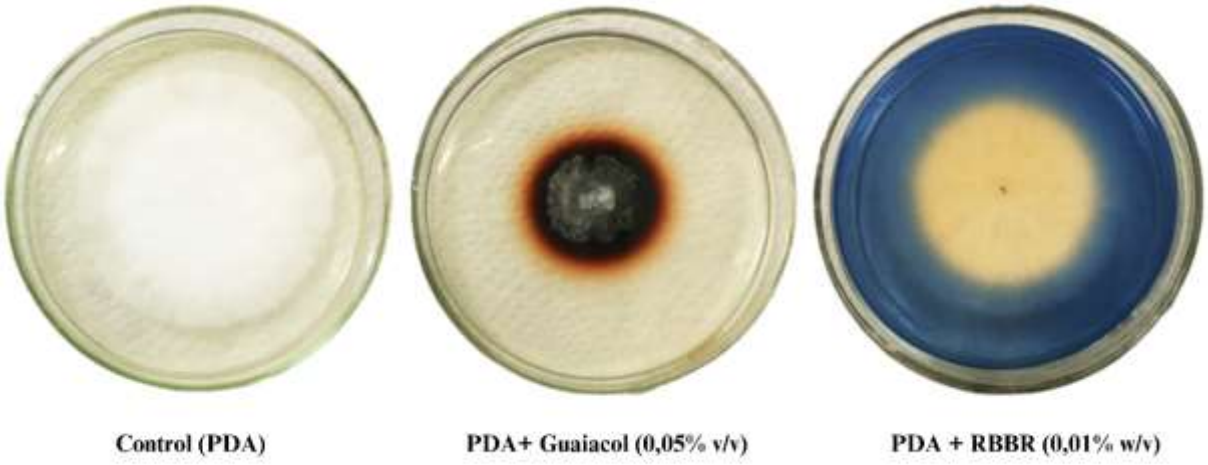
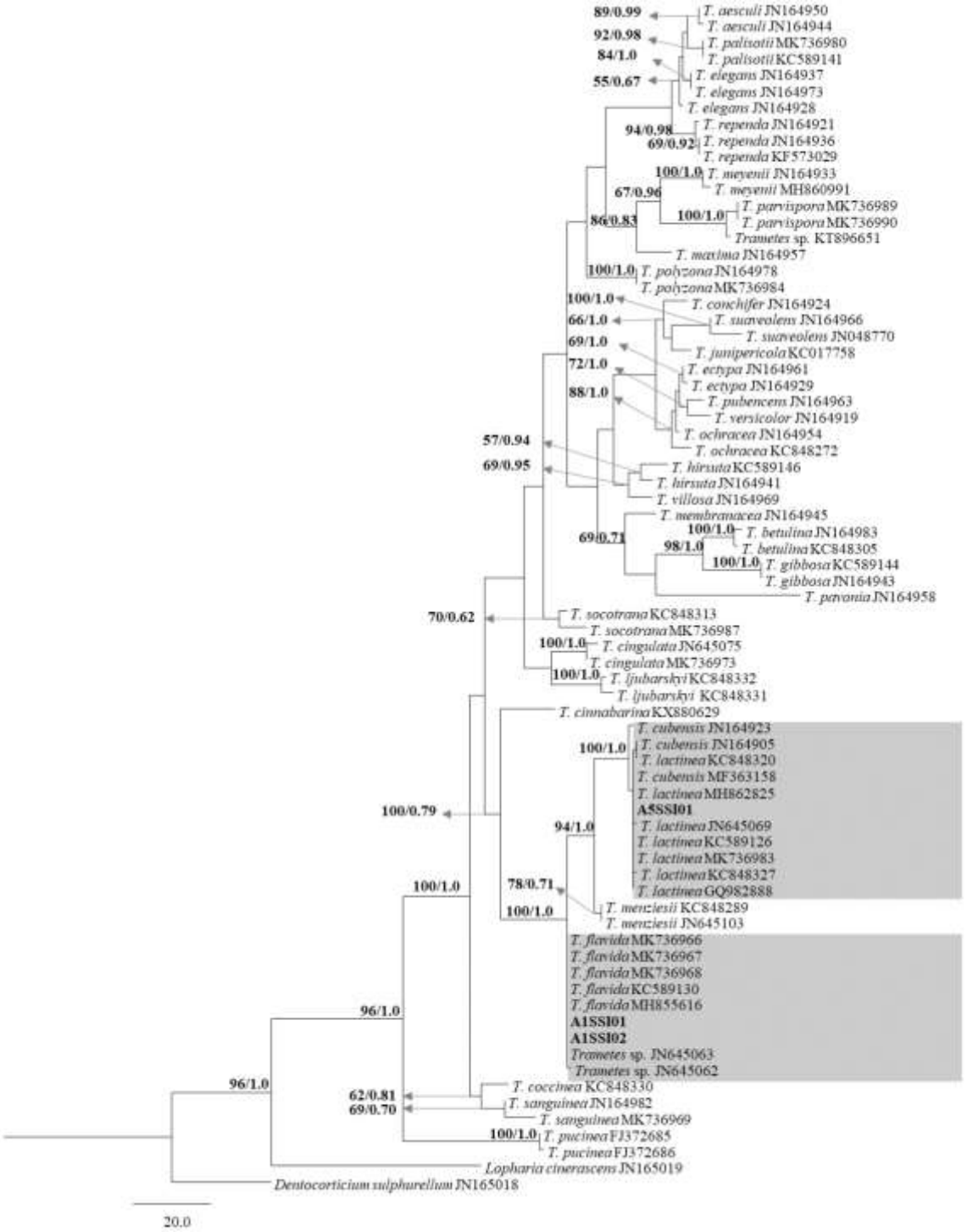


Figure 3. Phylogenetic tree based on MP and Bayesian analysis of ITS data. Bootstrap values (>55%) for PM analysis and PP values (>0.67) for Bayesian inference are indicated near branches (MP/PP). The bold isolates were sequenced in this study. *Dentocorticium sulphurellum* and *Lopharia cinerascens*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



The 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).

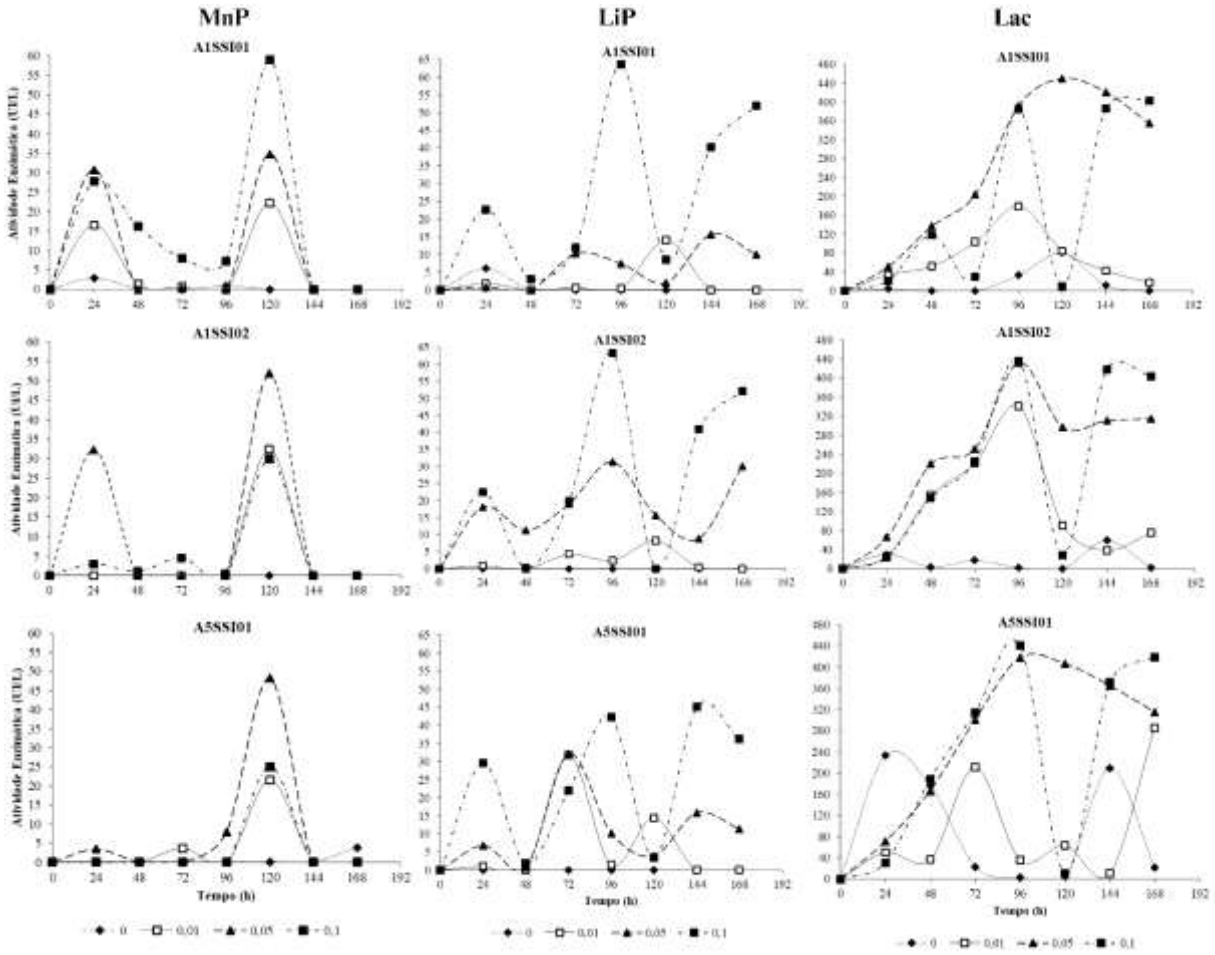
3.1 Enzymatic activity

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 $UI.mL^{-1}$ 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 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 $UI.mL^{-1}$, followed by A1SSI02 and A5SSI01 at 0.05% (51.89 and 48.29 $IU.mL^{-1}$, respectively) at 120h (day 5) after fermentation (Figure 4).

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.

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 colonies in this study, with activities oscillating between 42.25 and 63.64 UI.mL^{-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.mL^{-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.mL^{-1}), which was statistically equal to the activity of the A5SSI01 colony at 0.1% RBBR concentration (4th day; 440.63

UI.mL^{-1}) and A1SSI02 at 0.05 and 0.1% concentrations (432.43 and 435.60 UI.mL^{-1}), in both cases 96h (4th day) after the start of fermentation.

3.2 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).

Tabela 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.

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 (Dos Santos et al., 2018 and Monteiro et al., 2019).

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 and 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 and 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 ($\geq$ 57% for MP and $\geq$ 0.62 for MB) were similar to those found in other studies based on these sequences (Justo and Hibbett, 2011; Welti et al., 2012 and 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 et al., 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 for the production of 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

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Supplementary Material

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

Tabela 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.

