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Original article

Trametes lactinea and *T. villosa* collected in Brazil are able to discolor indigo carmine

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ABSTRACT

Dyes used in the textile industry contribute significantly to the increase of water pollution as they are disposed of, most of the time, without proper treatment. Indigo carmine is a synthetic dye widely used in the coloring of jeans and is considered difficult to remove, causing irreversible damage to the food chain in ecosystems. Mycomediation appears as an economical and sustainable way to treat textile effluents, and this work tested three strains of *Trametes* collected in Brazil against the ability to discolor the indigo carmine and also the activity of laccase, lignin and manganese peroxidases. The experiment was carried out in Kirk medium under static, non-sterile condition, at ± 28 °C for 120 h. *Trametes lactinea* (URM8350) discolored 81.40 % of the indigo carmine, *T. lactinea* (URM8350) 85.09 % and *T. villosa* (URM8022) 96.11 %. Laccase was detected in all specimens. Manganese peroxidase was detected in *T. villosa* and *T. lactinea* (URM8354), while lignin peroxidase was not detected in any of the isolates. The ability of *T. lactinea* to discolor dyes is reported for the first time. The discoloration rates demonstrate the ability of the strains to discolor carmine indigo and their promising use in the discoloration processes in wastewater from the textile segment.

Keywords: *Trametes*, Basidiomycota, indigo carmine, mycoremediation, pollutants, textile industry

Introduction

Population growth allied to industrial development has caused serious environmental problems, such as pollution of soil and water by chemicals (Zhang *et al*. 2011; Rodríguez-Couto 2017; Choi 2021). Among the pollutants, the effluents from paper, cellulose, textile and petrochemical industries and from alcohol distilleries contain aromatic, recalcitrant and xenobiotic compounds, responsible for the intense color and toxicity of wastewater (Sharma *et al*. 2011; Almeida *et al*. 2016; Chowdhury *et al*. 2020).

The textile sector is considered to be one of the largest sectors in the manufacturing industry in the world. In Brazil alone, the segment is responsible for generating 1.5 million direct jobs, being considered the largest textile chain in the West (Abit 2020) and employs 75 million people worldwide (De Oliveira *et al*. 2021). However, its expansion

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and maintenance cause damage to the environment, since the dyeing and washing processes of the fabric generate a large volume of effluents containing xenobiotic compounds, including dyes (Rodríguez-Couto 2009; Singh 2017).

Synthetic dyes are designed to resist discoloration, high temperatures and antioxidant chemicals. Therefore, they have a stable chemical structure, usually recalcitrant, highly toxic, mutagenic and carcinogenic (Baughman & Weber 1994; Vacchi *et al*. 2017; Berradi *et al*. 2019; Benkhaya *et al*. 2020). The presence of dyes in water bodies, even in small concentrations, interferes with the trophic chain in aquatic ecosystems, as it prevents the penetration of the light necessary for photosynthesis, thus causing serious environmental problems (Kunz *et al*. 2002; Berradi *et al*. 2019; Benkhaya *et al*. 2020; Chowdhury *et al*. 2020). The production of dyes reaches 7 x 10^7 tons per year in the world, of which Brazil accounts for 2.6 %. Of this production, 10-20% is transformed into wastewater (Carneiro & Zanoni 2016; Sen *et al*. 2016; Benkhaya *et al*. 2020). Indigo carmine synthetic dye belongs to the group of indigoids and has a ketone group (C = O) in its chemical structure. It is widely used in the food, paper, cellulose and textile industries, being indispensable in dyeing denim (Choi 2020 & 2021; Chowdhury *et al*. 2020). Considered chemically stable and difficult to remove when discarded in the environment (Guaratini & Zanoni 2000; Choi 2020), it is one of the main causes of wastewater coloring originated from textile effluents. The yarn dyeing process requires large amounts of water: it is estimated that for each kilogram of manufactured product, 200 to 400 liters of water are required, 88 % of which will be discarded with more than 10,000 by-products, as chlorinated compounds, salts, auxiliary chemicals, surfactants and especially dyes (Sen *et al*. 2016; Almeida *et al*. 2016; Singh 2017; Choi 2020; De Oliveira *et al*. 2021).

