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Neopestalotiopsis species presenting wide dye destaining activity: report of a mycelium-associated laccase

Miriam Marzall-Pereira[a], Daiani Cristina Savi[a], Elisandro Cesar Bruscato[a], Carolina Heyse Niebisch[b], Jaime Paba[b], Rodrigo Aluízio[a], Lisandra Santos Ferreira-Maba[a], Lygia Vitoria Galli-Terasawa[a], Chirlei Glienke[a], Vanessa Kava[a,*]

[a] Departamento de Genética, Universidade Federal do Paraná. Curitiba, PR, 81531-980, Brazil
[b] Departamento de Bioquímica, Universidade Federal do Paraná. Curitiba, PR, 81531-980, Brazil

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ABSTRACT

Wastewaters from textile dyeing industries represent an ecological concern, notably due to the known toxicity of azo dyes to the local microbiome and human health. Although physicochemical approaches are the rule for the treatment of industrial effluents, biological strategies such as enzyme-mediated dye destaining is a promising alternative. Notwithstanding a broad range of microorganisms, including fungi, algae, yeast, and bacteria, display dye-destaining properties, most of the literature has focused in ligninolytic fungi, leaving other classes of organisms somehow ignored. In this study, six endophytic strains isolated from *Maytenus ilicifolia* were studied for their destaining activity. The phylogenetic and morphological analysis allowed the identification of strain LGMF1504 as *Neopestalotiopsis* sp. LGMF1504 that decolorized several commercial dyes as the result of a mycelium-associated laccase. The enzyme expression was modulated by carbon and nitrogen content in the culture medium, it was weakly affected by the presence of aromatic compounds and metal ions while some common laccase mediators improved the destaining activity onto dye substrates. The best culture condition observed for laccase activity was a basic culture medium containing 5 g L^{-1} starch and 15 g L^{-1} ammonium tartrate. The laccase activity showed low substrate specificity and almost unaltered performance in a wide range of pH values and NaCl concentrations, suggesting the potential of *Neopestalotiopsis* sp. LGMF1504 for biodegradation approaches.

1. Introduction

Among the huge diversity of synthetic substances produced by humans, those resulting from the activity of the textile industry denote a high environmental concern (Khandare and Govindwar, 2015). Textile dyeing and processing of textile fibers result in the release of large volumes of effluents into water streams, which contain high loads of dyes and several other chemical compounds, representing a significant threat to environmental homeostasis (Saratale et al., 2011).

Several approaches are used to get rid of colored effluents; however, conventional physicochemical treatment is the rule. Although cheap, it does not usually eliminate dye toxicity (Khandare and Govindwar, 2015) and also yields a significant amount of sludge that needs a final destination (landfills and incinerators), adding costs and complexity to the process (Robinson et al., 2001). Among the strategies for the treatment of these wastewaters, biological approaches such as bioaccumulation (Hassan et al., 2018), biosorption (Almeida and Corso, 2014; Sinha and Osborne, 2016) and enzyme-mediated decolorization (Zilly et al., 2011; Singh et al., 2015; Niebisch et al., 2014; Shanmugam et al., 2017) are gaining attention because of reduced costs, minor consumption of reagents and increased biodegradability of the resulting by-products (Rauf and Salman, 2012).

The repertoire of enzymes exploited in the conversion of dyes into innocuous products includes basically peroxidases (Verma and Madamwar, 2002; Yang et al., 2003; Lopez et al., 2004) and laccases (lac) (EC 1.10.3.2) (Lin et al., 2003; Rodríguez et al., 2006; Champagne et al., 2013; Naranjo-Briceño et al., 2013; El-Rahim et al., 2017). These proteins belong to the so-called group of lignin-modifying-enzymes (LMEs) that catalyze the breakdown of lignin and are particularly effective in the biodegradation of pollutants of varied chemical nature (Harms et al., 2011; Sinha and Osborne, 2016). These enzymes have been described in plants (Hoopes and Dean, 2004), basidiomycetes

* Corresponding author at: P. O. Box 19071, Departamento de Genética, Universidade Federal do Paraná, Av. Cel. Francisco Heráclito dos Santos, 100 - Jardim das Américas, Curitiba, PR, 81531-980, Brazil.
E-mail address: vankava@ufpr.br (V. Kava).

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(Abadulla et al., 2000; Wesenberg et al., 2003; Ruhl et al., 2013), filamentous fungi (Ramos et al., 2011) and bacteria (Givaudan et al., 2004), and due to their relatively low substrate specificity, they represent a powerful tool to assess the treatment of industrial effluents (Singh et al., 2015). Although a significant amount of literature describes a wide variety of fungal sources of LMEs and their application in the treatment of colored effluents, there is still great need for enzymes with the ability to resist the extremely variable conditions of pH, temperature and salt concentration, typical of industrial wastewaters (Husain, 2006; Kumar et al., 2016a,b). These observations highlight the importance of the characterization of new fungal strains producing more active, stable and versatile catalysts.

Most of the available research focused in the characterization of enzymes derived from **white-**rot fungi (WRF), leaving other organisms, such as endophytic fungi, somehow ignored. Endophytes (endophytic fungi) colonize healthy tissues of plants without causing apparent symptoms of a disease (Wilson, 1995; Stone et al., 2000) and establishing a symbiotic relationship (Saunders et al., 2010). Endophytic fungi produce a wide range of enzymes such as amylases, pectinases, lipases, proteases (Sunitha et al., 2013) and cellulases (Bischoff et al., 2009). Although it has been described the production of ligninolytic enzymes by endophytic fungi (Oses et al., 2006; Wang et al., 2006; Fillat et al., 2016; Patil et al., 2016), few studies have been conducted regarding endophyte-mediated dye biodegradation (Afzal et al., 2014; Verma et al., 2015; Patil et al., 2016; Bulla et al., 2017).