Species	Isolate identification	Location	GenBank accession number
Dentocorticium sulphurellum	FP11801	USA	JN165018
Lopharia cinerascens	FP105043sp	USA	JN165019
T. aesculi	HHB4626sp	USA	JN164950
	FP105679sp	USA	JN164944
T. betulina	HHB9942sp	USA	JN164983
	Dai6847	China	KC848305
T. cingulata	MUCL:40167	Malawi	JN645075
	OAB0135	Benin	MK736973
T. cinnabarina	Dai 14386	China	KX880629
T. coccinea	Cui7096	China	KC848330
T. conchifer	FP106793sp	USA	JN164924
T. cubensis	TJV93_213sp	USA	JN164923
	AJ177	USA	JN164905
	UZ526_17	Malaysia	MF363158
T. ectypa	FP103976sp	USA	JN164961
	FP106037T	USA	JN164929
T. elegans	PR1133	Puerto Rico	JN164937
	FPRI10	Philippines	JN164973
	FP150762	Belize	JN164928
T. flavida	OAB0047	Benin	MK736966
	OAB0090	Benin	MK736967
	OAB0196	Benin	MK736968
	DMC811	Cameroon	KC589130
	CBS 158.35		MH855616
T. flavida	**A1SSI01**	**Brazil**	**OR888930**
	A1SSI02	**Brazil**	**OR888931**
T. gibbosa	DMC815	Cameroon	KC589144
	L11664sp	England	JN164943
T. hirsuta	DMC341	Cameroon	KC589146
	RLG5133T	USA	JN164941
T. junipericola	145295(O)		KC017758
T. lactinea	DMC346	Cameroon	KC589126
	CBS 109427	Taiwan	MH862825
	LIP:GUY09-110	French Guiana	JN645069
	Dai6865		KC848327
	OAB0232	Benin	MK736983
	BCC 33266	Thailand	GQ982888
	Yuan5493		KC848320
T. ljubarskyi	Wei1653		KC848332
	Li286		KC848331
T. maxima	OH189sp	Venezuela	JN164957

Species	Isolate identification	Location	GenBank accession number
T. membranacea	PRSC82	Puerto Rico	JN164945
T. menziesii	BRFM<FRA>:1368	Martinique	JN645103
	Dai6782		KC848289
T. meyenii		Philippines	JN164933
	CBS:453.76	India	MH860991
T. ochracea	HHB13445sp	USA	JN164954
	Dai2005	China	KC848272
T. palisotii	OAB0118	Benin	MK736980
	DMC816	Cameroon	KC589141
T. parvispora	OAB0022	Benin	MK736989
	OAB0023	Benin	MK736990
T. pavonia	FP103050sp	USA	JN164958
T. polyzona	BKW004	Ghana	JN164978
	OAB0092	Benin	MK736984
T. pubencens	FP101414sp	USA	JN164963
T. pucinea	BCC26408	Thailand	FJ372685
	BCC27595		FJ372686
T. rependa	FPRI390	Philippines	JN164921
	OH271sp	Venezuela	JN164936
	M0138339	Papua New Guinea	KF573029
T. sanguinea	OAB0088	Benin	MK736969
	PRSC95	Puerto Rico	JN164982
T. socotrana	BJFC12724	China	KC848313
	OAB0131	Benin	MK736987
T. suaveolens	FP102529sp	USA	JN164966
	Dai 10729	China	JN048770
T. versicolor	FP135156sp	USA	JN164919
T. villosa	FP71974R	USA	JN164969
Trametes sp.	LIP:GUY08-156	French Guiana	JN645062
	BC1	Finland	KT896651
	LIP:GUY08-167	French Guiana	JN645063
Trametes sp.	**A5SSI01**	**Brazil**	**OR888960**

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

Species	ITS1/5.8 S/ITS4																
	11	**34**	**103**	**104**	**117**	**118**	**119**	**120**	**146**	**155**	**156**	**174**	**291**	**381**	**383**	**384**	**412**
T. flavida **A1SSI01**	C	T	C	G	C	G	A	G	C	T	A	C	A	T	A	G	G
T. flavida **A1SSI02**	C	T	C	G	C	G	A	G	C	T	A	C	A	T	A	G	G
T. flavida KC589130	C	T	C	G	C	G	A	G	C	T	A	C	A	T	A	G	G
T. flavida MH855616	C	T	C	G	C	G	A	G	C	T	A	C	A	T	A	G	G
T. flavida MK736966	C	T	C	G	C	G	A	G	C	T	A	C	A	T	A	G	G
Trametes sp. **A5SSI01**	T	C	T	C	G	A	G	A	T	C	G	C	A	C	G	A	A
T. cubensis JN164905	T	C	T	C	G	A	G	A	T	C	G	C	A	C	G	A	A
T. cubensis JN164923	T	C	T	C	G	A	G	A	T	C	G	**T**	A	C	G	A	A
T. cubensis MF363158	T	C	T	C	G	A	G	A	T	C	**A**	C	A	C	G	A	A
T. lactinea GQ982888	T	C	T	C	G	A	G	A	T	C	G	C	A	C	G	A	A
T. lactinea JN645069	T	C	T	C	G	A	G	A	T	C	G	C	**G**	C	G	A	A
T. lactinea KC589126	T	C	T	C	G	A	G	A	T	C	**A**	C	A	C	G	A	A
T. lactinea KC848327	T	C	T	C	G	A	G	A	T	C	G	C	A	C	G	A	A
T. lactinea MH862825	T	C	T	C	G	A	G	A	T	C	G	C	A	C	G	A	A
T. lactinea MK736983	T	C	T	C	G	A	G	A	T	**T**	G	C	A	C	G	A	A

Note: The isolates identified in this study are in bold, and the unique nps of each species are highlighted.

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3.3 CAPÍTULO III

Título: Açaí seeds valorization as a substrate for the production of ligninolytic enzymes by Amazonian litter fungi

Valorização do caroço de açaí como substrato para a produção de enzimas ligninolíticas por fungos da serapilheira amazônica

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ABSTRACT
The objective of this research is to analyze the potential use of açaí seeds for the production of ligninolytic enzymes (LMEs) by fungi isolated from the leaf litter of Amazonian riparian forests. The study employed both solid-state fermentation (SSF) and submerged fermentation (SmF) to evaluate the enzymatic activity of the isolates. Results indicated that SSF favored the production of laccase (Lac) in Trametes spp. isolates, while Syncephalastrum sympodiale exhibited activity exclusively for manganese peroxidase (MnP) and lignin peroxidase (LiP). Among the isolates, A1SSI02 achieved the highest reduction in residual biomass (39.8%) under SSF conditions and the greatest decrease in lignin content of the degraded seed (24.3%). Microscopic analysis revealed structural alterations in the cell walls of the açaí seeds, attributed to the enzymatic action of the fungi. FTIR spectroscopy confirmed lignin degradation, showing changes in the functional groups of the substrate. These findings suggest that açaí seeds could serve as a sustainable substrate for LME production, offering a promising approach for managing this agro-industrial residue and unlocking potential biotechnological applications-particularly in light of the high Lac production observed in SSF with Trametes isolates. Additionally, this study reports the first occurrence of S. sympodiale in Amazonian leaf litter.

Keywords: Biodegradation, Ligninolytic Fungi, Agro-industrial Waste, Lignin Degradation, *Trametes* spp. and *Syncephalastrum sympodiale*

1. Introduction

Euterpe oleracea Mart. (açaí) is a native tropical palm that is distributed widely in the Amazon region. There are approximately 28 species in the *Euterpe* genus, including the *E. edulis* and *E. oleracea* species, highlighting the state of Pará as the main producer of the crop in Brazil [1,2]. Regarding productivity, data for the year 2023 indicates that the state achieved a production of 168 mil tons of fruit, which is equivalent to 70% of national production, with an income of R$ 6.5 billion for the Pará economy [2].

Nevertheless, in the processing of açaí fruit, only 15 to 20% is transformed into pulp, while the remaining 80 to 85% is made up of residual seed and fiber material.3 The high quantity of this waste represents an environmental burden for processors, since a large part of the plant material is converted into a by-product. A factor that worsens this production is the irregular disposal of the residual seed, causing environmental impacts [3,5], primarily in Belém metropolitan region.

The recycling of these residual fractions is important for the economy and sustainability of these açaí industries. In the past few years, studies have focused on alternatives to explore and produce value-added products from agro-industrial waste, especially food waste, which is widely generated and is a biodegradable source [3,6]. Although the composition of these residues varies, some exhibit significant potential for utilization. The açaí seed, for instance, comprises approximately 36% cellulose and 48% lignin, making it a promising raw material for diverse applications [7].

However, the main technological challenge in reusing this waste, especially cellulose, is the delignification stage [8]. Lignin, an aromatic polymer composed of phenylpropanoid subunits, forms a rigid matrix around crystalline cellulose microfibrils, due to its ether and carbon-carbon bonds which are difficult to degrade [9]. As an efficient, environmentally friendly and low-cost alternative, biological degradation processes involving fungi and bacteria stand out for their high selectivity and lower energy consumption, and are considered effective strategies for breaking down lignin and biotransforming these materials through the production of lignin-modifying enzymes (LMEs) [10-12].

The main enzymes released in this process and responsible for lignin degradation are manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Lac) [13]. Promising results have already been reported in previous studies using fungi to valorize waste through the production of enzymatic extracts and delignified biomass. For example, an isolate of *Inonotus* sp. was able to produce magnesia peroxidase (MnP) and lignin peroxidase (LiP) in corn cob and green tea waste, with respectively equal activities of MnP 18.5 $U.g^{-1}$ for tea, and 31.3 $U.g^{-}$

^{1} for corncob, and LiP of 2.4 and 2.5 U/g [14]. Meanwhile, the fermentation of rice straw, wheat straw, and corn stalks with *Cerrena unicolor* GC.u01 resulted in a lignin reduction of 24–34%, while laccase (Lac) activity reached peak values of up to 8.39 $U \cdot mL^{-1}$ [15].

Under these circumstances, the leaf litter in nature can represent a potential material for isolating fungi for isolating fungi involved in lignin degradation, since it harbors saprophytic fungi that produce ligninolytic enzymes, which are mainly responsible for breaking down the lignocellulosic matrix in this environment. Fungal screening has been strategy identifying species with a high capacity for producing these enzymes and, consequently, for degrading lignin [16]. Furthermore, the increase in the generation of agricultural waste reinforces the need for alternative routes for its conversion into by-products [17]. In view of this, this study aimed to select fungal isolates from leaf litter with the potential to produce ligninolytic enzymes and act in the deconstruction of the lignocellulosic matrix of the açaí seed.

2. Material and methods

2.1 Fungi isolates

There were four fungal isolates used in this study: A1SSI01, A1SSI02, A5SSI01 and A5SSI02. Colonies were isolated from samples of leaf litter, as described by De Sousa et al [18]. Leaf litter (1 g) was macerated in 100 mL of water and serially diluted (10^{-2}–10^{-4}). Aliquots (0.2 mL) of the last two dilutions were plated on PDA (potato dextrose agar) supplemented with 100 $\mu g \cdot mL^{-1}$ chloramphenicol and incubated at 28 °C for 48 h. Distinct colonies were purified on PDA (28 ± 2 °C, 12 h photoperiod, 7 days) and stored in sterile microtubes with distilled water (Castellani, 1939). Fungal isolates were qualitatively screened for enzymatic activity based on guaiacol oxidation and Remazol Brilliant Blue R (RBBR) decolorization. Colonies were grown on PDA containing 0.05% (v/v) guaiacol or 0.01% (w/v) RBBR at 28 °C for five days. The formation of brown (guaiacol) or clear (RBBR) halos indicated positive enzymatic activity, and such isolates were selected for further analysis.

The identification of the colonies was conducted using morphological slides and molecular techniques. Genomic DNA was extracted using the CTAB method (Cationic Hexadecyl Trimethyl Ammonium Bromid®, Sigma, USA) [19]. For the A1SSI01, A1SSI02, A5SSI01 isolates, only the LSU regions (DNA encoding the large ribosomal subunit (28S rRNA), primers LROR/LR5) were amplified by polymerase chain reaction (PCR) [19] and two protein-encoding genes RPB1 (RNA polymerase II large subunit, primers RPB1-Af/RPB1-Cr) [21] and RPB2 (RNA polymerase II - primers bRPB2-6 F and bRPB2-7.1R) [22], to complement the identification of the isolates carried out by De Sousa et al [18]. For the A5SSI02 isolate, the ITS (Internal Transcribed Spacer) region of the ribosomal DNA conserved 5.8S gene was amplified using primers ITS1/ITS 4 [23].

PCR took place in a Bio-Rad thermal cycler with initial denaturation at 94ºC for 30 seconds; 30 cycles at 94 ºC for 30 seconds, 54 ºC for 30 seconds and 72 ºC for 30 seconds; with final extension at 72 ºC / 3 minutes for the ITS, RPB1 and RPB2 regions. The temperature of the annealing process for the LSU regions was changed to 56 ºC for 30 seconds, in accordance with the other conditions of the cycles described above. The amplified products were subjected to gel electrophoresis with a 1 kb DNA marker. Purification of the PCR product and sequencing were carried out by ACTgene (Brazil). The sequences were manually edited, when necessary, in the SeqAssem software and compared with the reference sequences deposited in the Genbank database. Alignments were performed using the MAFFT online interface [24]. The phylogenetic trees were built using the software PAUP *, version 4.0 (Swofford, 2002) and MrBayes v. 3.2.1 [25], using the Maximum Parsimony (MP) and Bayesian Inference methods,

respectively. Branches support was established using 1000 bootstrap replicates for Maximum Parsimony and 10^6 posterior probability (pp) generation for Bayesian Inference analysis [26].

