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Laccase and lignin peroxidase production and decolorization of xenobiotic dyes by species of *Polyporaceae* (*Basidiomycota*) from South America

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ABSTRACT. *Polyporaceae* are known as wood decaying fungi with enzymatic production that plays an important role in environmental processes. Laccase (Lac) and lignin peroxidase (LiP) are two of the most explored enzymes and show significant industrial importance in degrading textile dyes, including Remazol Brilliant Blue R (RBBR), Crystal Violet (CV) and Congo Red (CR) that are some of the most commonly tested. Six strains representing six species of *Hexagonia* and *Trametes* (*Polyporaceae*) from South America were selected to evaluate Lac and LiP production and degradation of these dyes. The dataset of rDNA ITS confirmed the identity of the species, the amplification using LccF–LccR and Lip1–Lip2 primers detected Lac and LiP gene fragments in four and five strains, respectively, and the biostimulation essays of Lac and LiP showed the production of enzymes by all strains, being *T. versicolor* (VRTO 1064) and *H. hydnoides* (URM 9027) the best producers of Lac (117.839 U L^{-1}) and LiP (109.370 U L^{-1}), respectively. For RBBR, *T. villosa* (URM 8022) presented the best results (84.18 %). CR decolorization ranged from 61.37% to 68.74% by five strains, with lower results for *T. lactinea* (URM 8350) (19.9 %). Finally, *T. sanguinea* (URM 8774) had the best results for CV (85.06 %). *Trametes lactinea* (URM 8350), *T. versicolor* (VRTO 1064), and *T. villosa* (URM 8022) did not decolorize CV. Our results highlight the underexplored enzymatic potential of strains from South America and show that the strains here studied are promising alternatives to decolorize industrial textile dyes.

Keywords: Bioremediation; congo red; crystal violet; ligninolytic enzymes; remazol brilliant blue R.

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Introduction

Polyporaceae Fr. ex Corda are commonly known as wood decaying fungi characterized by high morphologically heterogeneity, with basidiomata of different colors, dimitic or trimitic hyphal system and cylindrical, broadly ellipsoid to subglobose basidiospores (Ryvarden & Johansen, 1980; Ryvarden, 1991; Hibbett & Donoghue, 1995; Ryvarden, 2015; Cui et al., 2019). Species of this family decompose the major compounds of wood, namely cellulose, hemicellulose, and lignin (Cui et al., 2019; Saini & Sharma, 2021; Suryadi et al., 2022).

The enzymatic production of *Polyporaceae* species plays an important role in environmental processes and the global carbon cycle (Othman et al., 2023). Among the enzymes produced by *Polyporaceae*, laccase EC 1.10.3.2 (Lac) and lignin peroxidase EC 1.11.1.14 (LiP) are two of the most explored in biodegradation studies (Avelino et al., 2020; Zambrano-Forero et al., 2021; Suryadi et al., 2022; Temporiti et al., 2022; Herath et al., 2024; Rosa et al., 2024). They have significant industrial importance due to their efficiency in degrading organic and phenolic compounds such as anthraquinone dyes (Avelino et al., 2020; Paredes et al., 2022; Herath et al., 2024; Rosa et al., 2024). The disposal of synthetic dyes in aquatic environments is responsible for altering the photosynthetic activity of organisms and causing the biomagnification of toxic compounds in the trophic chain, leading to recalcitrance and bioaccumulation and generating toxicity, mutagenicity and carcinogenicity (Lellis et al., 2019; Tkaczyk et al., 2020; Khan et al., 2020; Sudarshan et al., 2023). Remazol Brilliant Blue R (RBBR), Crystal Violet (CV) and Congo Red (CR) are some of the dyes most commonly used in the fabric dyeing process. They are relevant toxic dyes and, therefore, their removal from wastewater requires sustainable alternatives (Mani & Bharagava, 2016; Lellis et al., 2019; Hanafi & Sapawe, 2020; Mirza & Ahmad, 2020; Oladoye et al., 2022; Siddiqui et al., 2023).