There are numerous chemical and physical dye removal strategies implemented over the years. These include adsorption, flocculation, photodegradation, membrane filtration and coagulation (Adenan *et al*. 2020). The treatment of wastewater from the textile industry, especially discoloration, is expensive and not always effective as it can generate a large volume of sludge and generally requires the addition of other chemical additives dangerous to the environment (Singh 2017). Therefore, the search for low-cost biological alternatives is urgent. Biological removal of dyes can occur through three mechanisms: biosorption, bioaccumulation and/or biodegradation (Sen *et al*. 2016; Singh 2017; Chowdhury *et al*. 2020). Biosorption involves trapping the dye by binding the dye molecules to the functional groups present on the cell wall. Subsequently, the dyes are accumulated intracellularly in the living cells through a process known as bioaccumulation. The biodegradation process involves the breakdown of dye molecules by enzymes produced by microbial cells, where complete eradication of dyes is possible (Jasińska *et al*. 2015; Adenan *et al*. 2020). Mycoremediation emerges as an economically viable and ecologically effective biological alternative, as fungi are able to adapt to various pH and temperature ranges, in addition to producing extracellular lignolytic enzymes such as laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13), which can mineralize xenobiotic and recalcitrant compounds (Tien & Kirk 1984; Ellouze & Sayadi 2016; Sen *et al*. 2016; Singh 2017; Akhtar & Mannan 2020). White rot fungi, mainly Agaricomycetes, have been identified as a potentially efficient biological tool in the removal of synthetic dyes from textile effluents (Wesenberg *et al*. 2003; Ali 2010). Some studies have demonstrated the efficacy of *Trametes* species in the degradation of phenolic compounds in effluents from the paper industry, degradation of pentachlorophenol and synthetic dyes in textile effluents (Rodrígues-Couto 2009, Pinedo-Rivilla *et al*. 2009; Pandey *et al*. 2017). However, in Brazil, which has a high mycodiversity (Forzza *et al*. 2010; Maia *et al*. 2015), little is known about the potential for degradation and discoloration of the species collected in the country (Balan & Monteiro 2001; Lyra *et al*. 2009; Motato-Vasquéz *et al*. 2016; Araújo *et al*. 2020).

Thus, the aim of the present study was to test three strains of two species of *Trametes* collected in Northeast Brazil for the ability to remove the indigo carmine used in the customization of denim and to quantify lignolytic enzymes laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) produced after the experiment.

Materials and methods

Microorganism: collection and cultivation conditions

Specimens of *Trametes lactinea* (Berk.) Sacc. were collected on the campus of the Universidade Federal de Pernambuco (08°03'07"S 34°56'59"O, Atlantic Forest domain) in November 2019, while *T. villosa* (Sw.) Kreisel was collected in the Chapada Diamantina National Park (13°14'31"S, 41°40'7" O, Caatinga domain) in March 2015.

For culture, three fragments with a diameter of 5 mm were removed from the basidiomata and transferred to Petri dishes containing 2 % malt extract supplemented with chloramphenicol (20 mg L^{-1}). The plates were kept at 28 °C for 7 days or until mycelial development (Cavalcanti 1972; Stalpers 1978; Motato-Vásquez *et al*. 2016).

The cultures obtained were deposited in the Collection of Cultures Micoteca URM of the Department of Mycology of the Center Biosciences of the Federal University of Pernambuco under registration numbers URM8350 (*T. lactinea*), URM8354 (*T. lactinea*) and URM8022 (*T. villosa*).

Microorganism: identification

The morphological identification of the basidioma and DNA analyses followed the usual for this group (Gomes-

Silva *et al*. 2010; Verma *et al*. 2018; Xavier *et al*. 2020). The resulting ITS and LSU sequences were subjected to BLASTn search in NCBI to verify the closest identification match.