The *Pestalotiopsis* (Amphisphaeriaceae) genus has been evidenced as a rich source of enzymes with biotechnological applications, such as de biodegradation of polyurethane (Lii et al., 2017), fluorene (Kristanti and Hadibarata, 2015), asphalt (Yanto and Tachibana, 2014), petroleum (Yanto and Tachibana, 2013) and textile dyes (Hao et al., 2007; Yanto et al., 2014a). Furthermore, some *Pestalotiopsis* strains have been described as producers of LMEs (Hao et al., 2007; Saparrat and Hammer, 2006; Arfi et al., 2013; Feng et al., 2013). Phylogeny and morphological analyses of the *Pestalotiopsis* genus led to the addition of two novel genera namely Neopestalotiopsis and Pseudopestalotiopsis (Maharachchikumbura et al., 2014), raising the issue if they present similar potential in biotechnological applications as *Pestalotiopsis* (Maharachchikumbura et al., 2014).

In the present study, a set of six *Pestalotiopsis* strains isolated from *Maytenus ilicifolia* Mart. ex Reissek, a medicinal plant found in the Brazilian Atlantic forest, was evaluated for their dye-destaining activity. Isolate LGMF1504 showed the best results in dye-destaining assays and the expression and properties of the enzyme, involved in this activity, were also characterized. In addition, strain LGMF1504 was classified as *Neopestalotiopsis* sp. by morphological and phylogenetic analysis.

2. Materials and methods

2.1. Fungal isolates

Six endophytes from the LabGeM culture collection (http://www.labgem.ufpr.br) –at the Universidade Federal do Paraná (UFPR) (Curitiba, Brazil), named LGMF1499, LGMF1500, LGMF1501, LGMF1502, LGMF1503 and LGMF1504 were used in this study (Table S1). The endophytes were previously isolated from leaves of *Maytenus ilicifolia* specimens, located in the campus of the Centro Nacional de Pesquisa Florestal (CNPF) of Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Paraná, Brazil (lat -25.369227, long -49.189301). Pure isolates were maintained by serial cultivation on potato dextrose agar (PDA) at 28 °C.

2.2. Dyes and chemicals

2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), guaiacol and the reagents used as redox mediators were obtained from Sigma Chemical Company (St. Louis, MO, USA). Textile Remazol dyes reactive blue (RB220), reactive red (RR198) and reactive yellow (RY107) were kindly supplied by Dystar (São Paulo, Brazil) and Siderquímica (Curitiba, Brazil). Test batches had a minimum dye concentration of 85% (w/w). All chemicals used were of the highest available purity and analytical grade.

2.3. In culture decolorization screening

Fungal isolates were cultured on plates of solid minimal medium (SMM) containing (per liter) 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$ $7H_2O$, 0.02 g $ZnSO_4$ $7H_2O$, 10.0 g glucose, and 10.0 g bacteriological agar at pH 6.8. After ten days of growth (28 °C) on SMM, 4-mm-diameter plugs from culture borders were used to inoculate Petri dishes containing SMM and RB220, RR198 or RY107 reactive dyes (0.1 g L^{-1}). The decolorization zone was measured after ten days of growth (28 °C). The fungal strain exhibiting best activity was then cultivated in 5 mL replicates of liquid minimal medium (LMM, SMM without agar) containing the same concentration of reactive dye. After 15 days, the absorbance of culture supernatants was determined in an Epoch Microplate Spectrophotometer (BioTek, USA) at 600 nm for RB220, 512 nm for RR198 and 416 nm for RY107. A culture medium with no dye or inoculum was used as a blank, and a dye-containing medium without fungal inoculation was used as control. The percentage of in-culture decolorization after incubation was calculated as follows:

In culture decolorization (%) = [(Initial absorbance – Final absorbance) x 100] / Initial absorbance (1)

2.4. Dye destaining activity and LMEs assays

The fungal isolate that displayed the best performance in the in-culture decolorization assay was selected for further analysis. Thus, 5 mL samples from 15 days-old liquid cultures were centrifuged at 1500 rpm for 5 min. Supernatants were analyzed or stored at – 20 ºC. Pelleted mycelium was washed with distilled water, filtrated through previously dried and tared Whatman No. 1 filter papers and used in dye-destaining or enzyme activity tests. The mycelium was dried at 50 ºC, and the biomass content was determined.