Biotechnology investigations about *Hexagonia* and *Trametes*, two genera of *Polyporaceae*, have demonstrated the production and applicability of ligninolytic enzymes in the decolorization of industrial textile dyes, with promising results in the degradation of these compounds (Diorio et al., 2021; Laksmi et al., 2021; Munagapati et al., 2021; Ferreira-Silva et al., 2022; Thampraphaphon et al., 2022; Ajao et al., 2023; Mahdy & Suttinun, 2023; Herath et al., 2024; Martínez-Trujillo et al., 2024; Oktaviani et al., 2024; Tian et al., 2024). However, few South American species have been explored regarding their ability to produce these enzymes (Abrahão et al., 2008; Tortella et al., 2008; Fonseca et al., 2015; Araújo et al., 2020; Avelino et al., 2020; Ferreira-Silva et al., 2022; Paredes et al., 2022).

In this way, the present study aimed to analyze the production of Lac and LiP by strains of *Hexagonia* and *Trametes* species from Brazil and a species of *Trametes* from Chile and their use in the degradation of synthetic dyes, contributing to the knowledge about the applicability of South American strains in the bioremediation of these xenobiotic compounds.

Materials and methods

Polyporaceae strains

The strains *H. hydnoides* (URM 9027), *T. flavida* (URM 8773), *T. lactinea* (URM 8350), *T. sanguinea* (URM 8774), *T. villosa* (URM 8022) from Brazil deposited in the University Recife Mycology (URM) culture collection (Micoteca URM Profa. Maria Auxiliadora Cavalcanti) at the Federal University of Pernambuco, and *T. versicolor* (VRTO 1064) from Chile, deposited at the Universidad de La Frontera, were selected for molecular and enzymatic analysis after preliminary decolorization tests of RBBR on solid medium.

Molecular identification and detection of enzyme genes

Molecular analyses were carried out for the strains *H. hydnoides* (URM 9027), *T. flavida* (URM 8773), *T. lactinea* (URM 8350), *T. sanguinea* (URM 8774), *T. villosa* (URM 8022), and *T. versicolor* (VRTO 1064). The purified strains were determined by molecular techniques. DNA extraction was performed according to Góes-Neto et al. (2005) and the barcode marker ITS was amplified for taxonomic identification of the species (White et al., 1990; Lima-Júnior et al., 2014). Laccase and LiP genes fragments were amplified by primer pairs LccF–LccR and Lip1–Lip2, respectively, designed from the protein sequences performed according to Rahimlou et al. (2016).

The phylogenetic tree of ITS dataset was constructed based on Maximum Likelihood (ML) and Bayesian Analysis (BA) methods using the W-IQ-TREE software with 1000 bootstrap resamples (Trifinopoulos et al., 2016) for ML analyses and 1×10^6 generations with the MrBayes 3.2 software for BA analysis (Ronquist & Huelsenbeck, 2003). *Laetiporus montanus* Černý ex Tomšovský & Jankovský and *L. sulphureus* (Bull.) Murrill were used as outgroup following Cui et al. (2019). Sequences of each genus were recovered from GenBank using the BLAST platform and selected among those from type specimens or of material from the type locality or of reference material and with higher similarity with the sequences generated in the present study.