2.2 Fermentation of açaí seeds in solid and liquid phases

Residual açaí material used in the biodegradation process was supplied by producers in the city of Abaetetuba (1° 43′ 04″ S, 48° 52′ 58″ W), Acará (1° 57′ 39″ S, 48° 11′ 49″ W), Baião (02º47'26" S e 49º40'18" W), Cametá (02º14'40" S, 49º29'45" W) and Igarapé-Miri (01º 58' 30" S, 48º 57' 35" W). The seeds supplied belong to the *Euterpe oleraceae* species and were washed and mixed to make up a single residual sample. They were then oven-dried until they reached constant humidity and ground in a knife and hammer mill (Forage chopper – TRF 90® Trapp), with 2 mm particle formation. The solid-state fermentation (SSF) and submerged fermentation (SmF)of the açaí seed was performed to determine the degradation efficiency and ligninolytic activity of the isolates tested. To do this, the selected fungal isolates were pre-cultured on plates with PDA (potato-dextrose-agar) medium for 7 days at 28 ºC.

For the solid-state fermentation (SSF), 5g of ground seed was added to Petri dishes (90 mm) [14] and humidified to 3,75 Ml (75% m/v) with liquid medium containing glucose (1 $g.L^{-1}$), yeast extract (0.5 $g.L^{-1}$) NaCl (0.2 $g.L^{-1}$), KH_2PO_4 (0.1 $g.L^{-1}$), $MgSO_4.7H_2O$ (0.1 $g.L^{-1}$), $CaCl_2.2H_2O$ (0.05 $g.L^{-1}$) and $CuSO_4.5H_2O$ (0.37 $g.L^{-1}$), autoclaved and the pH of the liquid medium adjusted to 5.5 [27]. Afterwards, five discs of mycelium (5 mm) of each isolate obtained from the active edge of colonies that had been growing for 7 days in PDA medium were transferred to the respective plates for each treatment. Petri dishes containing açaí pits inoculated with the respective isolates (A1SSI01, A1SSI02, A5SSI01 and A5SSI02) were incubated at 35 ºC for 14 days. Petri dishes with açaí pits without inoculum were used as a control treatment.

During the submerged fermentation (SmF), the same medium as described for humidifying the ground seed in the SSF above was used and a volume of 100 mL was added to 250 ml Erlenmeyer flasks. Each Erlenmeyer flask was supplemented with 5g of ground açaí seeds. Five discs of mycelia (5 mm) from each colony were also transferred to this medium, according to Linares [50], obtained from the edge of colonies with 7 days of growth. The flasks were incubated in a shaker (Orbitek, Scigenics Biotech) at 33 ºC ± 2 ºC and 120 rpm for 14 days (Fig 1).

Samples were collected at 24-hour intervals in each of the two trials by destructive sampling, with three plate for SSF and three flasks for SmF being collected from each treatment. The SSF samples obtained were placed in glass vials containing 50 mL of sodium acetate buffer (pH:

5.5; 0.1 M), under agitation at 120 rpm for 1 hr. After this period, the SSF 5 mL crude extract samples were filtered, centrifuged and the supernatant collected. For SmF, the supernatant was filtered, 5 mL crude extract centrifuged and collected. The supernatant centrifuged extract from each treatment was tested for Lac, LiP and MnP activity (Fig. 1).

Figure 1. Schematic process of solid-state fermentation (SSF) and submerged fermentation (SmF) of the açaí seed and extraction of the enzymatic substrate.

2.3 Ligninolytic enzyme assays

The Lac activity was measured using guaiacol as a substrate. The brownish-red coloration developed due to guaiacol oxidation was detected and measured by absorbance at 450 nm [28]. The reaction mixture (5 mL) was prepared with 1 mL of sample supernatant, 1 mL of guaiacol (50mM) and 3 mL of sodium acetate buffer (0.1 M; pH 5.0). MnP activity was estimated by oxidizing phenol red with the addition of MnSO4 and the absorbance read at 610 nm [29]. The sample was prepared with 800 µL of phenol red (0.01% m/v), 100 µL of sodium lactate (0.25 M), 200 µL of bovine albumin (0.5% m/v), 50 µL of $MnSO_4$ (2mM), 50 µL of

hydrogen peroxide (H_2O_2) + sodium succinate buffer (20 mM, pH 4.5) and 500 µL of enzyme source. LiP activity was measured by oxidizing veratryl alcohol in the presence of H_2O_2 and the absorbance was read at 310 nm in quartz cuvettes [30]. The reaction blend contained 1 mL of sodium tartrate buffer (125 mM and pH 3.0), 500 µL of veratryl alcohol (4 mM), 500 µL of H_2O_2 (2 mM) and 500 µL of aliquots of enzyme source. Enzyme activities were calculated and the results expressed in $U.mL^{-1}$.

2.4 Biomass loss and determination of lignin percentage

The mycelia were harvested each day from the broth by filtering through filter paper and the remaining biomass was dried in an oven at 60 ºC for 5 to 6 h [27]. The estimated biomass reduction of the degraded pit was determined everyday by the difference with the untreated pit over the course of the experiment.

To determine the lignin content (%) in seeds treated and untreated with the isolates, samples were obtained at the end of the 14 days of fermentation of the treatments, dried in ovens and submitted and initially determined the extractive content in samples of approximately 2 g (dry basis) according to the TAPPI T 204 cm-97 standard [31]. Successive extractions were carried out in a soxhlet-type reflux system, using toluene, ethanol and hot water as solvents, respectively, until no color was seen in the soxhlet chamber (approximately 6 - 8 h). The insoluble lignin of extractive-free samples was measured according to the TAPPI T222 standard [32], with modifications presented in Araujo et al. [33]. The samples weighing 0.35 g (dry basis) were hydrolyzed in 3 mL of sulfuric acid solution (72%) for 1 h at 30 °C on a hot plate under constant magnetic stirring. Subsequently, the resulting mixture was diluted with distilled water and placed in an autoclave for 1 hour at 120 °C and a pressure of 1.2 $kg.cm^{-2}$. The solution was then vacuum filtered through previously weighed 02 pore filters. The total solids obtained during filtration correspond to insoluble lignin, while acid-soluble lignin was obtained by reading the absorbance at 205 nm of the filtrate [34] using a spectrophotometer. Total lignin corresponded to the sum of insoluble lignin and soluble lignin.

2.5 Physical and chemical composition of the degraded seed

In order to observe the physical changes, the degraded and non-degraded açaí seed samples were oven dried until they reached a constant mass and subjected to scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) analysis. For SEM analysis, the samples were mounted on a conductive carbon tape and coated with gold using a cathodoluminescence coating module (Quorum, model Q150T-ES) for 30 seconds. Photographic images were obtained using secondary electrons and backscattered electrons. The equipment used was a Zeiss SEM model SIGMA-VP. The operating conditions were: electron

beam current = 80 μA, constant accelerating voltage of 10 kV and 15 kV, working distance between 12 and 14.5 mm. FTIR analysis was used to characterize the functional groups present in the degraded and non-degraded samples. The Agilent Technologies FTIR spectrophotometer (CARY 630 FTIR, Santa Clara, CA, USA), equipped with a ZnSe crystal ATR system and set to 32 scans, with a resolution of 4 cm-1 was used for this analysis. The spectral range was 4000 cm^{-1} to 650 cm^{-1} with special attention given to the region commonly referred to as the "fingerprint" (2000 cm^{-1} to 700 cm^{-1}) [35]. And Spectragryph v1.2.16.1 (Spectroscopy Ninja) software was used to analyze the spectra generated by each treatment in this experiment.

2.6 Data analysis

Three treatments were evaluated: submerged fermentation, solid-state fermentation, and unfermented seeds (control). All assays—enzyme activity, lignin, glucose, and biomass loss—were performed in triplicate (n = 3). Data on enzymatic activity, biomass loss, and lignin content were analyzed by ANOVA using RStudio v.4.2.2, and means were compared by Tukey's test at a 5% significance level ($p < 0.05$). FTIR spectra were interpreted by comparison with reference spectra from the literature.

3. Results and discussion

3.1 Fungi identification

Bayesian Inference (BI) and Maximum Parsimony (MP) analysis have generated phylogenetic trees with similar and consistent topologies for all isolates. The partition homogeneity test showed that the combined ITS and LSU, and ITS, LSU, RPB1 and RPB2 datasets for isolates A1SSI01, A1SSI02 and A5SSI01 did not present significant conflicts (p=0.01) and could therefore be combined. The concatenated ITS-LSU trees built by MP presented a dataset with a total of 1362 characters, of which 1048 were constant, 87 were non-informative parsimony and 227 were informative parsimony and were compared to 56 reference sequences available in GenBank for the genus, totaling a dataset of 59 sequences for this region (Table S1).

For ITS-LSU-RPB1-RPB2, the concatenated trees consisted of 3550 characters, of which 2081 were constant, 411 were non-informative parsimony variables and 1058 were informative parsimony, and were compared to 25 reference sequences available on GenBank for the genus, totaling a dataset of 26 sequences for this region (Table S1). The phylogenetic tree connected to the ITS and LSU regions reaffirmed the identity of isolates A1SSI01 and A1SSI02 as *Trametes flavida* [18], presenting a well-supported branch, with bootstrap and pp values equal to 100% and 1.0, respectively, as shown in Fig. 1A. Nevertheless, the ITS and LSU regions were not sufficient to separate the *T. cubensis*, *T. lactinea* and *T. orientalis* species (Fig. 2A) [36-39].

The concatenated analysis carried out for the four gene regions ITS, LSU, RPB1 and RPB2 carried out for isolate A5SSI01 with 26 reference sequences obtained from GenBank, it was possible to visualize in Figure 1B that the isolate shows genetic proximity to the species *T. cubensis* and *T. orientalis.* However, it forms a distinct clade, separate from these species, supported by a branch with a high support value (100% bootstrap and 1.0 pp), suggesting that it is probably an isolate of the *T. lactinea* species. Despite this, it is necessary to sequence the RPB1 and RPB2 regions of more isolates of the *T. lactinea* species in order to accurately compare the sequences obtained in this study, as there are no reference sequences of this region available in GenBank for isolates of the species (Fig. 2B).

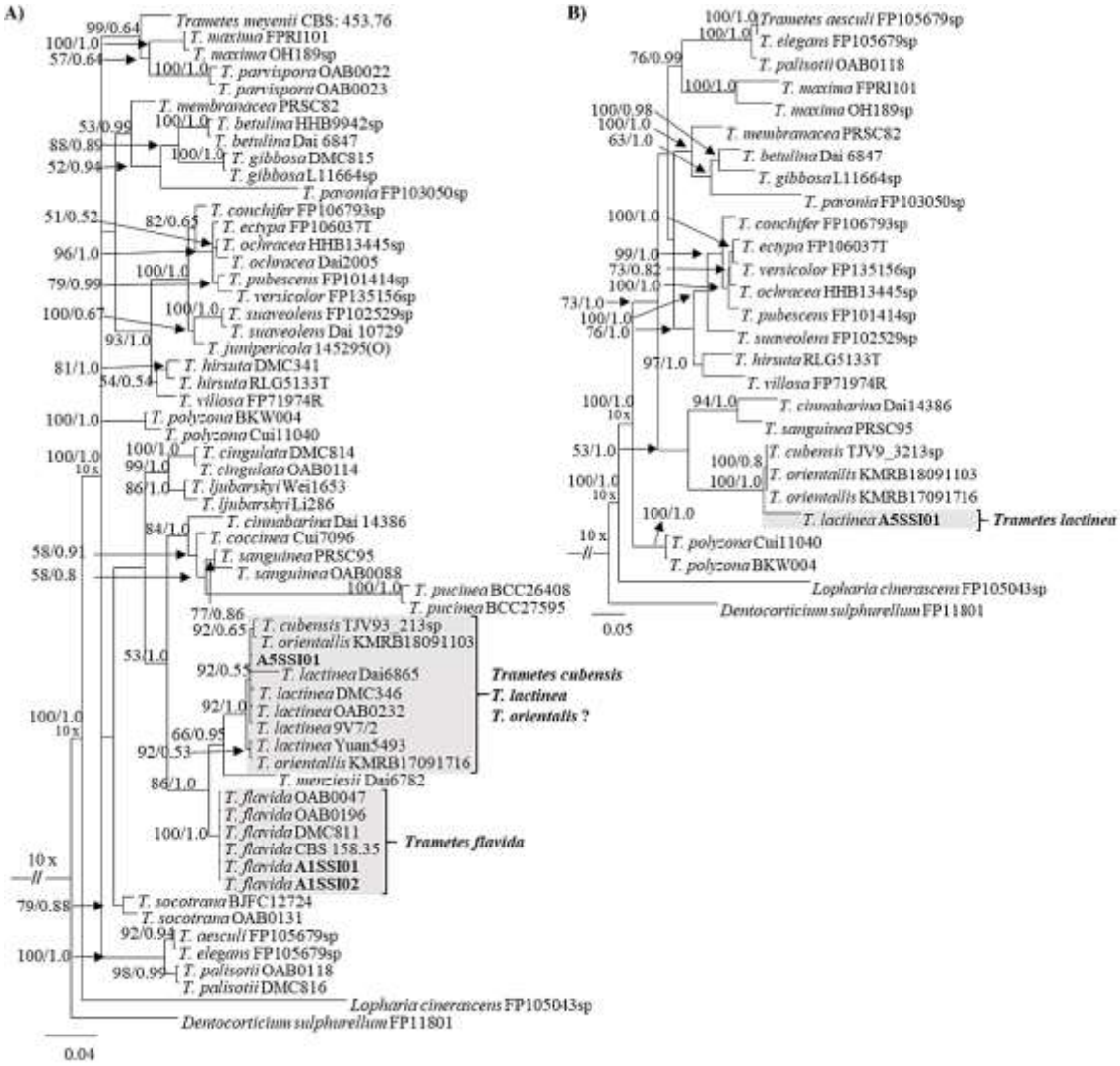

Figure 2. MP/IB *Trametes* spp. phylogeny based on the concatenated ITS-LSU dataset (A). MP/IB *Trametes* spp. phylogeny based on the concatenated ITS- LSU-RPB1-RPB2 dataset. Branch support values given as BS/PP (B). All clades where the newly generated sequences clustered are highlighted in gray. Taxonomic names are preceded by the voucher or isolate number.