Culture, supernatants, and mycelia were submitted to dye destaining assays using RB220 as substrate, to identify the fungal fraction responsible for decolorization.. Mycelium samples derived from 5 mL culture replicates were added to 1.1 mL of salt medium (SM: LMM without carbon nor nitrogen sources) containing RB220 dye (0.1 g L^{-1}) and incubated for 24 h at room temperature. Mycelium was autoclaved (121 °C, 20 min) or used fresh in the presence and absence of sodium azide (20 mM). Dye destaining assays of soluble fractions were performed adding 1 mL of culture supernatants to 0.1 mL of SM containing RB220 dye (final concentration 0.1 g L^{-1}), and the resulting mixture was incubated at room temperature. The absorbance was determined at 600 nm on a spectrophotometer. The resulting dye consumption was described as "destaining activity units" (DAU). One DAU was defined as the decrease of 0.001 absorbance units per milligram of mycelium h^{-1}, calculated as follows:

DAU = (|Initial absorbance - Final absorbance|/ 0.001) / (incubation time (h) x biomass (mg)) (2)

The involvement of LMEs in dye destaining was monitored through ABTS (ε = 36,000M^{-1} cm^{-1}) oxidation, in the presence or absence of manganese ions or peroxide. Total oxidase activity was determined incubating mycelium in a final reaction volume of 1.1 mL containing 0.3 mM ABTS, 50 mM acetate buffer (pH 5.0), 20 mM $MnSO_4$ and 0.05 mM H_2O_2. Oxidation of ABTS was monitored at 5 min intervals, for 20 min at 28 °C. Lac activity was determined under the same conditions without manganese ions and peroxide. One enzyme unit (EU) was

defined as the amount of enzyme capable of oxidizing 1 μmol of substrate per minute.

Once the enzymatic activity and the fungal fraction responsible for dye destaining were determined, the effect of several culture conditions on the production of the enzyme was evaluated.

2.5. Effect of culture conditions on enzyme production

2.5.1. Carbon and nitrogen content

Four carbohydrates (glucose, fructose, starch, and galactose) one polyalcohol (sorbitol) and three nitrogen compounds (sodium nitrate, ammonium tartrate, and urea) were used to substitute the original sources of carbon and nitrogen in LMM. The concentrations used for each substrate were 5, 10 and 15 g L^{-1}. After 15 days of growth, mycelium samples were submitted to the dye-destaining assay and the corresponding carbon source yielding the best results was then used to test the effect of nitrogen sources. All experiments to determine the effect of culture on the enzyme production and biomass yield were performed in five replicates.

2.5.2. Culture supplementation with aromatic compounds and metal ions

In order to increase enzyme expression, the fungus was grown for 10 days in 5 mL replicates of optimized liquid culture medium supplemented with 0.1 mM copper sulfate, 0.25 mM manganese sulfate and 1 mM concentration of one of several aromatic compounds: veratryl alcohol (3,4-dimethoxy benzyl alcohol), veratraldehyde, catechol (benzene-1,2-diol), vanillin, p-anisidine (p-methoxy aniline) or ferulic acid (propenoic acid). Aromatic compounds were dissolved in distilled water or 50% (v/v) ethanol (p-anisidine and ferulic acid). All solutions were sterilized through Millipore membranes (0.45 μm). Fresh mycelium was collected and destaining or lac activity was determined.

2.6. Effect of pH, salt concentration and redox mediators in dye destaining

Mycelium dye destaining activity was analyzed in different pH values, salt concentration and in the presence of redox mediators. Mycelium derived from 15 days-old liquid cultures carried out under optimized conditions (carbon and nitrogen sources) were washed twice in salt medium and then submitted to the dye-destaining assay using RB220 (0.1 g L^{-1}) as substrate. The pH of the reaction mixtures varied from 2.0 to 9.0 (intervals of 1.0 unit) using 50 mM citrate-phosphate or 50 mM glycine-NaOH buffer. Different NaCl concentrations (0.05, 0.1, 0.2, 0.4, 0.5 and 0.6 M) were subsequently tested in the previous resulting best pH value. The UV–Vis absorbance was determined after 24 h incubation at 28 °C.

The effect of redox mediators in the mycelium dye destaining activity was verified using nine dyes and four redox mediators. The following textiles dyes were used: reactive yellow 84 (RY84), Sidercron red (PF3B), reactive red 120 (RR120), reactive blue 198 (RB198), reactive yellow 107 (RY107), acid black 194 (AB194) and acid blue 193 (AB193). Two laboratory dyes: methylene blue - MB, malachite green - MG were also tested (Table S2). Redox mediators added to the mixture assay were p-coumaric acid, vanillin, acetosyringone, or syringaldehyde. The mycelium was added to the SM assay mixture with the corresponding dye (0.1 g L^{-1}) and a particular redox mediator (1 mM) in a final volume of 1.1 mL and incubated for 24 h at 28 °C.

For all assays, decolorization percentage (Eq. 1) was determined by monitoring the decrease in the absorbance at the maximum absorption visible wavelength (λ max) of each dye (Table S2) after incubation at 28 °C for 24 h.

2.7. Identification

2.7.1. Multilocus sequence analysis

For DNA extraction fungal the isolate that showed the best activity was grown on PDA medium for three days at 28 °C. The mycelium was harvested, lyophilized and ground with mortar and pestle under liquid nitrogen. Genomic DNA was extracted using UltraCleanMicrobial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA).

Multilocus analyses were performed using sequences of the 5.8S nrDNA region with two flanking internal transcribed spacers (ITS), β-tubulin region (TUB) and elongation factor 1-alpha (EF-1α). PCRs were performed using primers V9G and ITS4 (White et al., 1990), Bt2a and Bt2b (Glass and Donaldson, 1995) and EF1-728 F and EF-2 (O'Donnell and Cigelnik, 1997; Carbone and Kohn, 1999), respectively. Amplification products were electrophoresed in a 1% (w/v) agarose gel with Low DNA Mass Ladder (Invitrogen, Carlsbad, CA, USA) as molecular mass standard. PCR products were purified by ethanol precipitation and directly sequenced using a DYEnamic ET Dye Terminator Kit (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA). The products of sequencing reactions were purified using Sephadex gel G-50 column (GE Healthcare, Piscataway, NJ, USA) in ELISA microplates, and subjected to electrophoresis on the automated MegaBACE DNA sequencer (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA). The resulting sequences were inspected using the BioEdit program, version 7.2.5 (Hall, 2013).