Laccase and lignin peroxidase activity

The enzymatic activity and decolorization tests were carried out in triplicate. The strains were cultured in test tubes with 20 mL^{-1} of malt extract medium for 20 days at room temperature (26 ± 2°C). Each sample was centrifuged at 13.000 rmp to separate the biomass, and the supernatant was collected for analysis. To the determination of Lac activity (EC 1.10.3.2), 0,5 mM of 2,2'-azino-bis-ethylbenthiazoline (ABTS) was diluted in 100 mM of sodium acetate tampon (pH 5.0) and extracted according to Buswell et al. (1995). The final volume of the reaction mixture was 1 mL^{-1} (800 µL ABTS, 100 µL sodium acetate, and 100 µL enzymatic extract). The oxidation of ABTS was measured by monitoring the increase of absorbance at 420 nm after 5 min. To determine LiP activity (EC 1.11.1.14), the oxidation of veratryl alcohol was performed according to Buswell et al. (1995), consisting of a mixture of 200 mL^{-1} containing 125 mM sodium tartrate buffer (pH 3.0), 100 mL^{-1} of veratryl alcohol (10 mM), 100 mL^{-1} of hydrogen peroxide (2 mM), and 100 mL^{-1} of enzyme extract. The reaction was initiated by the addition of hydrogen peroxide, and the absorbance was determined at 310 nm until 5 min. For this reaction, one unit of enzyme activity was defined as 1.0 mmol of product formed (ABTS and veratraldehyde, respectively) per min under assay conditions.

Decolorization

Tubes containing 10 mL^{-1} of the industrial textile dyes RBBR, CR and CV in distilled water (0.05 g L^{-1}) were prepared. The tubes were autoclaved and two discs with 0.5 mm of the strains were inoculated and incubated at 25°C (± 2°C) for 9 days. The standard curve of the maximum wavelength of dyes and the decolorization analysis was obtained in a HP-8453/UV-Visible spectrophotometer. The experiment was performed in triplicate (Ferreira-Silva et al., 2022).

Statistical analysis

The data were analyzed using the Kruskal-Walllis test to assess whether there were statistically significant differences between the medians. Pairwise comparisons using the Wilcoxon rank test were also performed to evaluate statistical significance between the strains. All tests were performed in the R environment (R Core Team 2023).

Results and discussion

Molecular identification and detection of enzyme genes

The dataset of rDNA ITS regions included 23 sequences, with 635 nucleotide sites. The best evolutionary model estimated for the alignment according to BIC was K2P + G4. The ML and BA analyses produced similar topologies, and the ML tree was chosen to represent the phylogenetic placement of the specimens (Figure 1). The six specimens were placed in two clades representing six species: *Hexagonia hydnoides* (URM 9027), *T. flavida* (URM 8773), *T. lactinea* (URM 8350), *T. sanguinea* (URM 8774), *T. versicolor* (VRTO 1064), and *T. villosa* (URM 8022).