Phylogenetic analysis of the ITS region (sequencing performed in duplicate for the ITS region of this isolate) from both directions for isolate A5SSI02 has generated phylogenetic trees with similar topology using the MP and BI methods. The sequences were compared to 23 reference sequences available in GenBank for the genus, totaling a dataset of 25 sequences for this region (Table S2). The MP analysis obtained to generate the ITS region tree was carried out with a total of 581 sites, 342 constant sites, 72 non-informative variable sites and 167 informative variable sites. In the tree, 9 species formed eight main clades. The sequences of isolate A5SSI01 were grouped into a monophyletic clade corresponding to the species *Syncephalastrum sympodiale* H. Zhao, Y.C. Dai & X.Y. Liu, supported by a bootstrap value equal to 98% and pp equal to 1.0, reporting the first occurrence of this species in Brazil (Fig. 3A). The results found by the phylogenetic tree obtained in this study are similar to those

presented for the genus by Zhao et al [40] in a new classification of the species of the phylum Mucoromycota.

It also presents morphological characteristics similar to the species recently described by Zhao et al [40] in China, isolated from soil samples in Beijing and from a mushroom in the Shennongjia National Nature Reserve. The colony's morphological characteristics are similar to those described by the authors for the species, such as initial mycelial growth which is white in color and gradually turns greyish. The hyphae are hyaline and aerial, with the presence of erect to curved sporangiophores, generally with the presence of sympodial branching and basal rhizoids. And the sporangiospores have a rough texture, with striations, and shapes that vary from subglobose to ovoid or ellipsoid (Fig. 3B).

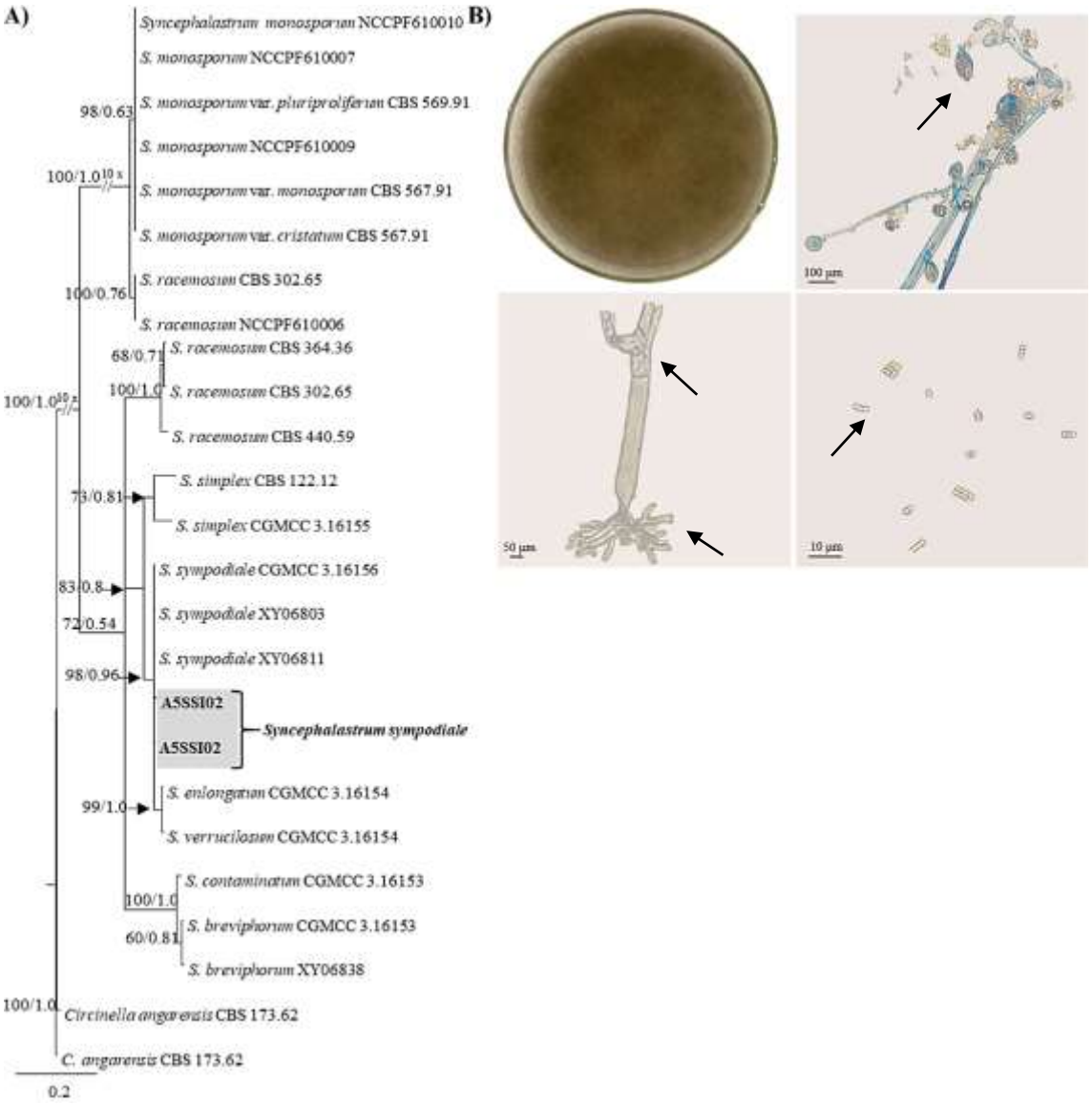

Figure 3. Phylogenetic tree of *Syncephalastrum* based on ITS rDNA sequences, with *Circinella angarensis* as outgroup. Bootstrap values for MP and posterior probability for BI (A). And morphological characteristics of A5SSI02 growing on PDA; sporangiophores with branches, sporangiospores and vesicles stained with 0.1% lacto-phenol; basal rhizoid and sporangiospores (B).

3.2 Enzymatic production

The activity of the ligninolytic enzymes in media containing açaí residue in SmF and SSF form showed that the fungus *S. sympodiale* (A5SSI02) did not have the capacity to produce Lac in both types of fermentations containing the açaí seed as a substrate, as shown in Table 1. The isolates of *T. flavida* (A1SSI01 and A1SSI02) and *T. lactinea* (A5SSI01) showed higher Lac production compared to the other enzymes (Table 1).

Table 1 shows that Lac activity at the end of the test was, on average, higher in SSF from the açaí seed, especially in the presence of *T. flavida* isolates A1SSI01 and A1SSI02, with values of 313.39 and 312.22 U.mL^{-1}, respectively. However, this activity did not differ statistically from the values observed in SmF for the same isolates, which showed activities equal to 303.40 and 270.57 U.mL^{-1}, respectively. Analysis of daily Lac activity revealed differences in the enzyme production peaks between the fermentation types. The highest value recorded was 797.73 U.mL^{-1} at nine days after the start of SSF for the *T. flavida* A1SSI01 isolate, which was statistically higher than the other enzyme activities recorded on the same day, as shown in Fig. 4.

The A1SSI02 isolate of *T. flavida* was responsible for the second highest Lac levels in SSF, with activities of 752.30 and 735.01 U.mL-^{1}, higher than the other treatments at eight and ten days after the start of SSF, respectively. Meanwhile, the highest Lac level was 446.15 U.mL^{-1}, associated with *T. lactinea* (A5SSI01) eight days after the start of the experiment (Fig.3). For this isolate, it was also possible to observe that at the end of the 14 days of fermentation, there was no significant difference in Lac activity in both fermentation states, with averages of 217.20 U.mL^{-1} and 190.8207 U.mL-^{1} in SmF and SSF, respectively, as shown in Table 1.

Table 1. A comparison of the average enzymatic activity of ligninolytic fungi after a submerged fermentation (SmF) and solid-state fermentation (SSF) of 14 days using agro-industrial açaí seed waste.

Species	Isolated	Fermentation	Enzyme activity (U.mL^{-1})*					
			Lac		MnP		LiP	
T. flavida	A1SSI01	SmF	303.4000	ab	17.2082	b	32.2186	ab
	A1SSI01	SSF	313.3944	a	13.1069	b	26.4711	ab
	A1SSI02	SmF	270.5707	ab	11.5124	b	32.7103	ab
	A1SSI02	SSF	312.2155	ab	11.0065	b	24.3852	b
T. lactinea	A5SSI01	SmF	217.1969	abc	17.9347	b	28.3952	ab
	A5SSI01	SSF	190.8207	c	12.9034	b	24.9599	b
S. sympodiale	A5SSI02	SmF	0	d	38.2671	a	46.8216	a
	A5SSI02	SSF	0	d	15.5841	b	23.2292	b

*Treatments with the same letter do not differ significantly from each other by Tukey's test at 5%.

Regarding MnP activity, the overall average enzyme activity recorded for the fungi studied revealed that the highest detection was associated with the SmF of the *S. sympodiale* isolate (A5SSI02) compared to the *Trametes* spp. isolates, with no statistical difference between the

average activity of the other treatments (Table 1). However, the data analyzed for MnP activity over the days of fermentation showed that the isolates of *T. flavida* and *T. lactinea* had higher MnP activity in the first two days of fermentation. The highest MnP peak recorded in this study was for isolate A5SSI01 (*T. lactinea*), reaching 87.44 $U.mL^{-1}$, followed by A5SSI02 with 76.00 $U.mL^{-1}$, after 24 hours and seven days from the start of SmF, respectively, as seen in Fig. 4. For *S. sympodiale* (A5SSI02), there were still records of activities greater than 65 $U.mL^{-1}$ in SmF at six and ten days after the start of the experiment (Fig. 4).

In the LiP analysis, the average general behavior of the enzyme activity was similar to that of MnP, as shown in Table 1. Thus, the *S. sympodiale* isolate showed the highest average LiP, equal to 46.82 $U.mL^{-1}$ at the end of the 14 days of fermentation, which was higher than the other isolates and treatments tested. For the *Trametes* spp. isolates, it can be seen in Fig. 3 that although there was no statistical difference between the averages of LiP activity at the end of fermentation, there were differences in activity over the days of fermentation. The highest values obtained for LiP associated with fermentations with *Trametes* isolates were 53.54; 57.52 and 45.05 $U.mL^{-1}$, respectively, for A1SSI01, A1SSI02 (*T. flavida*) and A5SSI01 (*T. lactinea*) after eight days in SSF and 62.86; 68.67 and 45.45 $UI.mL^{-1}$ in LF. However, the highest peak of enzymatic activity for LiP was recorded 10 days after the start of SmF of the açaí seed in the presence of *S. sympodiale* (A5SSI02), with this activity being equal to 96.80 $U.mL^{-1}$, higher than the other activities recorded for this evaluation period (Fig. 4).