Qualitative tests for phenoloxidase

The qualitative analysis of phenoloxidase activity was verified using the Bavendamm method, which allows observing the production of cellular oxidase such as laccase, lignin peroxidase and manganese peroxidase, in addition to tyrosine and catechol oxidase (Nobles 1965; Melo & Azevedo 2008). In our assay, an agar block with diameter of 5 mm was removed from colonies with 7 days of cultivation and transferred to the center of the Petri dishes with diameter of 90 mm containing solid malt agar medium plus tannic acid (0.5 %). The control was prepared under the same conditions without tannic acid. All procedures were performed under aseptic conditions. After 3 days of incubation, the formation of a brown halo was observed in the colony reverse, indicating a positive reaction to produce phenoloxidases. These halos were measured with the aid of a digital caliper. The enzyme index was measured through the relationship between the average diameter of the degradation zone and the average diameter of the colony, expressed in millimeters (Hankin & Anagnostakis 1975; Silva *et al*. 2019).

Discoloration tests

The indigo carmine dye was of analytical grade purchased from Sigma-Aldrich Corporation, St. Louis, Missouri, USA and used at a concentration of 50 mg L^{-1}. The experiment was carried out in Erlenmeyer flasks (250 mL) containing 50 mL of Kirk medium without sterilization (Kirk & Farrell 1987) plus 5 disks of the fungal mycelium with diameter of 5 mm grown in 2 % malt extract after 7 days. The vials were kept for 120 h at ± 28 °C under static condition; 2 mL aliquots were removed from the broth and centrifuged at 1500 rpm for 15 min at 4 °C. The percentage of discoloration (D %) was calculated according to equation: D % = [(Abscontrol-Abstest)/Abscontol)] × 100, by which abscontrol (absorbance of the control) and abstest (absorbance with fungal treatment) denote the percentage of discoloration the at 610 nm. As a control, Kirk medium was used with the dye without fungal inoculum. The experiments were carried out in triplicate.

The discolored broth was used to quantify the production of the enzymes laccase, manganese peroxidase and lignin peroxidase.

Enzymatic assays

The enzymatic activity of the laccase was verified by measuring the oxidation of ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) 0.5 mM in 100 mM sodium acetate buffer (pH 5) plus the enzyme broth. The final volume of the reaction was 1 mL (800 µL of ABTS + 100 µL of sodium acetate buffer + 100 µL of crude extract). Activity was calculated based on ABTS molar absorptivity (ε420nm = 36,000 M^{-1}. Cm^{-1}) (Bourbonnais *et al*. 1997; Boran 2019). The activity of lignin peroxidase was verified through the oxidation of the mixture composed of 375 µL of 0.25 M sodium tartrate buffer at pH 3.0; 125 µL of 10 mM veratryl alcohol; 50 µL of 2 mM hydrogen peroxide and 500 µL of enzymatic extract. The reaction was monitored on a spectrophotometer at a length of 310 nm (ε310nm = 9,300 M^{-1}. Cm^{-1}) (Tien & Kirk 1984). The reaction mixture for manganese peroxidase (1mL) was composed of 100 µL of phenol red (0.01 %), 100 µL of sodium lactate (25 mM), 50 µL of MnS04 (100 mM), 200 µL of egg albumin (0.5 %), 50 µL of H202 (100 µM) in 20 mM sodium succinate buffer (pH 4.5) and 500 µL of enzymatic extract. The reactions were carried out at 30 °C for 5 minutes and stopped with the addition of 40µL of 2N NaOH. The absorbance was monitored at 610 nm (Kuwahara *et al*. 1984). A unit of enzymatic activity was defined as 1 µmol of the product formed per minute. All tests were performed in triplicate.

Statistical analysis

The decolorization test was carried out in triplicate. The data were analyzed using analysis of variance (ANOVA) with the software Statistical Package for the Social Sciences (SPSS) version 24.0. The Tukey-Kramer multiple comparison test (honestly significant difference, HSD, $P < 0.05$) or paired t test ($P < 0.05$) was also performed to evaluate statistical significance between the mean values.

Results and Discussion

Morphological and molecular identification

The specimens were morphologically identified as *Trametes lactinea* (URM8350, URM8354) and *T. villosa* (URM8022). DNA analyses resulted in one ITS sequence for each specimen *T. lactinea* URM8350 (MW578797), *T. lactinea* URM8354 (MW578798) and *T. villosa* URM8022 (MW578795) and LSU sequences for both *T. lactinea* URM8350 (MW553720), *T. lactinea* URM8354 (MW553721) and *T. villosa* URM8022 (MW553718). BLASTn search confirmed the original identifications.