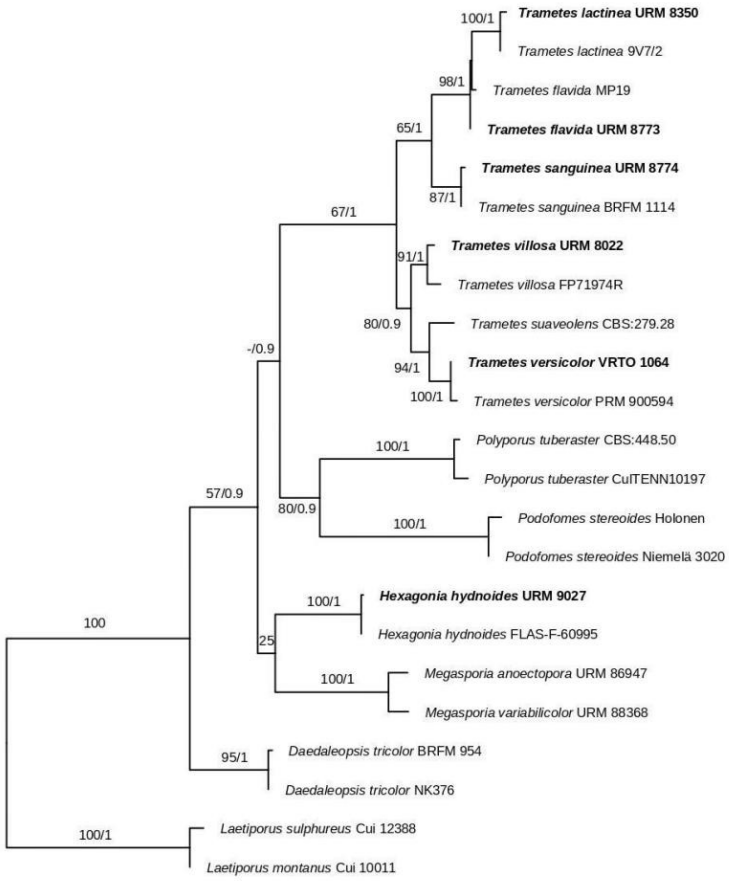

Figure 1. Maximum likelihood (ML) tree based on the ITS region for strains of the genera *Hexagonia* and *Trametes* from the URM culture collection. Bootstrap values above 50 % and Bayesian posterior probability above 0.9 are shown.

Genes coding for Lac were detected in *T. lactinea* (URM 8350), *T. sanguinea* (URM 8774), *T. versicolor* (VRTO 1064), and *T. villosa* (URM 8022), while LiP was detected in all strains, except in *H. hydnoides* (URM 9027) (Figure 2).

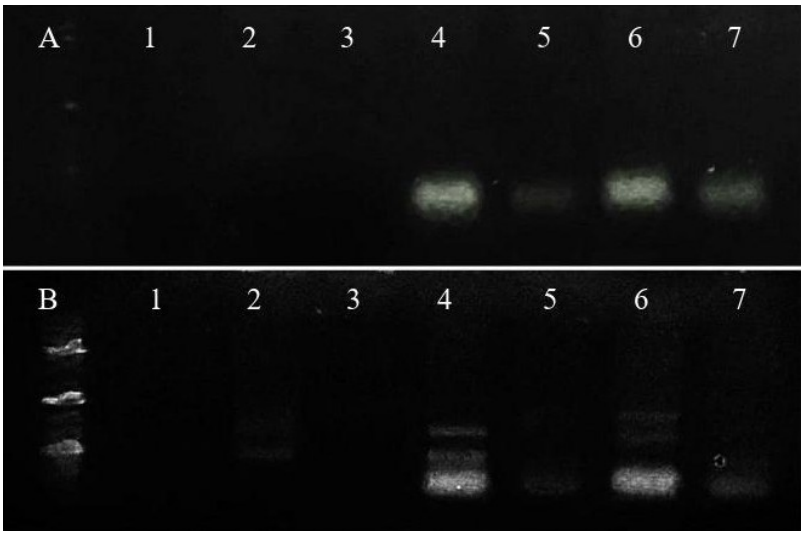

Figure 2. Gel electrophoresis of PCR purified Lac and LiP. (A) Lac gene amplification; (B) LiP gene amplification. Left to right: DNA ladder, (1) control reaction mixture without DNA, (2) *T. flavida* (URM 8773), (3) *H. hydnoides* (URM 9027), (4) *T. lactinea* (URM 8350), (5) *T. versicolor* (VRTO 1064), (6) *T. sanguinea* (URM 8774), and (7) *T. villosa* (URM 8022).

Laccase and Lignin peroxidase activity

In the Lac biostimulation essay, all strains presented activity within 20 days of growth (Table 1). *Trametes versicolor* (VRTO 1064) presented the best result, followed by *T. lactinea* (URM 8350), while *T. sanguinea* (URM 8774) presented the lowest activity (Table 1, Figure 3). *Hexagonia hydnoides* (URM 9027) had the best result in the LiP essay, much higher when compared to the other strains (Table 1, Figure 3).

Table 1. Laccase and LiP production and percentage of decolorization of RBBR, CR and CV dyes by *Hexagonia* and *Trametes* strains.