Results of this investigation revealed that for the isolates of *T. flavida* and *T. lactinea*, the highest activities found in fermentations of açaí waste were Lac, similar to the result for *T. hirsuta* in fermentations with sorghum waste biomass, as demonstrated by Andriani et al. [41]. Lac production is mainly associated with wood rot fungi, such as white rot [42], such as isolates A1SSI01, A1SSI02 and A5SSI01. In these fungi, Lac plays a role in their metabolism, such as in the process of sporulation, pigment production, basidiocarp formation and plant pathogenesis [43]. A further relevant point to highlight the higher production of this enzyme in *Trametes* is the fact that lignin is an inducer for this activity [44], contributing to its higher production in

açaí seeds, even when compared to the results obtained with RBBR in the study conducted by De Sousa et al. [18] with these isolates.

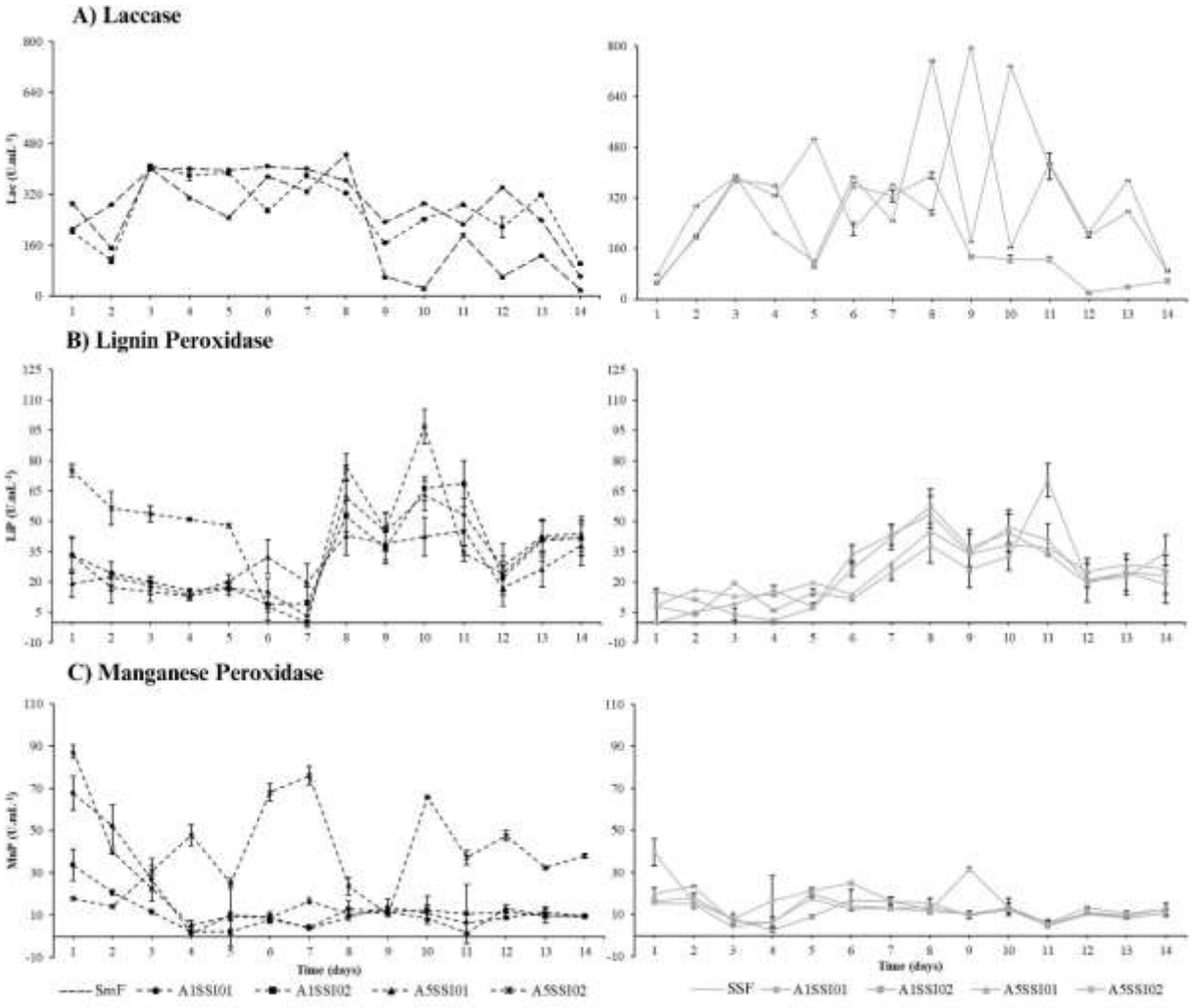

Figure 4. Enzyme Lacase (Lac) (A); Lignin peroxidase (LiP) (B) and Manganese peroxidase (MnP) (C). Enzymatic activities of isolates of *T. flavida* (A1SSI01, A1SSI02), *T. lactinea* (A5SSI01) and *S. sympodiale* (A5SSI02) in submerged fermentation (SmF) and solid-state fermentation (SSF) of açaí seeds.

The reduction in enzyme activity over time, such as Lac after 9 days of incubation, is directly related to a decrease in the nutritional resources available in the culture medium. During the growth of the fungus, there is a direct relationship between the increase in biomass and the consumption of carbon sources, such a glucose, which are progressively consumed, reducing the availability of these essential substrates for enzyme production. Results like these have already been reported, where the rapid and efficient consumption of the substrate results in high production and enzyme activity [45-47]. In addition, daily glucose measurement data indicates that glucose decreases over time during the fermentation process, which may further help to explain the drop in enzyme activity observed (Fig. S1).

The reduced LiP activities in *Trametes* colonies, compared to Lac, might be related to two factors: i. LiP is stored in large quantities intracellularly, despite its extracellular role, which may result in the absence of activity detection or low production in fermentations [48]; ii. the function of LiP in the breakdown of lignin has an additional role in its fragments initially released by MnP, this enzyme being reported in some white rot fungi highly active in decomposer fungi as non-essential in the biotransformation of lignin [49]. In the case of MnP, the decreased production in *Trametes* spp. isolates compared to Lac activity may be related to the increase in phenolic compounds during degradation, which may have negatively influenced MnP production, making it toxic to cells and decreasing enzyme activity, as observed in the fungus *Phanerochaete chrysosporium* [50].

For *Trametes* isolates, SSF favored Lac activity, but showed no significant differences for the enzymes MnP and LiP. This is the main fermentation used when using lignocellulosic biomass as a carbon source for enzymatic production by *T. versicolor* [51]. The use of SSF can facilitate the process of experimental scale-up at an industrial level, as it has advantages such as the need for low quantities of chemicals for preparing the media, high enzyme productivity, low waste flow and low water consumption. In addition, it is a sustainable method due to the use of lignocellulosic waste, such as the açaí seed [52].

Although Lac was not present in *S. sympodiale*, considerably higher LiP and MnP enzyme activity values were recorded compared to data obtained in SSF of grape pomace with Muiromycota *Rhizopus oryzae* [53]. The absence of the Lac enzyme has already been reported for soft rot species in Muiromycota, such as the case of *R. oryzae* in the study carried out by Kasonga et al. [54], when verifying the ligninolytic activities of this fungus in the removal of drugs from wastewater. Additionally, it was observed in this research that higher production of MnP by *R. oryzae* compared to LiP was recorded around nine days after fermentation [54], as shown for these enzymes in ours for the açaí kernel treated with *S. sympodiale* (A5SSI02 - 7 and 9 days after fermentation). The enzymatic activities of MnP and LiP for *S. sympodiale* tend to increase over the course of fermentation, improving the efficiency of lignin degradation and the release of phenolic compounds from its structure, as demonstrated by Šelo et al. [53].

3.3 Characterization of the degraded seed

The data on estimated biomass loss indicated that the isolate of *T. flavida* A1SSI02 in SSF was solely responsible for the greatest loss of biomass in açaí seeds, with an average reduction of 0.14 $g.day^{-1}$, totaling a reduction of up to 39.9% of the biomass of the degraded residue (1.99 g) at the end of the experiment, as shown in Table 2. Broadly speaking, the açaí seed SSF had

a greater loss of biomass associated with isolates of the basidiomycete *Trametes*, a white rot fungus, compared to the ascomycete *S. sympodiale*. This latter in turn had a reduction in biomass in the treated residue of 23% in SSF and 18.7% in SmF (Table 2). Mass loss associated with the control treatment can be linked to the loss of extractives in the SmF over the days of fermentation and in the SSF to the sodium acetate extraction stage.

Table 2. A comparison of the estimated biomass loss of the seed degraded by ligninolytic fungi after 14 days of submerged fermentation (SmF) and solid-state fermentation (SSF).

Species	Isolated	Fermentation	Estimated Biomass Loss*			
			Mean (g/day)	Total (g and %)		
Control	-	SmF	0.0143	0.200	4.0	b
	-	SSF	0.0143	0.200	4.0	b
T. flavida	A1SSI01	SmF	0.0619	0.867	17.3	ab
	A1SSI01	SSF	0.0974	1.364	27.3	ab
	A1SSI02	SmF	0.1190	1.667	33.3	a
	A1SSI02	SSF	0.1424	1.994	39.9	a
T. lactinea	A5SSI01	SmF	0.0964	1.350	27.0	ab
	A5SSI01	SSF	0.1284	1.797	35.9	a
S. sympodiale	A5SSI02	SmF	0.0667	0.933	18.7	ab
	A5SSI02	SSF	0.0964	1.148	23.0	ab

*Treatments with the same letter do not differ significantly from each other by Tukey's test at 5%.

An analysis to establish the percentage of lignin indicated that its content decreased in the samples treated with the isolates at the end of the 14 days of fermentation, as shown in the graph in Fig. 5. The highest reduction found in the açaí seed fermentation treatments was associated with the SSF of *T. flavida* (A1SSI02), which reduced the lignin content by 24.3% compared to the control. The other isolates differed statistically from the control, but had higher lignin values than the above treatment, reducing the lignin content by between 5 and 17% compared to the control (Fig. 5).

Larger mass and lignin loss associated with SSF with *T. flavida* (A1SSI02) is related to the ability of white rot fungi to degrade cellulose and be more efficient at degrading lignin, compared to brown rot and soft rot fungi, which mainly attack cellulose but are less effective at degrading lignin [55]. In relation to soft rot fungi, such as the *S. sympodiale* (A5SSI02), which despite secreting enzymes responsible for lignin degradation, generally have a low capacity for degrading this phenolic macromolecule and take longer to degrade lignin [56] compared to white rot fungi, such as the *T. flavida* (A1SSI01 and A1SSI02) and *T. lactinea* (A5SS101). Remarkably, lignin degradation is 3 to 5 times lower in the *S. sympodiale* (A5SSI02) compared to the isolate with the highest degradation in this study, *T. flavida* (A1SSI02).

During the 14 days of fermentation, the percentage of lignin degraded was higher than the data found by Del Cerro et al. [57] when evaluating the degradation of lignin in *Populus*

trichocarpa wood by isolates of *Gelatoporia subvermispora* FP-105752 and *Trametes versicolor* FP-101664. The mentioned study found that in SSF the fungi were able to reduce the lignin content by 4.8 ± 0.9% and 11.8 ± 1.0%, respectively, after 14 days of cultivation, lower than the greatest reduction found by our study in the same cultivation time and type of fermentation with açaí seeds.