DNA sequences obtained in this study were compared to those previously described in databases from GenBank, Mycobank or derived from other *Neopestalotiopsis*, *Pseudopestalotiopsis*, and *Pestalotiopsis* studies (Table S1). Sequences were aligned by ClustalW (Thompson et al., 1994) in the MEGA software, version 7 (Kumar et al., 2016a,b). Suitable substitutions models were determined for single (ITS), and multilocus analyses (ITS, EF-1αand TUB) were performed using R software (R Core Team, 2017). Bayesian inference analyses using MrBayes software, version 3.2.1 (Ronquist and Huelsenbeck, 2003) were performed implementing GTR + I and GTR + I + gamma substitution models for ITS and multilocus (ITS, EF-1α and TUB) alignments, respectively. Four simultaneous Markov Chain Monte Carlo (MCMC) chains were run from random trees for 10.000.000 generations and sampled every 1.000 generations. The temperature value was lowered to 0.15, burn-in was set to 0.25. We obtained maximum likelihood branch supports with the ultrafast bootstrap (Hoang et al., 2018) implemented in the IQ-TREE software (Nguyen et al., 2015) which, based on BIC, implemented the GTR + I + gamma substitution model for tree reconstruction. The consensus tree was obtained after 1000 bootstraps. Comparisons of sequences similarity were estimated using R software (R Core Team, 2017), Pegas Package (Paradis, 2010), and WriteXLS package. Informative sites were retrieved using MEGA software, version 7 (Kumar et al., 2016a,b).

2.7.2. Morphological analysis

Following the method described by Maharachchikumbura et al. (2012), the morphological characterization of isolate that showed the best destaining activity was conducted on 2% PDA at 22 °C with a 12 h photoperiod for 7–12 days. Micro-cultures (Kern and Blevins, 1999) were prepared to observe the development of fungal microstructures. Microscopic preparations of fungal structures in distilled water were submitted to 30 measurements each, in an Axio Imager Z2 (Carl Zeiss, Jena, DE) microscope, equipped with Metafer 4/V Slide software (Metasystems, Altlussheim, DE), using differential phase contrast (DIC) illumination and ImageJ software support.

2.8. Statistical analysis

Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey or Kruskal-Wallis tests, through the GraphPad Prism software package, version 6.0 (GraphPad Software Inc.). Differences were considered significant when $p \leq 0.05$.

3. Results and discussion

3.1. Fungal isolates present dye-destaining activity

The ability of six endophytic isolates to act onto reactive dyes was assessed through two experimental approaches: in-culture decolorization and dye-destaining assays. For in-culture decolorization, the fungus was cultivated in liquid or solid dye-containing media, and the absorbance was determined after 15 days of growth. In dye-destaining assays, the fungus was grown for 15 days, and its mycelium and culture supernatants were collected and incubated with the dye substrate for 24 h. The difference in absorbance in each test was used to determine decolorization percentage or destaining activity units, DAU (using Eq. 1 and Eq. 2 respectively).

Initially, in-culture decolorization was assessed in a solid medium, containing RR198, RY107, and RB220 dyes as substrates, by monitoring the halo of decolorization. RB220 dye decolorization was observed in cultures of LGMF1499, LGMF1502, LGMF1500, LGMF1501 and LGMF1504 (data not shown). The strain LGMF1504 was selected for further investigation since it developed the clearest destaining halo in solid cultures. UV–vis spectra for RB220 demonstrated a 96% reduction of the original absorbance peak at 600 nm and a 67% reduction at 280 nm (Fig. 1A). These results suggest that decolorization was accompanied by the reduction of azo-linkages and loss of aromatic rings, respectively (Niebisch et al., 2014). In-culture decolorization of RR198 and RY107 dyes resulted in lower activity (56 ± and 47% respectively) (Fig. 1B).

3.2. LGMF1504 identification

Strain LGMF1504 was identified through multilocus analysis of ITS, EF-1α and TUB regions (Fig. 2). The multilocus alignment comprised 71 strains, corresponding to 40 taxa. A total of 1380 characters were analyzed, with 902 conserved characters, 221 characters were parsimony informative. The tree topology obtained (Fig. 2) agreed with phylogenetic analysis performed by Maharachchikumbura et al. (2014) for the *Neopestalotiopsis* genus.

Isolate LGMF1504 is phylogenetically related to *N. honoluluana* and *N. zimbabwana,* as observed in Fig. 2. The sequence of type strain *N. honoluluana* CBS 114,495 presented 99.71% similarity to LGMF1504 and *N. zimbabwana* showed 99.28% of similarity, which impossibility the identification of LGMF1504 at the species level. *N. honoluluana* (Maharachch., K.D. Hyde & Crous) was described by authors confined to *Telopea* sp., in Hawaii, and *N. zimbabwana (*Maharachch., K.D. Hyde & Crous) was isolated as a pathogen on leaves of *Leucospermum cuneiforme* in Zimbabwe

Once *Neopestalotiopsis* was recently segregated from Pestalotiopsis (Maharachchikumbura et al., 2014), the biotechnological potential of this new genus remains unrecognized. To address this question, we reclassified, through ITS phylogeny analyses, five *Pestalotiopsis* isolates that presented biodegradation potential in recent literature, namely NCi6, BM-04, NG007, NIOCC#C3 and J63 (Arfi et al., 2013; Naranjo-Briceño et al., 2013; Yanto and Tachibana, 2013, 2014; Yanto et al., 2014a) (Supplementary Fig. S1). Thus, strains NCi6, BM-04, and NG007 belong to the *Neopestalotiopsis* genus. NCi6 and BM-04 were able to produce LMEs (Arfi et al., 2013; Naranjo-Briceño et al., 2013) and NG007 degraded asphalt (Yanto and Tachibana, 2014), petroleum (Yanto and Tachibana, 2013) and textile dyes (Yanto et al., 2014a, 2014b). Considering the data appointed by literature, the *Neopestalotiopsis* genus may also represent a tangible biological reservoir to be explored for biodegradation of dyes and maybe other pollutants.