Species	Voucher	Lac (U L^{-1})	LiP (U L^{-1})	% RBBR	% CR	% CV
H. hydnoides	URM 9027	33.456	109.370	62.17	64.2	10.01
T. flavida	URM 8773	27.901	5.734	62.17	68.74	79.87
T. lactinea	URM 8350	74.753	8.172	1.06	19.9	–
T. sanguinea	URM 8774	7.808	8.602	59.4	66.42	85.06
T. villosa	URM 8022	23.765	6.810	84.18	66.53	–
T. versicolor	VRTO 1064	117.839	6.989	4.7	61.37	–

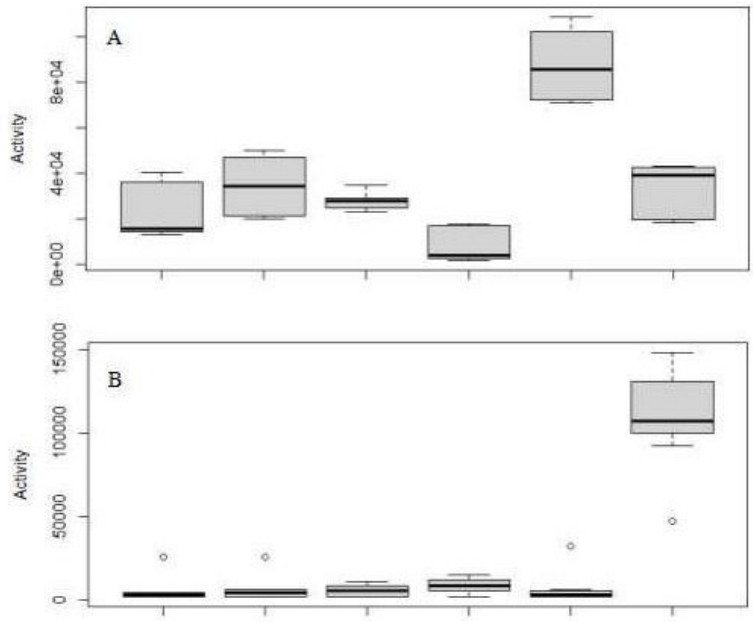

Figure 3. Laccase (A) and LiP (B) production after 20 days of experiment for the strais. Left to right: Trametes villosa (URM 8022), T. lactinea (URM 8350), T. flavida (URM 8773), T. sanguinea (URM 8774), T. versicolor (VRTO 1064) and H. hydnoides (URM 9027). *T. villosa* (URM 8022), *T. lactinea* (URM 8350), *T. flavida* (URM 8773), *T. sanguinea* (URM 8774), *T. versicolor* (VRTO 1064), and *H. hydnoides* (URM 9027).

The Kruskal-Wallis rank test showed a significant difference between Lac and LiP activity among the strains ($p < 0.01$ and $p < 0.003$, respectively) (Tables 2 and 3). The higher difference was detected between *T. flavida* (URM 8773) and *T. sanguinea* (URM 8774), and *T. sanguinea* (URM 8774) and *H. hydnoides* (URM 9027) to Lac activity (Table 2) and between *T. versicolor* (VRTO 1064) and *H. hydnoides* (URM 9027) to Lip activity (Table 3).

Table 2. Pairwise comparisons of Lac activity using the Wilcoxon rank test. P-value between strains are presented in the table and significant values are marked in bold.

	T. villosa URM 8022	*T. lactinea* URM 8350	*T. flavida* URM 8773	*T. sanguinea* URM 8774	*T. versicolor* VRTO 1064
T. lactinea URM 8350	1.0000	-	-	-	-
T. flavida URM 8773	1.0000	1.0000	-	-	-
T. sanguinea URM 8774	0.4056	0.0266	0.0061	-	-
T. versicolor VRTO 1064	0.0261	0.0750	0.0263	0.0263	-
H. hydnoides URM 9027	0.7754	1.0000	1.0000	0.0061	0.0263

Table 3. Pairwise comparisons of LiP activity using the Wilcoxon rank test. P-value between the strains are presented in the table and significant values are marked in bold.