Detection of phenoloxidases

All strains tested showed a dark amber halo in three days of the experiment, evidenced by the degradation of tannic acid and the production of phenoloxidases: diameter of 80 mm for *T. villosa* (URM8022), of 90 mm for *T. lactinea* (URM8350) and of 80 mm for *T. lactinea* (URM8354). According to Bavendamm (1928), these amber-colored diffusion zones around the fungal colony are the result of the oxidation of phenolic acid produced by extracellular

phenoloxidases. The detection of phenoloxidases in microorganisms is used as a way to select promising strains with the potential for degradation of complex compounds to be used in studies of degradation of recalcitrant compounds. The production of phenoloxidase complex enzymes is associated with the discoloration of synthetic dyes due to the similarity in the chemical structure of the dyes and the components of lignin (Melo & Azevedo 2008; Arora & Sharma 2010; Sen *et al*. 2016; Singh 2017).

Discoloration of indigo carmine

Trametes villosa (URM8022), *T. lactinea* (URM8350) and *T. lactinea* were all able to degrade indigo carmine (Fig. 1) at high rates (Tab. 1). The results referring to the percentage of discoloration were submitted to analysis of variance (ANOVA) and the effects were considered significant for $p < 0.05$. All groups showed values of F (26.60) greater than the values, indicating that there is a significant difference in all experiments performed in the present work.

Species of *Trametes* are well studied for discoloration of various synthetic dyes: *T. trogii* discolored 97 % of the remazol brilliant blue (Zouari-Mechichi *et al*. 2006); *T. hirsuta*, 94 % indigo carmine, 85 % of Bromophenol Blue, 41 % of Methyl Orange and 47 % Poly R-478 (Rodríguez-Couto *et al*. 2006); *T. membranacea*, 99.2 % of bromophenol blue and 71.8% of methylene blue (Lyra *et al*. 2009); *T. trogii,* 8 % of indigo carmine in the first hour of experiment (Grassi *et al*. 2011), 69% of Janus Green and 6 % of Poly R-478 (Levin *et al*. 2010); *T. pubescens*, 59 % of Bemaplex Navy M-T and 50 % of Bezaktiv Blue BA (Rodríguez-Couto 2014); *T. versicolor,* 44.74% of blue indigo 24 hours after the maximum recorded activity of laccase (Lopes *et al*. 2014) and 93.5 % of Remazol Brilliant Yellow 3-GL (Asgher *et al*. 2016); *T. ljubarskyi*, 97.7 % of reactive violet 5 (Goh *et al*. 2017); *T. villosa,* 93.8 % of acid orange 142 (Ortiz-Monsalve *et al*. 2019); and *T. polyzona,* 90 % at 100 mg L^{-1}, 91 % at 150mg L^{-1} and 93 % at 200 mg L^{-1} of indigo carmine (Uribe-Arizmendi *et al*. 2020). However, *T. lactinea* has not been tested before for discoloration of indigo carmine. Also, studies of discoloration of indigo carmine using species, not only of *Trametes*, collected in Brazil are scarce.

Lyra *et al*. (2009) found that *T. membranacea* collected in the Atlantic Forest was able to discolor 99.2 % of the bromophenol blue and 71.8 % of the methylene blue in 10 days, while Lopes *et al*. (2014) obtained efficient results in 44.78 % in 5 days. More recently, Ortiz-Monsalve *et al*. (2019) tested *T. villosa*, also collected in the Atlantic Forest, for discoloration of acid orange 142 and observed discoloration of 93.8 % in 264 h of incubation. To date, our study is the first report of discoloration of indigo carmine and quantification of lignolytic enzymes using species of *Trametes* collected in Brazil.