3.3. A mycelium-associated laccase-like enzyme is responsible for dye-destaining

In order to determine the localization of the enzyme responsible for dye decolorization in *Neopestalotiopsis* sp. LGMF1504, culture supernatants, and fungal biomass were submitted to dye-destaining assays (Fig. 3A). Supernatants did not present any activity, while an efficient decolorization (60%) was observed when mycelium was used. The addition of sodium azide, a common lac inhibitor, and the previous biomass autoclavation totally depleted the destaining activity (Fig. 3A). Moreover, neither the addition of peroxide or manganese ions changed the rate of ABTS oxidation by the fungal biomass (Fig. 3B). These results support the assertion that a laccase-like enzyme associated with the mycelium was responsible for dye consumption, disregarding the participation of peroxidases and biosorption events in the process. Considering endophytic fungi, few species were described as producers of lac with dye destaining activity (Patil et al., 2016; Bulla et al., 2017), being most of them referred as secreted enzymes (Arfi et al., 2013; Baldrian, 2006) and this assertion applies to all laccases described in *Pestalotiopsis* strains (Feng et al., 2013; Verma et al., 2010; Arfi et al., 2013; Naranjo-Briceño et al., 2013; Yanto and Tachibana, 2014). Mycelium associated laccases are commonly described in ligninolytic fungi where they are required to access cellulose as carbon source (Pakhadnia et al., 2009; Svobodová et al., 2008; Tapia-Tussell et al., 2011). In *Cryptococcus neoformans,* a lac related to the mycelium is described as a virulence factor (Zhu et al., 2001). The presence of lac in endophytic fungi, such as *Neopstalotiopsis* sp. LGMF1504, may be involved in host penetration and protection against plant defenses (Doss, 1999), which is indispensable for the endophyte-plant relationship.

3.4. Carbon and nitrogen culture content alters the expression of laccase

The evaluation of culture conditions aimed to select the best source and concentration of carbon and nitrogen for lac production. It

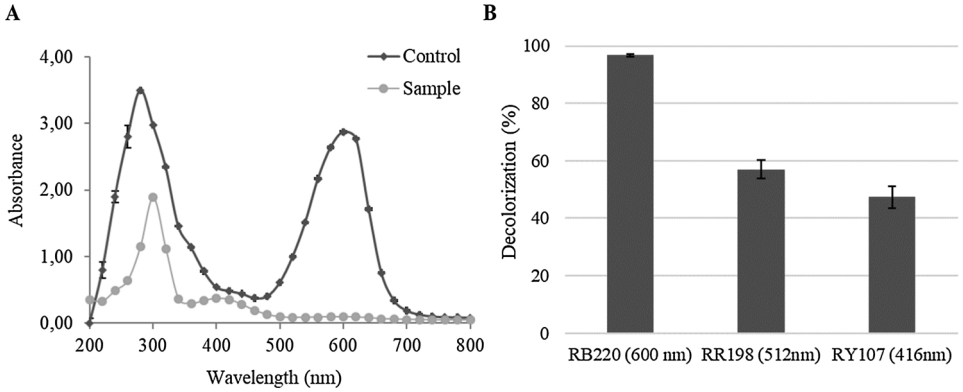

Fig. 1. A. UV–vis spectra of the RB220 dye (0.1 g L^{-1}) before and after incubation with LGMF1504 strain in liquid minimal medium (LMM). B. Decolorization of RB220, RR198 and RY107 dyes (0.1 g L^{-1}) by LGMF1504 strain after 15 days of growth in the presence of reactive dyes.