	T. villosa URM 8022	*T. lactinea* URM 8350	*T. flavida* URM 8773	*T. sanguinea* URM 8774	*T. versicolor* VRTO 1064
T. lactinea URM 8350	1.000	-	-	-	-
T. flavida URM 8773	1.000	1.000	-	-	-
T. sanguinea URM 8774	1.000	1.000	1.000	-	-
T. versicolor VRTO 1064	1.000	1.000	1.000	1.000	-
H. hydnoides URM 9027	0.047	0.084	0.050	0.334	0.019

Laccases and peroxidases are extracellular enzymes considered the two most important subclasses of fungal enzymes used in the degradation of xenobiotic compounds. Among them, Lac and LiP are commonly reported to be related to the dye decolorization process because of their catalytic characteristic and broad-spectrum nonspecific degradative ability (Levin et al., 2019; Anita et al., 2020; Saha & Mukhopadhyay, 2020; Uribe-Arizmendi et al., 2020; Laksmi et al., 2021; Ferreira-Silva et al., 2022; Ajao et al., 2023; Laothanachareon et al., 2023; Herath et al., 2024; Martínez-Trujillo et al., 2024; Rosa et al., 2024; Tian et al., 2024).

Previous studies indicate that the production rate of Lac and LiP varies according to the species or methodology used during the analyses (Abrahão et al., 2008; Tortella et al., 2008; Mounguengui et al., 2013; Avelino et al., 2020; Uribe-Arizmendi et al., 2020; Laksmi et al., 2021; Brazkova et al., 2022; Ferreira-Silva et al., 2022; Suryadi et al., 2022; Ajao et al., 2023; Mahdy & Suttinun, 2023; Oktaviani et al., 2024). *Trametes hirsuta* D7 produced a maximum Lac activity of 148 + 27 U L^{-1} in Alam et al. (2021), while Avelino et al. (2020) obtained a production of 35,915 U L^{-1} for Lac and < 10 U L^{-1} for LiP with a consortium of different species of *Trametes*. Further, *T. lactinea* (URM 8350) and *T. villosa* (URM 8022) presented a rate of 27,833 and 0.250 U L^{-1} of Lac, respectively, in the work of Ferreira-Silva et al. (2022).

In tests using farnesol in pineapple waste in solid-state fermentation, Backes et al. (2023) obtained 130.95 ± 2.20 U g^{-1} with *T. sanguinea* strains, while *T. versicolor* produced 77.88 ± 5.62 U g^{-1} under the same conditions. In a study employing the inducers acid cellulignin (CA), $MnSO_4$ (Mn^{2+}), $CuSO_4 \cdot 5H_2O$ (Cu^{2+}), veratryl alcohol (VA), Tween 80 (T80), and the carbon-to-nitrogen ratio (C/N), Souza-Junior et al. (2022) reported high production levels of Lac (64,580 U L^{-1}) and LiP (80.72 U L^{-1}) by *T. sanguinea*, highlighting the potential for optimization as a strategy to enhance ligninolytic enzyme yields. *Trametes flavida* also showed promising enzymatic activity, with Lac (452.13 U L^{-1}) and LiP ranging from 42.25 to 63.64 U L^{-1}, as reported by Sousa et al. (2024) using a strain isolated from the Brazilian Amazon rainforest.

Hexagonia has been much less explored in terms of enzymatic activity compared to *Trametes* (Abrahão et al., 2008; Yadav et al., 2010; Kandasamy et al., 2016; Mounguengui et al., 2013; Arumugam & Uthandi, 2024). *Hexagonia hirta* did not produce significant levels of LiP in the study by Abrahão et al. (2008), whereas *H. tenuis* reached 2.90 U mL^{-1} according to Yadav et al. (2010). However, the *H. hirta* strain MSF2 produced up to 1585.24 U/g of Lac in a study focused on optimizing enzyme production in solid-state fermentation (Arumugam & Uthandi, 2024), suggesting that appropriate cultivation conditions can greatly influence enzyme output.

Decolorization

The wavelength with the highest optical density was considered the maximum wavelength (λmax). The λmax was 594 nm for RBBR, 502 nm for CR and 575 nm for CV (Figure 4).