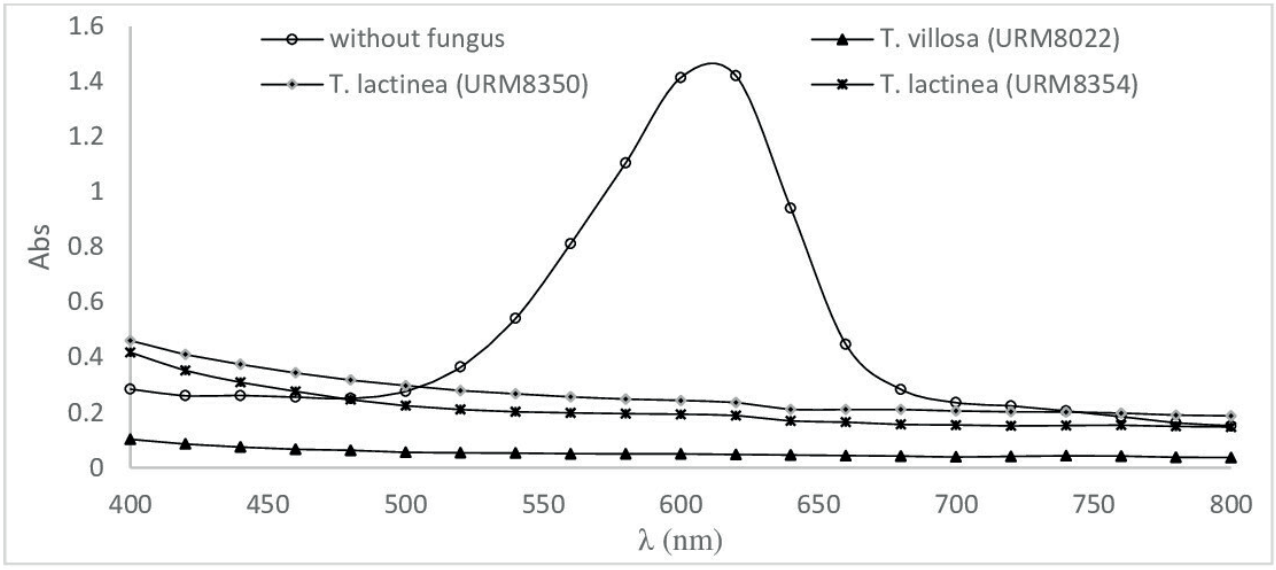

Figure 1. Discoloration of the indigo carmine dye (50 mg L^{-1}) by three strains of *Trametes* during 120 h at 28 °C under static condition.

Table 1. Percentage of discoloration and production of laccase (Lac) and manganese peroxidase (MnP) enzymes in units per liter (U/L) by *Trametes* strains after decolorization of indigo carmine dye for 120 h.

Fungi	Lac (U/L)	MnP (U/L)	% Discoloration
T. villosa (URM8022)	27.833 ± 0.031	3.408.065 ± 31.70	96.11 ± 0.86
T. lactinea (URM8350)	0.250 ± 0.002	-	81.40 ± 3.40
T. lactinea (URM8354)	0.750 ± 0.003	3.677.125 ± 25.36	85.09 ± 2.73

(-) Not detected

Quantification of detected enzymes

In the present study, the production of enzymes was detected (Tab. 1). The low enzyme indices observed for laccase may be related to the presence of the dye, as found by Novotný *et al*. (2001), who observed that the presence of dye decreased the detection rates of laccase and manganese peroxidase in a lineage of *Irpex lacteus*, as well as the mycelial development of the fungus. Trombini & Obara-Doi (2012) obtained 99.97 % of discoloration using *Ganoderma* sp. and low laccase indices, showing the action of another enzyme or other mechanisms involved in the discoloration process. Dye discoloration process may involve the participation of enzymes as well as the association of other mechanisms such as adsorption involved in the discoloration process (Novotný *et al*. 2001; Rodríguez-Couto *et al*. 2004; Srinivasan & Viraraghavan 2010). Several studies indicate that laccase acts as the enzyme responsible for discoloration (Levin *et al*. 2004; Rodríguez-Couto *et al*. 2004; Rodriguéz-Couto *et al*. 2006; Zeng *et al*. 2011; Yuan *et al*. 2012; Younes *et al*. 2015; Orzechowski *et al*. 2018; Uribe-Arizmendi *et al*. 2020). However, the participation of manganese peroxidase has also been observed in some discoloration studies (Eichlerová *et al*. 2007; Grassi *et al*. 2011; Li *et al*. 2015; Zhang *et al*. 2016).