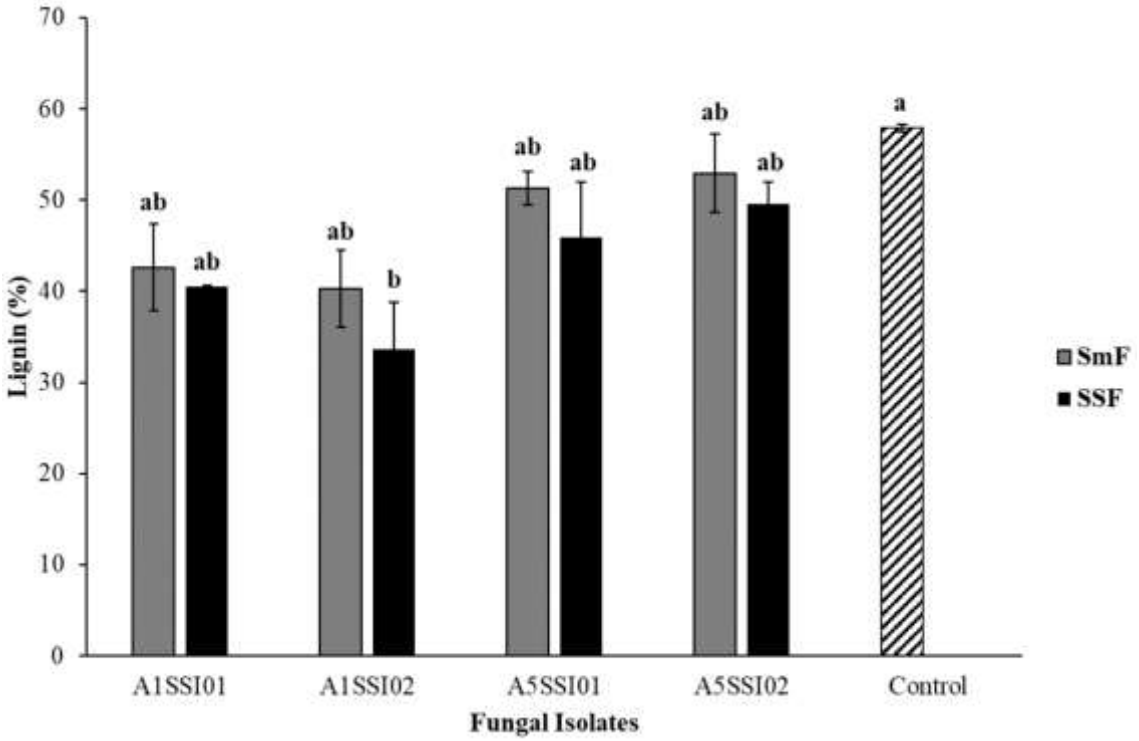

Figure 5. Lignin levels recorded for açaí seed samples at the end of submerged fermentation (SmF)) and solid-state fermentation (SSF) using isolates of *Trametes flavida* (A1SSI01 and A1SSI02), *T. lactinea* (A5SSI01) and *S. sympodiale* (A5SSI02) compared to the control treatment (untreated açaí seed).

The surface and morphology of the degraded and non-degraded pits were analyzed using SEM before and after the fermentations, as can be seen in Fig. 6. The SEM image of the control treatment (non-degraded pit) shows a regular and compact structure, with no grooves or pores on the upper surface (Fig. 5A). On the other hand, the SEM images of the açaí seeds treated with isolates of *Trametes* spp. and *S. sympodiale* after 14 days of fermentation revealed significant structural changes. These changes include cell wall disorganization, cellulose ruptures, irregular texture and surface openings, allowing internal visualization of the cell wall structure of the degraded material (Fig. 6B and C).

Breaks in the cell wall was noted both in samples treated with isolates of *Trametes* spp. and with *S. sympodiale* (Fig. 6B and C). The fungal hyphae of the *Trametes* isolates can also be seen penetrating the interior of the compact structure, increasing the surface area available for subsequent enzymatic decomposition. For the pits treated with *S. sympodiale*, the presence of

its spores was also observed in the material analyzed. The presence of these fungal structures can be seen in Fig. 6B and C.

Alterations caused by the enzymes were detected in the SEM analysis, showing that the açaí residue treated with the selected fungi showed exposure of the internal structures when compared to the untreated sample. These modifications indicate a reduction in the lignin content and that the isolates A1SSI01, A1SSI02, A5SSI01 and A5SSI02 used lignin as a natural substrate and inducer for the production of ligninolytic enzymes such as Lac, LiP and MnP. In addition, the removal of lignin causes the structure of the cellulose-hemicellulose-lignin matrix to break down, disorganizing the agricultural waste used. This result was consistent with previous studies carried out using Lac fungi to degrade corn cobs and rice straw [58] and with results recorded for the biodegradation of sorghum biomass by *T. hirsuta* and corn stalks by *Trametes* sp. [41,59].

These results indicate that the use of açaí residue as a readily available raw material for the production of ligninolytic enzymes favors greater production of this enzyme under the conditions in which the test was carried out, especially Lac in SSF using isolates of *T. flavida*. The use of the açaí seed to produce Lac by *T. flavida* is a viable solution for industrial applications, and an interesting alternative for the environmental management of this waste, which contributes greatly to the environment.

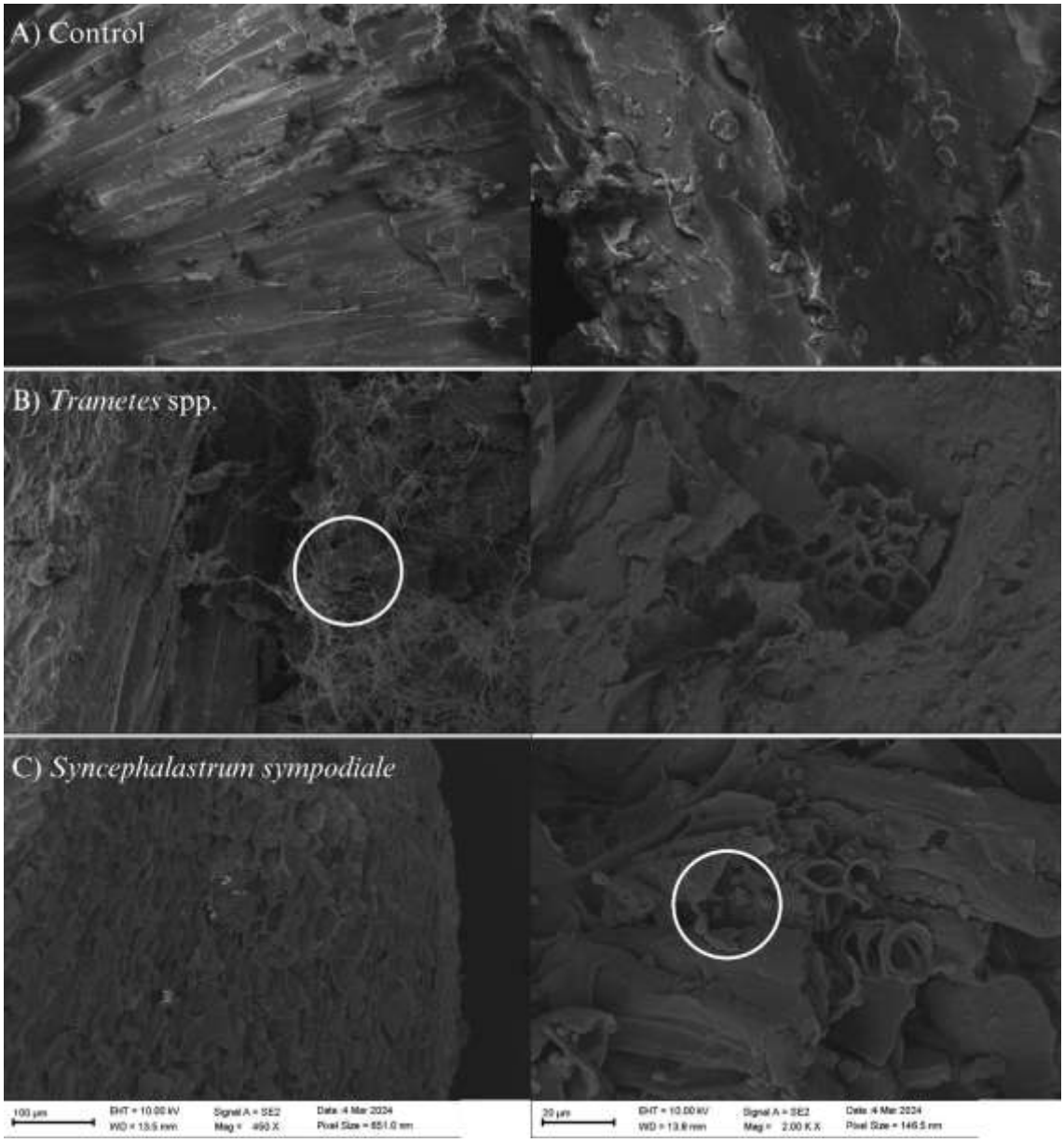

Figure 6. Scanning Electron Microscopy (SEM) images showing ruptures in the structure of the açaí kernel biotreated with the respective fungi: control treatment (A), kernel treated with Trametes spp. (B), kernel treated with S. sympodiale (C). The photograph was taken on the 14th day of cultivation. Highlight in figure B: hyphae of *Trametes* spp. isolates Highlight in figure C: *S. sympodiale* spores.

FT-MIR assessment evaluated the structural changes that occurred in the lignocellulosic material of the açaí seed after treatment with the isolated fungi. The Fig. 7 shows the results obtained, with absorption peaks in the 3340 cm^{-1} region that can be attributed to the presence of -OH groups [60]. The intense bands in the 2920 cm^{-1} region are due to the stretching of C-sp3 bonds that are present in the CH_2, CH_3 groups, while the band resulting from the stretching vibration at 2858 cm^{-1} is due to the presence of the methoxyl group (CH_3O) in the lignin structure [61,62]. The presence of aromatic rings is confirmed by the absorption bands at 1610 cm^{-1} typical of conjugated double bonds and by the band at 1515 cm^{-1}, both of which originate from the vibration of aromatic nuclei. At 1720 cm^{-1} there are bands corresponding to the

carbonyl groups. In addition, the presence of the guiacil and syringyl groups can be seen in the absorptions recorded at 1238 and 1367, respectively [63].

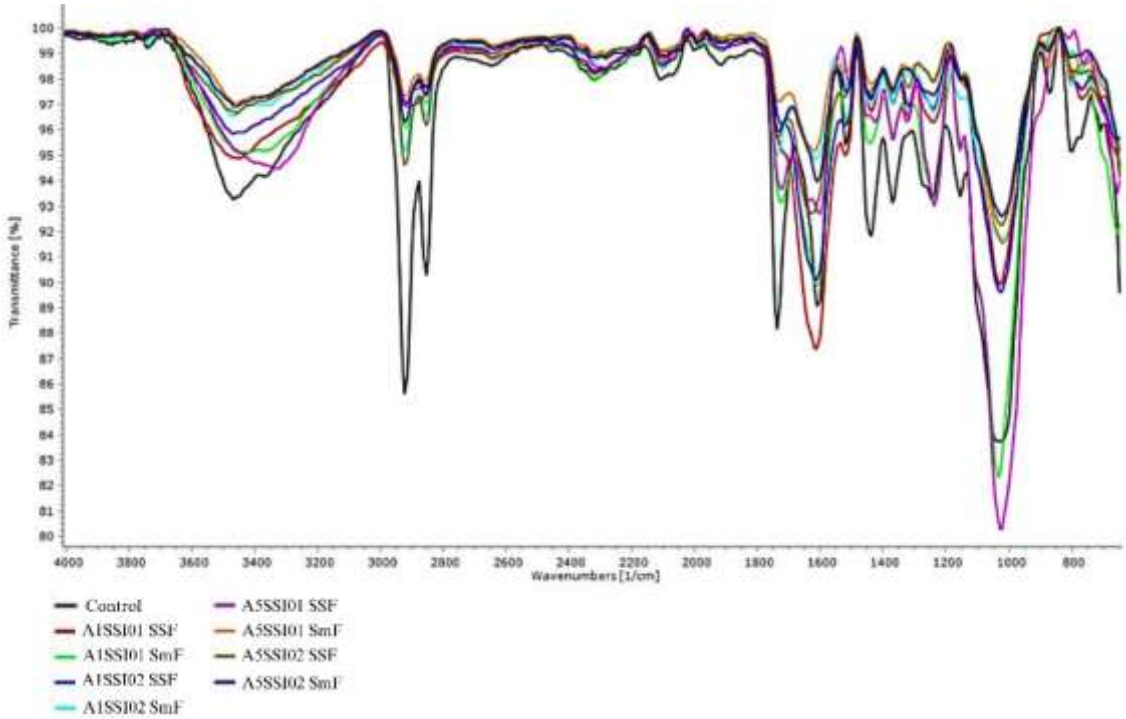

Figure 7. Infrared spectrums of the samples treated with the isolated fungi, where isolates *Trametes flavida* A1SS101 and A1SSI02, *T. lactinea* A5SSI01 and *Syncephalastrum sympodiale* A5SSI02; SSF stands for solid-state fermentation and SmF submerged fermentation. And the control treatment refers to the non-treated buck.

The degradation process of lignin by the action of the enzymes secreted by the fungi is evident after 14 days of fermentation when there is a reduction in the intensity of the bands compared to the control group. The reduction in intensity is more noticeable in the sample treated with isolate A1SSI02, which also revealed a lower lignin content and greater enzymatic activity. The effect of treatment with this isolate led to the breaking of aromatic ring bonds, which resulted in a reduction in the intensity of the band at 1610 cm^{-1} [60]. The reduction in intensity of other absorption bands may have originated from the process of breaking bonds by the action of the enzymes secreted by the fungi, for example, at 1029 cm^{-1} the vibration of C-O bonds of aliphatic esters decreases with treatment by the different strains used [64].