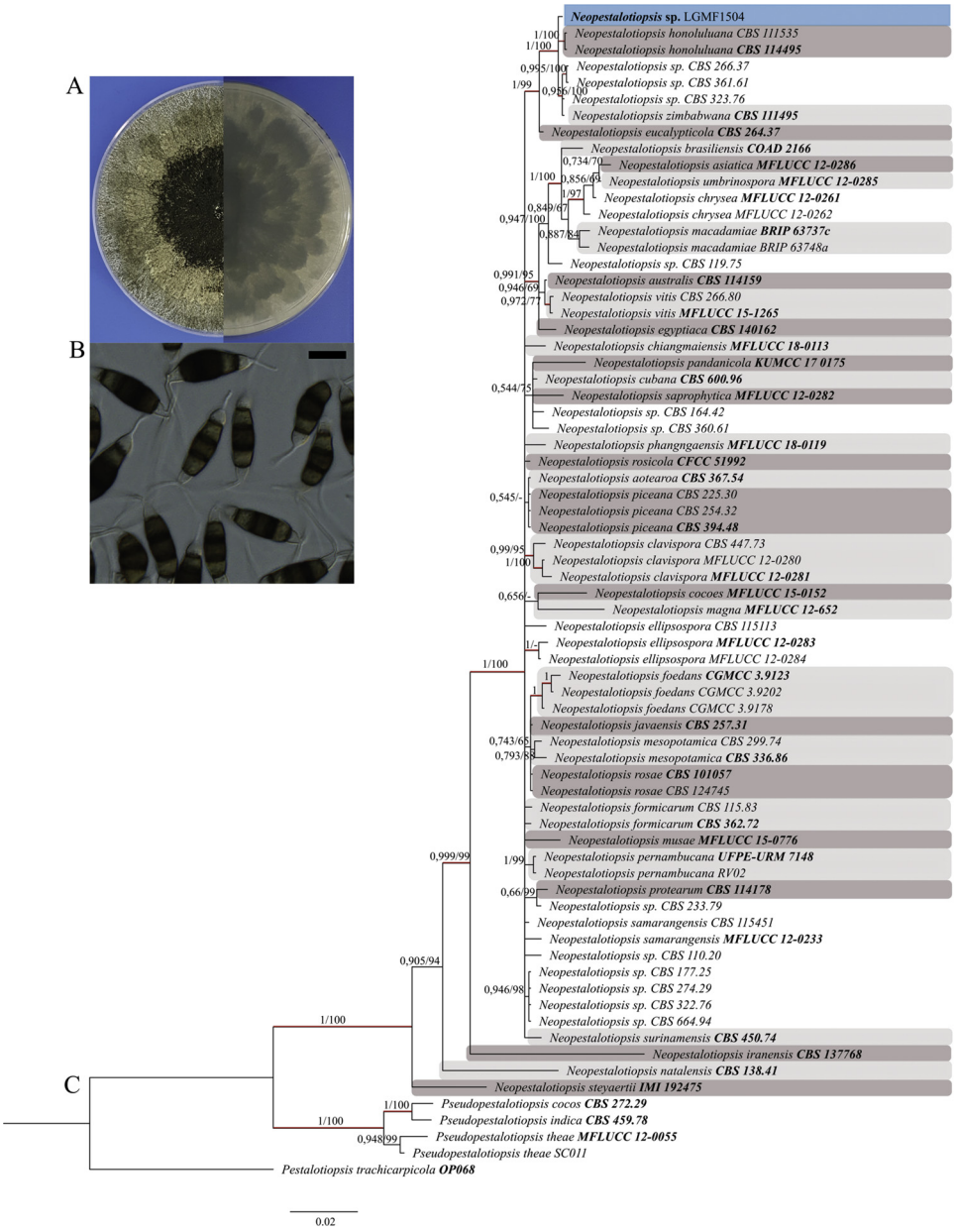

Fig. 2. Morphological characteristic of strain LGMF1504. A. Conidia. B. Upper surface and reverse view of a 10d-old culture on 2% PDA at 22 ºC (12 h photoperiod) Scale bars = 10 µm culture. C. Consensus phylogenetic tree (GTR + G+I) from the Bayesian and Maximum Likelihood analyses of the combined (ITS + TUB + TEF) alignment of *Neopestalotiopsis* species. Note: Red lines indicate Bayesian posterior probabilities above 95%. Ex-types strain accession numbers are printed in bold. The scale bar represents the expected number of changes per site. The tree was rooted to *Pestalotiopsis trachicarpicola* (OP068).

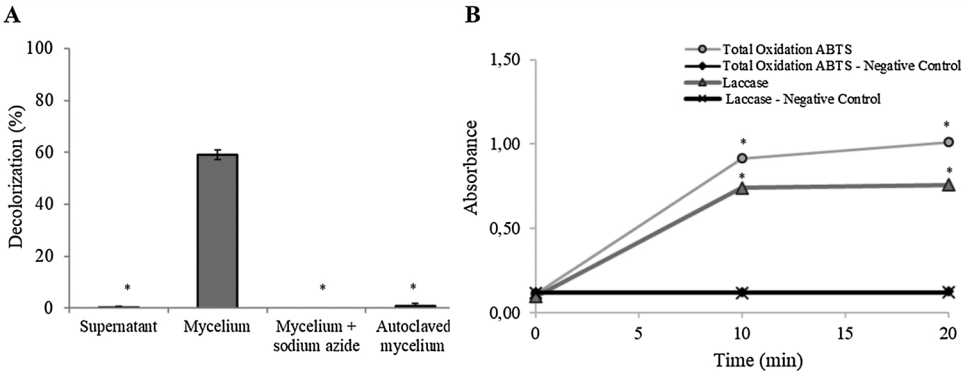

Fig. 3. A Destaining of RB220 dye by the LGMF1504 strain culture supernatant and mycelia measured after 24 h. The activity of the biomass was also assessed in the presence of sodium azide and after biomass autoclavation. B Oxidation of ABTS by mycelium in the presence and absence of manganese sulphate and hydrogen peroxide.