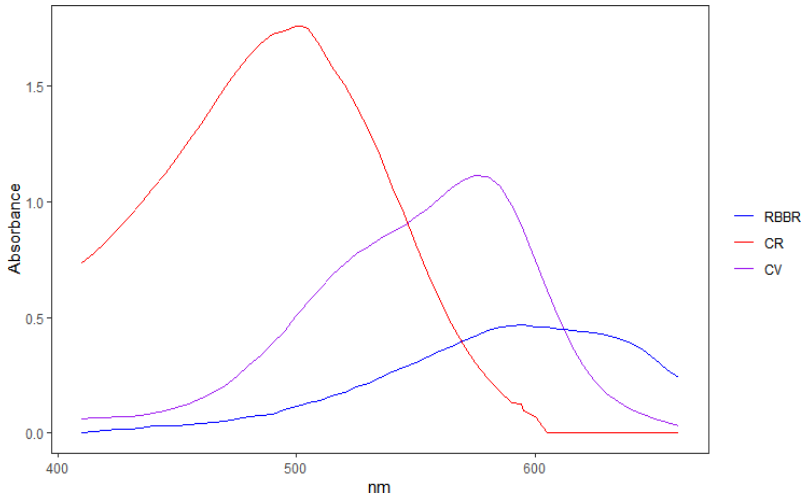

Figure 4. Standard curve of the maximum wavelength of RBBR, CR and CV.

All strains were able to degrade RBBR and CR (Figure 5), while only three decolorized CV (Table 1). For RBBR, *T. villosa* (URM 8022) presented the best results, while *T. lactinea* (URM 8350) and *T. versicolor* (VRTO 1064) the worst. CR decolorization ranged from 61.37% to 68.74% by all strains except for *T. lactinea* (URM 8350), which had the lowest result (Table 1). For CV, *T. sanguinea* (URM 8774) and *T. flavida* (URM 8773) had the best results, while *H. hydnoides* (URM 9027) the worst (Tabel 1). *Trametes lactinea* (URM 8350) and *T. villosa* (URM 8022) not only did not decolorize CV, but also produced a CV concentration higher than 100%. Possibly these strains did not use the dye in their metabolic process and consumed only water when growing, thus increasing the dye concentration in the solution. Interestingly, *T. villosa* (URM 8022), that did not present results for CV, and *T. lactinea* (URM 8350), which presented the worst results for RBBR and CR, were tested by Ferreira-Silva et al. (2022) and showed decolorization rates of 96.11% and 81.4%, respectively, for indigo carmine dye.

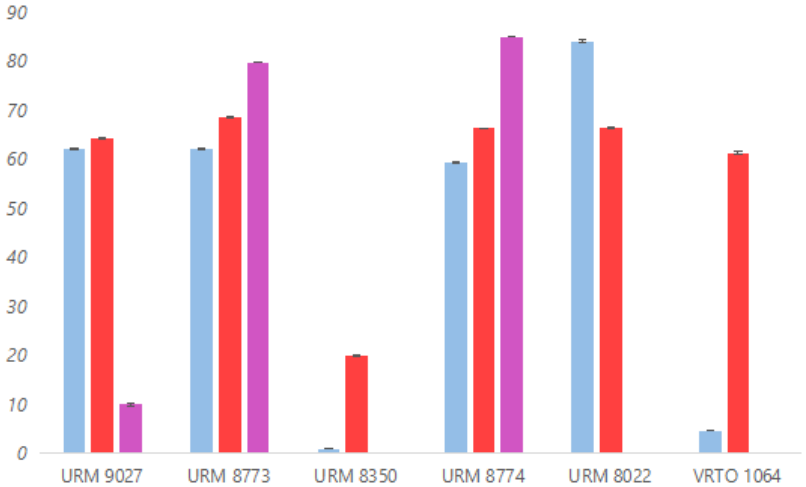

Figure 5. Decolorization of RBBR (blue), CR (red) and CV (purple) dyes by the strains *H. hydnoides* (URM 9027), *T. flavida* (URM 8773), *T. lactinea* (URM 8350), *T. sanguinea* (URM 8774), *T. villosa* (URM 8022), and *T. versicolor* (VRTO 1064).