In the present study, the indices of discoloration of indigo carmine were well above the rate observed by Lopes *et al*. (2014), Rodríguez-Couto (2014) and Levin *et al*. (2010). The discoloration time observed in the present study was relatively better if compared to other studies. Uribe-Arizmendi *et al*. (2020) carried out their experiments in 21 days (*T. polyzona,* 90 % at 100 mg L^{-1}, 91 % at 150mg L^{-1} and 93 % at 200 mg L^{-1} of indigo carmine), Ortiz-Monsalve *et al*. (2019) in 264 h (*T. villosa*, 93.8 % of acid orange 142), Lyra *et al*. (2009) in 10 days (*T. membranacea*, 99.2 % of bromophenol blue and 71.8 % of methylene blue), and Zouari-Mechichi *et al*. (2006) after three weeks (*T. trogii*, 97 % of the remazol brilliant blue). Generally, studies that report good results of dye discoloration in a shorter time are those that use optimization of the enzymes of interest with addition of the dye after enzymatic production, commonly laccase and/or manganese peroxidase (Campos *et al*. 2001; Rodriguez-Couto *et al*. 2006; Li *et al*. 2015; Wang *et al*. 2019; Xu *et al*. 2020).

The chemical treatment processes of indigo carmine generate potentially dangerous by-products and sludge, causing serious environmental pollution. The treatment with indigoids using the enzymatic arsenal of fungal species has been considered a promising strategy at an environmental and economic level (Nyanhongo *et al*. 2007; Mugdha & Usha 2012; Li *et al*. 2015). Species belonging to the genus *Trametes* can produce multiple isoforms of Lac and MnP expressed under different cultivation conditions. However, LiP, when observed, is produced in low quantities (Choi 2021). Lacase contains copper polyphenoloxidases, produces four free electrons that react with phenolic and non-phenolic molecules and is one of the few enzymes capable of catalyzing the reduction of four electrons of molecular oxygen to water and even produced in small quantities can act in the degradation of recalcitrant compounds. The catalytic efficacy of Lac and MnP in the removal of recalcitrant compounds is due to the high redox potential, activity and stability of these enzymes, whether in a raw or purified state. However, other enzymes may be involved in the discoloration process (Nyanhongo *et al*. 2007; Campos *et al*. 2016; Zheng *et al*. 2017; Xu *et al*. 2020; Choi 2021).

Species of Agaricomycetes that cause white rot have an arsenal of degradable lignolytic enzymes that can be used in bioremediation processes. These enzymes are expressed according to the composition of the substrate and the lineage used. The interest in identifying promising strains has been increasing as an effort to minimize and/or treat environments polluted or degraded by anthropic action. Knowing the enzymatic potential of neotropical species is essential in view of the fungal megadiversity in these still unexplored but threatened environments. The results obtained here proved that the *T. lactinea* strains (URM8350), *T. lactinea* (URM8354) e *T. villosa* (URM8022), collected in the Northeast of Brazil, showed a significant percentage of indigo carmine discoloration in a short period of time and at a low cost. In this work, it was possible to detect the production of Lac and MnP after dye removal, but LiP was not detected under the conditions of this experiment. The present work allowed, therefore, to identify promising strains of the genus *Trametes* that can be used to remove synthetic dye from textile effluents. Future studies of enzyme optimization and growing conditions need to be better studied for use on an industrial scale. The understanding of the enzymatic mechanisms acting in the discoloration process presented in the present study, needs to be elucidated. This study presented the first report of use for removing a synthetic dye from the *T. lactinea* strain. The results presented in this work, even if preliminary, show the potential of the studied strains. The strains *T. lactinea* (URM8350), *T. lactinea* (URM8354) and *T. villosa* (URM8022), collected in Northeastern Brazil, showed significant percentage of discoloration of indigo carmine in a short time and at a low cost and their Lac and MnP were efficient in discoloration of the dye. The present work allowed, thus, the identification of promising strains of the genus *Trametes* that can be used in the treatment of textile effluents. Further studies will be necessary to verify the toxicity level of the discoloration product.

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