4. Conclusions

This study advances the understanding of ligninolytic enzyme production by demonstrating that saprophytic basidiomycetes of the *Trametes* genus and the mucoromycete *Syncephalastrum sympodiale*, isolated from Amazonian leaf litter, can efficiently use açaí seed as a substrate. The findings highlight the biotechnological potential of this abundant agro-industrial residue for sustainable enzyme production, particularly under solid-state fermentation conditions. Furthermore, the molecular data generated contribute to the taxonomic resolution within the *Trametes* complex and provide the first record of *S. sympodiale* associated with riparian forest litter in the Amazon. Collectively, these results expand the knowledge of fungal biodiversity and valorization of regional residues, offering new perspectives for environmentally sustainable bioprocesses.

Supporting Information

Figure S1. Estimation of glucose in submerged fermentation (SmF) and solid-state fermentation (SSF) solid (SSF) of açaí seeds for the isolates A1SSI01 (*T. flavida*) (A); A1SSI02 (*T. flavida*) (B); A5SSI01 (*T. lactinea*) (C) and A5SSI02 (*S. sympodiale*) (D).

Table S1. The species, isolate identification and GenBank reference sequence numbers of the ITS, LSU, RPB1 and RPB2 regions of the genus *Trametes* and the subgroups *Dentocorticium sulphurellum* and *Lopharia cinerascens* which were used in this research.

Table S2. The species, isolate identification and GenBank access numbers of the reference sequences of the ITS region of the genus *Syncephalastrum* and the *Circinella angarensis* subgroup included in this research.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Iêda A. L. de Sousa. The first draft of the manuscript was written by Iêda A. L. de Sousa and all authors commented on previous

versions of the manuscript Alessandra de J. Boari contributed to the molecular analysis and identification of the fungal isolates in this study. Elesandra da S. Araujo and Lina Bufalino contributed to the analysis and quantification of lignin from açaí seeds. Renan C. e Silva and Nelson R. Ferreira analyzed and interpreted the Fourier transform infrared spectroscopy (FTIR) data. And Alberdan S. Santos was responsible for supervising the research and revising the manuscript. All authors have read and approved the final version of the manuscript.

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Declarations

Data Availability Genetic sequence data obtained for the identification of the fungal isolates in this study have been deposited in GenBank NCBI, under the deposit numbers OR888930, PQ815174, OR888931, PQ815175, OR888960, PQ815176, PQ819839, PQ819840, PQ815171 and PQ815172. Copies of the data can be obtained free of charge from https://www.ncbi.nlm.nih.gov/genbank/. The authors declare All other relevant data generated and analysed during this study, which include date experimental, scanning electron microscopy images and infrared spectrums, are included in this article and its supplementary information. Source data are provided with this paper. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Competing interests The authors declare no competing interests.

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Figure S1. Estimation of glucose in submerged fermentation (SmF) and solid-state fermentation (SSF) solid (SSF) of açaí seeds for the isolates A1SSI01 (*T. flavida*) (A); A1SSI02 (*T. flavida*) (B); A5SSI01 (*T. lactinea*) (C) and A5SSI02 (*S. sympodiale*) (D).

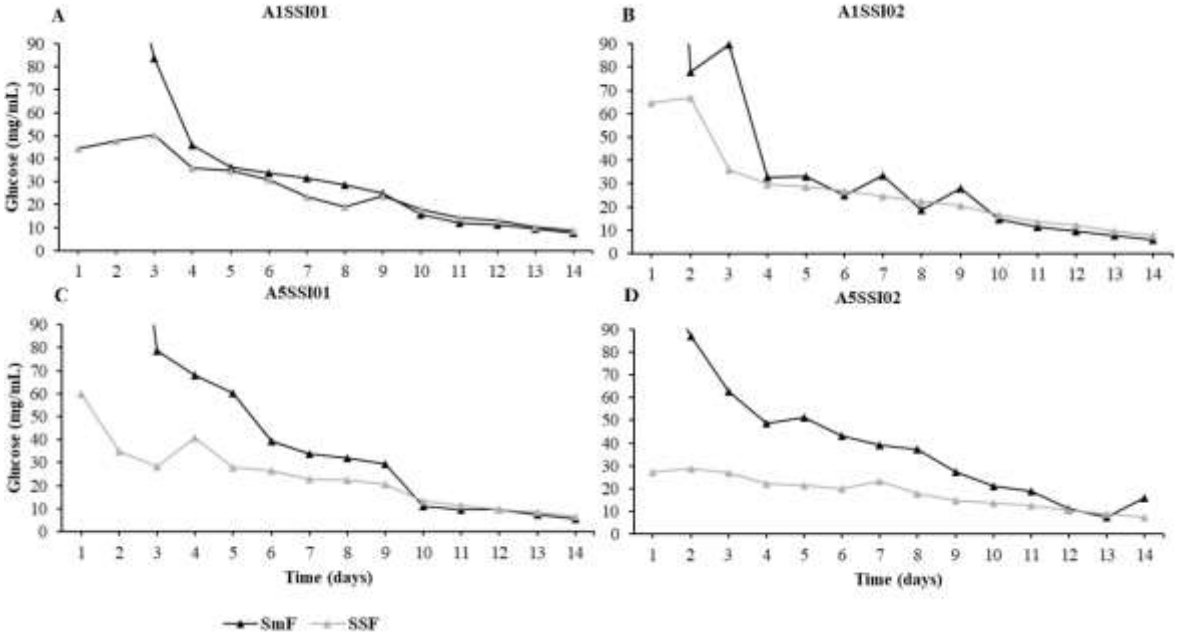

Tabela S1. The species, isolate identification and GenBank reference sequence numbers of the ITS, LSU, RPB1 and RPB2 regions of the genus *Trametes* and the subgroups *Dentocorticium sulphurellum* and *Lopharia cinerascens* which were used in this research.

Species	Isolate	Location	Accession Genbank			
			ITS	LSU	RPB1	RPB2
Dentocorticium sulphurellum	FP11801	USA	JN165018	JN164815	JN164841	JN164876
Lopharia cinerascens	FP105043sp	USA	JN165019	JN164813	JN164840	JN164874
Trametes aesculi	FP105679sp	USA	JN164944	JN164799	JN164833	JN164861
T. betulina	HHB9942sp	USA	JN164983	JN164794		
	Dai6847	China	KC848305	KC848390	JN164822	JN164860
T. cingulata	DMC814	Cameroon	KC589133	KC589159		
	OAB0114	Benin	MK736971	MK736950		
T. cinnabarina	Dai 14386	China	KX880629	KX880667	KX880818	KX880854
T. coccinea	Cui7096	China	KC848330	KC848414		
T. conchifer	FP106793sp	USA	JN164924	JN164797	JN164823	JN164849
T. cubensis	TJV93_213sp	USA	JN164923	JN164798	JN164834	JN164865
T. ectypa	FP106037T	USA	JN164929	JN164803	JN164824	JN164848
T. elegans	FP105679sp	USA: Georgia	JN164944	JN164799	JN164833	JN164861
T. flavida	OAB0047	Benin	MK736966	MK736946		
	OAB0196	Benin	MK736968	MK736947		
	DMC811	Cameroon	KC589130	KC589156		
	CBS 158.35		MH855616	MH867126		
T. flavida	**A1SSI01**	**Brazil**	**OR888930**	**PQ815174**		
	A1SSI02	**Brazil**	**OR888931**	**PQ815175**		

Species	Isolate	Location	Accession Genbank			
			ITS	LSU	RPB1	RPB2
T. gibbosa	DMC815	Cameroon	KC589144	KC589164		
	L11664sp	England	JN164943	JN164800	JN164831	JN164859
T. hirsuta	DMC341	Cameroon	KC589146	KC589166		
	RLG5133T	USA	JN164941	JN164801	JN164829	JN164854
T. junipericola	145295(O)		KC017758	KC017763		
	DMC346	Cameroon	KC589126	KC589152		
	Dai6865		KC848327	KC848411		
T. lactinea	OAB0232	Benin	MK736983	MK736948		
	9V7/2	Thailand	GQ982888	GQ982881		
	Yuan5493		KC848320	KC848404		
T. lactinea	**A5SSI01**	**Brazil**	**OR888960**	**PQ815176**	**PQ819839**	**PQ819840**
T. ljubarskyi	Wei1653		KC848332	KC848416		
	Li286		KC848331	KC848415		
T. maxima	FPRI101	USA	JN164933	JN164802	JN164836	JN164863
	OH189sp	Venezuela	JN164957	JN164804	JN164816	JN164864
T. membranacea	PRSC82	Puerto Rico	JN164945	JN164805	JN164832	JN164857
T. menziesii	Dai6782		KC848289	KC848374		
T. meyenii	CBS:453.76	India	MH860991	MH872762		
T. ochracea	HHB13445sp	USA	JN164954	JN164812	JN164826	JN164852
	Dai2005	China	KC848272	KC848357		

Species	Isolate	Location	Accession Genbank			
			ITS	LSU	RPB1	RPB2
T. orientallis	KMRB18091103	South Korea	ON402820	ON402841	OP216061	OQ167981
	KMRB17091716	South Korea	ON402821	ON402842	OP216062	OQ167980
T. palisotii	OAB0118	Benin	MK736980	MK736956	MK802884	MK802882
	DMC816	Cameroon	KC589141	KC589162		
T. parvispora	OAB0022	Benin	MK736989	MK736964		
	OAB0023	Benin	MK736990	MK736965		
T. pavonia	FP103050sp	USA	JN164958	JN164806	JN164835	JN164862
T. polyzona	BKW004	Ghana	JN164978	JN164790	JN164844 e JN164846	JN164856
	Cui 11040	China	KX880647	KX880689	KX880836	KR610849
T. pubencens	FP101414sp	USA	JN164963	JN164811	JN164827	JN164851
T. pucinea	BCC26408	Thailand	FJ372685	FJ372707		
	BCC27595		FJ372686	FJ372708		
T. sanguinea	OAB0088	Benin	MK736969	MK736949		
	PRSC95	Puerto Rico	JN164982	JN164795	JN164842	JN164858
T. socotrana	BJFC12724	China	KC848313	KC848397		
	OAB0131	Benin	MK736987	MK736962		
T. suaveolens	FP102529sp	USA	JN164966	JN164807	JN164828	JN164853
	Dai 10729	China	JN048770	JN048789		
T. versicolor	FP135156sp	USA	JN164919	JN164809	JN164825	JN164850
T. villosa	FP71974R	USA	JN164969	JN164810	JN164830	JN164855

The isolates in bold have been sequenced in this research for the LSU, RPB1 and RPB2 areas.

Tabela S2. The species, isolate identification and GenBank access numbers of the reference sequences of the ITS region of the genus *Syncephalastrum* and the *Circinella angarensis* subgroup included in this research.

Species	Isolate	Location	Accession Genbank ITS
Circinella angarensis	CBS 173.62	USA	MH858136.1
	CBS 172.62	USA	MH858135.1
S. breviphorum	XY06838	China	OL678217.1
	CGMCC 3.16153	China	OL678218.1
S. contaminatum	UoMD18-1[a]	Australia	MK799842.1
S. elongatum	CGMCC 3.16154	China	OL678219.1
S. monosporum	NCCPF610010	India	MN192179.1
	NCCPF610010	India	MN192176.1
	NCCPF610009	India	MN192177.1
	CBS122.12	Switzerland	MH854610.1
S. monosporum var. *cristatum*	CBS 568.91	-	HM849720.1
S. monosporum var. *monosporum*	CBS 567.91	China	NG069822.1
S. monosporum var. *pluriproliferum*	CBS 569.91	China	NG069823.1
S. racemosum	CBS 302.65	Brazil	MH858577.1
	NCCPF610006	India	MN192182.1
	CBS 364.36	-	MH855824.1
	CBS 440.59	USA	MH857909.1
	CBS 302.65	-	HM849721.1
S. simplex	CGMCC 3.16155	China	OL678220.1
S. sympodiale	CGMCC 3.16156	China	OL678221.1
	XY06811	China	OL678223.1
	XY06803	China	OL678222.1
S. sympodiale	**A5SSI02.1**	**Brazil**	**PQ815171**
	A5SSI02.2	**Brazil**	**PQ815172**
S. verrucilosum	CBS 213.78	China	OL678219.1

Isolates in bold were sequenced in this research.