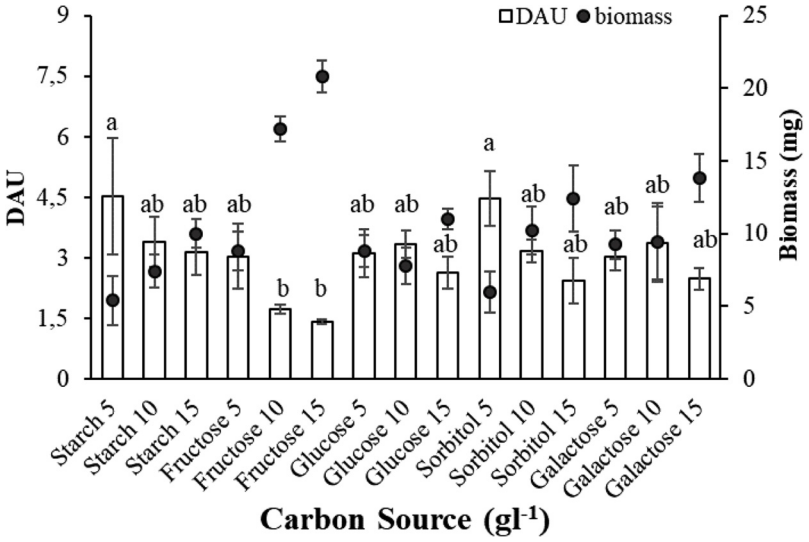

Fig. 4. Effect of carbon culture content on dye-destaining and fungal growth. LGMF1504 mycelia derived from cultures containing different sources and concentrations of carbon were incubated with an RB220 dye solution (0.1 g L^{-1}) for 24 h, and the dye-destaining activity (DAU) was calculated. Error bars represent the standard deviation of the mean (SD, n = 5). The Kruskal Wallis test is significant at $p >$ 005.

considered the balance among the enzyme activity and the yield of fungal biomass, and it was described as DAUs. (See Eq. 2). The amount of available carbon in culture media displayed a positive correlation to cell growth (biomass) in all assessed conditions since it was observed a higher biomass yield in culture media with increasing carbon content (Fig. 4 and Table S3). This was more apparent when using fructose, starch, and sorbitol as carbon source. However, the improvement in the biomass yield was not accompanied by an increase in the destaining activity (Fig. 4). Mycelium derived from medium containing the lowest assessed concentration of sorbitol and starch (5 g L^{-1}) presented the highest DAU values (4.47 and 4.53, respectively). In contrast, fungal growth in media containing 10 and 15 g L^{-1} fructose showed the lowest DAU values (1.73 and 1.42, respectively). Starch was selected for further evaluations rather than sorbitol for being the most abundant industrial waste in textile industries (Sarayu and Sandhya, 2012) and therefore, it could be reused from industrial wastes in biodegradation processes.

The variation of nitrogen content displayed low correlation with the yield of biomass and destaining activity (Table S4). It means that biomass yield and destaining activity remained basically unaltered in all experiments; nonetheless each nitrogen compound showed a particular profile (Fig. 5). Thus, sodium nitrate followed by ammonium tartrate-based media induced a relatively high biomass production, while urea-based medium behaves as a poor substrate for fungal growth. In contrast, the overall observed DAU values were similar in all types and concentrations of nitrogen sources (Tukey test, $p > 0.05$) (Fig. 5).

The relation between lac production and nitrogen/carbon content in the culture medium is highly variable among fungal species (Kenkebashvili et al., 2009). While early studies suggested that starvation (low carbon and nitrogen content) results in a better lac production (Kirk and Farrell, 1987; Asgher et al., 2008; Piscitelli et al., 2011), other authors show that lac genes present variable expression in non-limiting carbon and nitrogen conditions (Yang et al., 2016). Working with a sea mud derived *Pestalotiopsis* isolate, Feng et al (2013) found that three g·L^{-1} maltose and eight g·L^{-1} ammonium sulfate was the best mix of sources for lac production. Hao et al. (2007) using a different *Pestalotiopsis* isolate, showed that lac formation was strongly affected by substrates in the culture medium. Those sources, rapidly and efficiently absorbed by the organism, stimulated a high level of lac activity.

Additionally, increasing the glucose concentration from 5 to 20 g L^{-1} resulted in a more than five-fold improvement in lac production but a further increase resulted in lower enzyme activity. Ammonium tartrate was described as the best nitrogen source for lac production as it was observed in the present *Neopestalotiopsis* isolate. The variable effect of culture media content in lac production observed in fungal

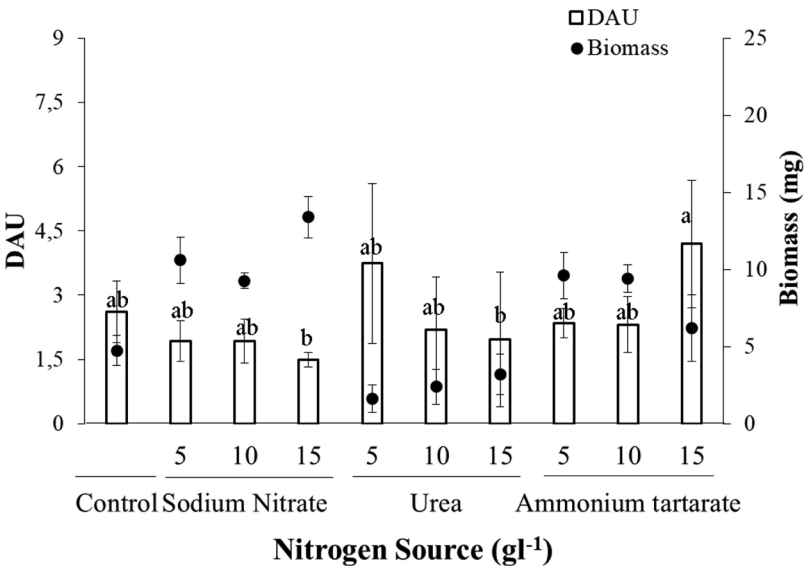

Fig. 5. Effect of nitrogen culture content on dye-destaining and fungal growth. LGMF1504 mycelia derived from cultures containing different sources and concentrations of nitrogen were incubated with an RB220 dye solution (0.1 g L^{-1}) for 24 h, and the dye-destaining activity (DAU) was calculated. Error bars represent the standard deviation of the mean (SD, n = 5). DAU: differences to control were not significant (Tukey test, $p > 0.05$).