The decolorization rate is closely associated with enzyme production. However, our results showed that some strains, such as *T. flavida* (URM 8773) and *T. sanguinea* (URM 8774), which were not among the top producers of Lac and LiP, as well as *H. hydnoides* (URM 9027), in which the corresponding Lac and LiP genes were not detected, still exhibited significant decolorization rates across all dyes tested (Figure 5). In this case, the production of other enzymes not evaluated in this study were possibly responsible for the high rates of decolorization, suggesting the need to perform a more detailed enzymatic screening in future studies, for example, with manganese peroxidase (MnP), versatile peroxidases, and dye-decolorizing peroxidases, which are mainly produced by fungi and depend on the substrate as the reducing agent (Temporiti et al., 2022; Rosa et al., 2024; Tian et al., 2024).

Species of *Trametes* are commonly studied with different methodologies, including free or immobilized enzymes that can be used for decolorization of synthetic industrial dyes, including RBBR (Anita et al., 2020; Avelino et al., 2020; Alam et al., 2021; Laksmi et al., 2021; Mahdy & Suttinun, 2023; Oktaviani et al., 2024), CR (Yang et al., 2009; Bhattacharya & Das, 2011; Si & Cui, 2011; Munagapati et al., 2021; Diorio et al., 2021), and CV (Moturi & Charya, 2009; Yang et al., 2009; Si & Cui, 2011; Moldes et al., 2012; Ajao et al., 2023). In contrast, *Hexagonia* species have not yet been widely explored, and only two species have been studied so far, *H. apiaria* and *H. hirta*, with good results for the decolorization of CR, Reactive Blue 4, Orange G, and Orange II by solid-state fermentation (Abrahão et al., 2008) or in the presence of different substrates (Mounguengui et al., 2013).

Optimization of cultivation conditions, like immobilization of strains and enrichment of the culture medium, is an alternative to improve experimental results. Mahdy & Suttinun (2023) reported that veratryl alcohol and glucose supplementation accelerated the rate of *T. hirsuta* decolorization by up to 97% RBBR removal. Saha & Mukhopadhyay (2020) showed that the use of ABTS as the electron mediator also helped in enhancing the oxidizing capability of the enzyme with CR decolorization by *T. versicolor*. Similarly, the use of $CuSO_4$ as an inducer provided higher percentages in tests of RBBR decolorization (Laksmi et al., 2021). The use of activated Light Expanded Clay Aggregate (LECA) for immobilization of strains enhanced the activity and stability of enzymes and produced decolorization rates of 99.29% (Anita et al., 2020) and 95% (Alam et al., 2021) of RBBR by strains of *T. hirsuta*. In a study about adsorption of CR by biomass of *T. versicolor* in aqueous medium, Munagapati et al. (2021) showed that agitation speed, contact time, adsorbate concentration, and temperature standards improved the results and that the strain could be used as an efficient low-cost sorbent.

Fungi strains used in dye decolorization are an alternative for the bioremediation of contaminated water because the specimens may have an increased growth under a dye stress environment with the addition of nutrients (Tiwariet al., 2023). Previous studies have already shown the efficiency of fungi in the textile effluent treatment process and their use as a sustainable alternative for the recovery of contaminated water bodies (Alexander & Thatheyus, 2021; Paredes et al., 2022; Herath et al., 2024; Rosa et al., 2024).

Conclusion

The results highlight the underexplored enzymatic potential of Lac and LiP by strains of *Polyporaceae* from South America and show that species of *Hexagonia* and *Trametes*, particularly *T. flavida* (URM 8773) and *T. sanguinea* (URM 8774), are promising alternatives for the treatment of the industrial textile dyes RBBR, CV and CR.

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