4. DISCUSSÃO INTEGRADORA

A serapilheira das florestas amazônicas, especialmente das formações ciliares, abriga uma rica diversidade de microrganismos com potencial biotecnológico ainda pouco explorado. Os fungos ligninolíticos isolados neste estudo, principalmente do gênero *Trametes* e da espécie *Syncephalastrum sympodiale*, demonstraram capacidade relevante de produzir enzimas oxidativas associadas à degradação da lignina, Lac, MnP e LiP. Esses resultados reforçam achados da literatura que destacam a Amazônia como um reservatório de microrganismos com elevada eficiência catalítica, capazes de atuar sobre substratos complexos, como os resíduos lignocelulósicos gerados pela agroindústria regional.

No segundo capítulo, observou-se que apenas os isolados de *Trametes* foram capazes de descolorir o corante sintético RBBR, sugerindo especificidade e eficiência na produção de lacase, uma enzima fundamental em processos de oxidação e biodescoloração. Esses resultados corroboram estudos anteriores que atribuem ao gênero *Trametes* um papel fundamental em processos de biorremediação, especialmente devido à sua habilidade de utilizar compostos fenólicos e estruturas aromáticas como fonte de carbono. A atividade significativa de Lac nos ensaios com RBBR destaca seu potencial para tratamento de efluentes contaminados com corantes, cuja estrutura química se assemelha à da lignina, indicando aplicabilidade em diferentes contextos ambientais.

O terceiro capítulo aprofundou o potencial da produção enzimática destes fungos com uso do caroço de açaí como substrato para fermentação sólida e submersa, evidenciando que a fermentação sólida (SSF) foi mais eficiente na indução de enzimas ligninolíticas, especialmente Lac. Os isolados de *Trametes* spp. mostraram elevada atividade enzimática e promoveram alterações morfológicas e químicas significativas nos resíduos tratados, conforme demonstrado por microscopia eletrônica e espectroscopia FTIR. O isolado de *S. sympodiale* apresentou produção restrita a MnP e LiP, mas contribuiu no processo de degradação. O uso de caroço de açaí como substrato representa uma estratégia sustentável, alinhando manejo de resíduos e geração de bioprodutos com valor agregado.

Com base nos resultados da análise FT-MIR, é possível inferir a presença de compostos fenólicos residuais no substrato após a ação dos fungos ligninolíticos. As bandas características associadas a grupos aromáticos, carbonílicos, hidroxilas e metoxilas, ainda que com intensidades reduzidas, indicam que a degradação da lignina gerou subprodutos fenólicos. Moléculas fenólicas solúveis possuem reconhecidas propriedades biológicas ativas, incluindo atividade antioxidante, antimicrobiana, anti-inflamatória e até potencial anticancerígeno, o que amplia as possibilidades de aplicação em setores como alimentos, cosméticos, farmacêutico e

biomateriais. Assim, além da função ambiental de biorremediação, os sistemas fúngicos ligninolíticos explorados nesta pesquisa podem contribuir para a geração de produtos de valor agregado a partir de resíduos amazônicos, alinhando-se aos princípios da bioeconomia e da valorização de subprodutos com funções bioativas.

Esses achados, quando integrados, demonstram não apenas a viabilidade de uso de fungos ligninolíticos amazônicos em processos de biorremediação e valorização de resíduos, mas também ressaltam a importância da conservação desses ecossistemas como fonte de recursos genéticos com ampla aplicabilidade biotecnológica. A identificação de *Trametes flavida* e *T. lactinea*, bem como o primeiro registro de *S. sympodiale* em serapilheira amazônica, contribuem para o conhecimento taxonômico e ecológico desses grupos fúngicos, revelando o potencial oculto de espécies pouco documentadas.

Os resultados obtidos consolidam o papel dos fungos amazônicos como agentes promissores na bioeconomia regional, com aplicações que podem ir desde o tratamento de efluentes industriais até o aproveitamento de resíduos lignocelulósicos como fontes para enzimas e compostos de interesse comercial. O desafio futuro está em expandir a escala desses processos, avaliar a estabilidade e o rendimento enzimático em condições industriais e promover políticas que incentivem o uso sustentável da biodiversidade microbiana amazônica em cadeias produtivas inovadoras e ambientalmente responsáveis.

5. CONCLUSÕES

Este trabalho demonstrou o potencial biotecnológico de fungos ligninolíticos isolados da serapilheira de floresta ciliar amazônica, com destaque para os gêneros *Trametes* e *Syncephalastrum*, na biodegradação de resíduos lignocelulósicos e sintéticos. A capacidade desses isolados de produzir enzimas oxidativas, como lacase (Lac), manganês peroxidase (MnP) e lignina peroxidase (LiP), foi confirmada em diferentes condições de fermentação, revelando sua aplicabilidade em processos sustentáveis voltados para a gestão de resíduos agroindustriais e o tratamento de efluentes coloridos por corantes recalcitrantes.

Os isolados de *Trametes flavida* e *T. lactinea* apresentaram destaque tanto na atividade enzimática quanto na eficiência de descoloração do corante RBBR e na degradação do caroço de açaí. A fermentação em estado sólido (SSF) com caroço de açaí como substrato revelou-se uma estratégia eficaz para indução de enzimas ligninolíticas, especialmente Lac, além de representar uma alternativa ecologicamente viável para o aproveitamento de um resíduo abundante na região amazônica. A espécie *Syncephalastrum sympodiale*, por sua vez, apresentou atividade enzimática complementar, contribuindo para o processo de degradação da lignina, e foi registrada pela primeira vez em material de serapilheira da Amazônia, ampliando o conhecimento sobre a diversidade microbiana local. E as análises morfológicas e espectroscópicas confirmaram alterações estruturais e químicas nos substratos tratados com estes fungos.

Assim, esta tese além de gerar conhecimento sobre a respeito da diversidade de fungos amazônicos, contribui para o seu aproveitamento em aplicações práticas, como a produção de enzimas industriais e a remediação de poluentes. Como perspectivas futuras, sugere-se a otimização dos processos fermentativos em escala piloto, o isolamento e purificação das enzimas com maior atividade, além da avaliação da estabilidade dessas enzimas em diferentes condições industriais. Tais avanços permitirão a transição dos resultados obtidos em laboratório para aplicações comerciais sustentáveis, promovendo o uso racional da biodiversidade amazônica. Outra perspectiva relevante é a avaliação do potencial antioxidante ou antimicrobiano dos subprodutos gerados, especialmente os compostos fenólicos, que podem apresentar propriedades bioativas com aplicações nos setores farmacêutico, cosmético e alimentício.

A continuidade dessas linhas de pesquisa poderá não apenas ampliar o conhecimento sobre a biodiversidade microbiana amazônica, mas também contribuir para o desenvolvimento de soluções sustentáveis baseadas na bioeconomia, valorizando recursos locais com foco na conservação ambiental e inovação tecnológica.

6. ANEXOS

Figura 1. Artigo de revisão oriundo do desenvolvimento do projeto de tese.

Revista CADERNO PEDAGÓGICO

REVISTA CADERNO PEDAGÓGICO – Studies Publicações Ltda.
ISSN: 1983-0882

Fungi from litter in Amazonian forest ecosystems and biocatalytic aspects in the biotransformation of residues: a review

Fungos do serapilheira de ecossistemas florestais amazônicos e aspectos biocatalíticos na biotransformação de resíduos: uma revisão

Hongos de la hojarasca de ecosistemas forestales amazónicos y aspectos biocatalíticos en la biotransformación de residuos: una revisión

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ABSTRACT

Decomposition of Amazonian litter is driven by microorganisms, particularly fungi from the Basidiomycota and Ascomycota, which produce ligninolytic enzymes such as manganese peroxidases, lignin peroxidase, and laccase. These fungi exhibit remarkable capabilities in degrading agro-industrial waste and treating contaminated wastewater, offering a sustainable approach to pollution mitigation. Research has unveiled substantial fungal diversity, notably within the Chaetosphaeriaceae and Russulaceae families. Ligninolytic enzymes demonstrate adaptability and catalytic efficiency, enabling the breakdown of complex lignin bonds into simpler molecules. This process generates compounds, including phenolic acids, methyl gallate, and vanillin, which have significant applications in the food, cosmetics, and pharmaceutical industries. Utilization of agro-industrial waste as substrates for enzyme production not only reduces costs but also fosters sustainable technological solutions. Furthermore,

Figura 2. Primeiro artigo de resultados oriundo do desenvolvimento do projeto de tese.

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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

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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

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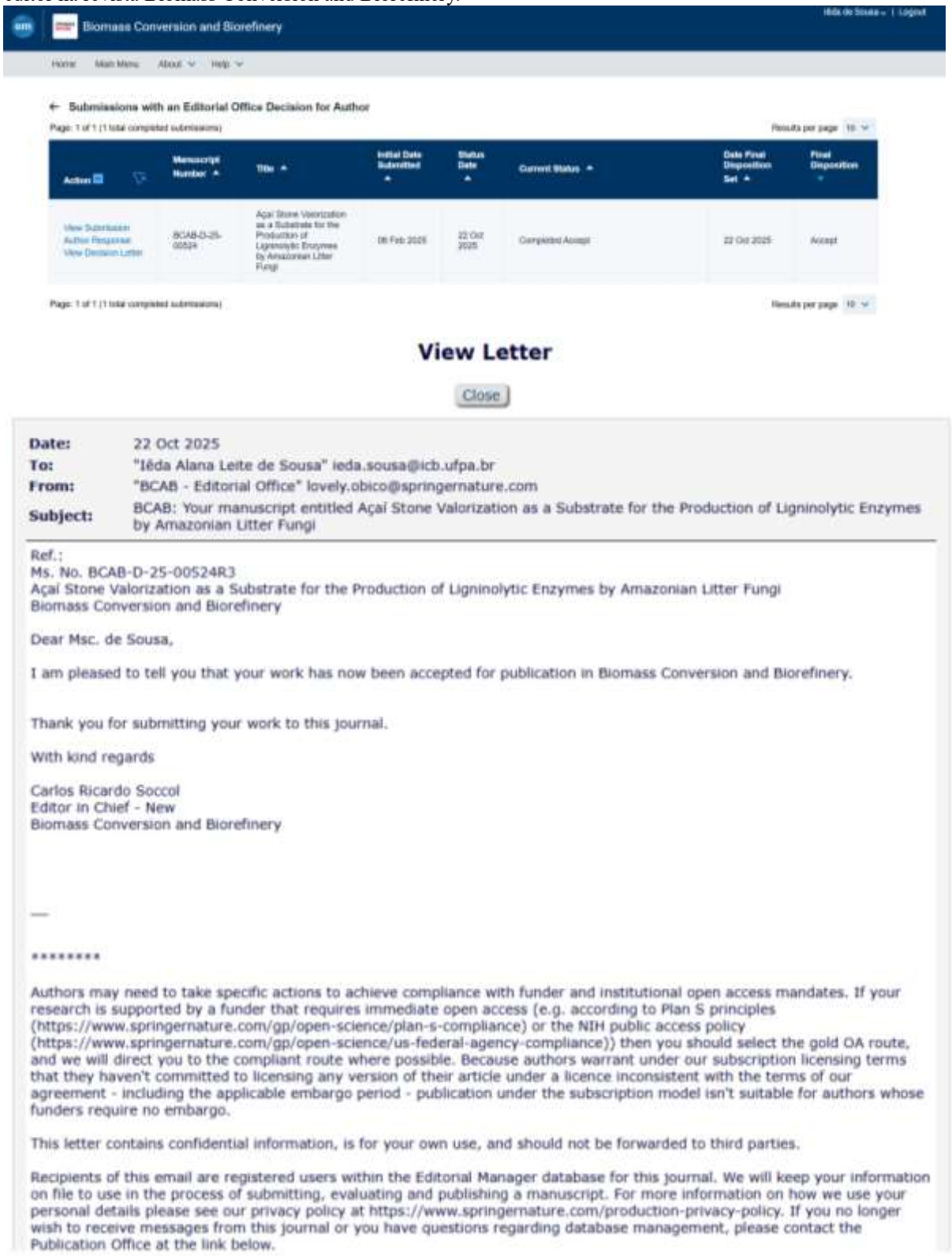
Figura 3. Segundo artigo de resultados oriundo do desenvolvimento do projeto de tese em fase de revisão pelo editor na revista Biomass Conversion and Biorefinery.



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With kind regards

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