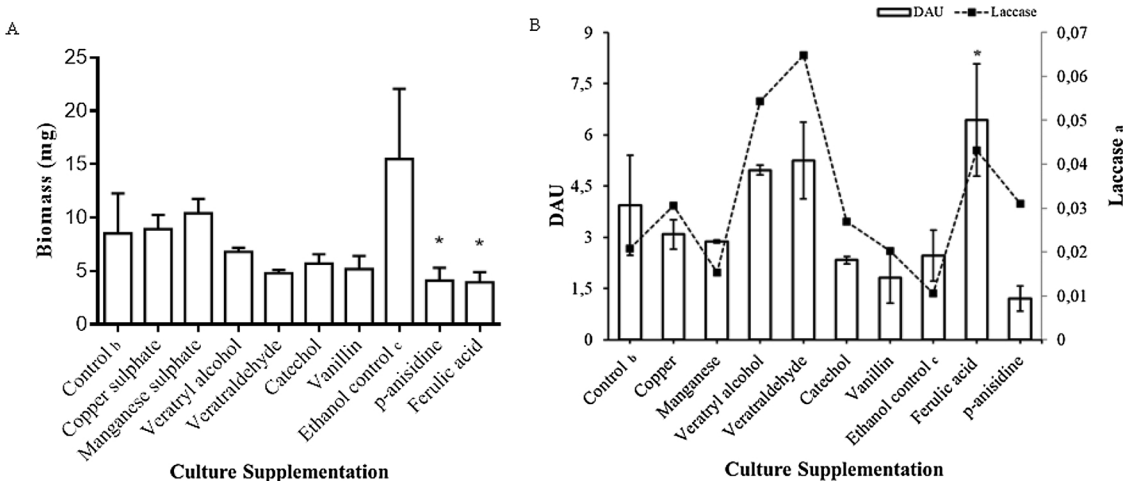

Fig. 6. Effect of the culture supplementation with aromatic compounds and metal ions in LGMF1504 growth **(A)** and the production of laccase and dye destaining **(B).** The fungus was incubated with an RB220 dye solution (0.1 g L^{-1}) for 24 h, and the dye-destaining activity (DAU) was calculated. Laccase activity was determined by the ABTS oxidation after 30 min incubation. Controls were cultivated without supplementation.
Notes: $_a$: Values show mEU mg biomass^{-1}. $_b$ Copper sulphate, manganese sulphate, veratryl alcohol, veratraldehyde, catechol, and vanillin were dissolved with water. $_c$ p-anisidine and ferulic acid were dissolved in ethanol. Control cultures were added of the same volume of the respective solvent. Error bars represent the standard deviation of the mean (SD, n = 3).

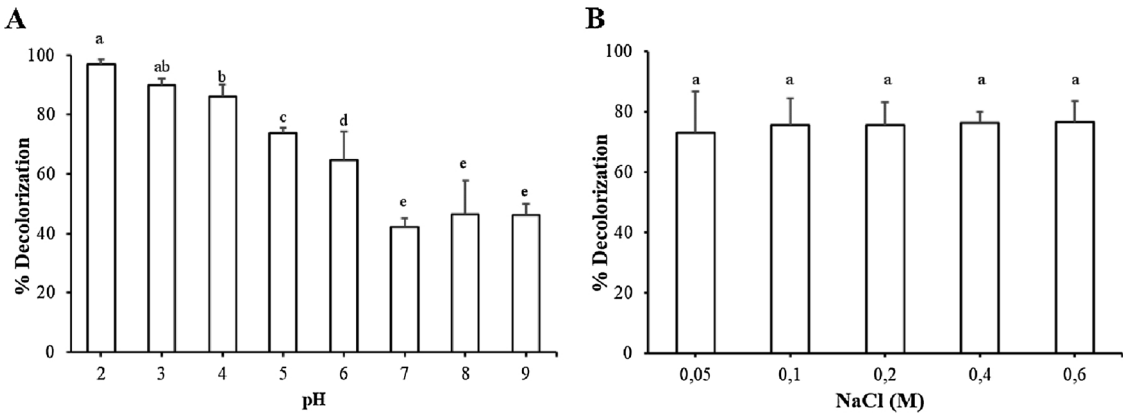

Fig. 7. Effect of pH **(A)** and Salt (NaCl) **(B)** in the decolorization of RB220 by the LGMF1504 mycelium-associated laccase, after 24 h of incubation Error bars represent the standard deviation of the mean (SD, n = 4).

isolates as well as the description of multiple fungal genes (including lac genes) containing responsive elements to nitrogen and carbon (Soden and Dobson, 2003) make that nowadays it is accepted that culture conditions must be particularly determined for each isolate (Piscitelli et al., 2011). In the present study, optimized culture media for lac expression in the LGMF1504 isolate contained 5 g L^{-1}starch and 15 g L^{-1} ammonium tartrate.

3.5. Lac expression was responsive to culture supplementation with some aromatic compounds

Lignin-related aromatic compounds and metal ions are routinely used to boost lac production (Niladevi e Prema, 2008; Majeau et al., 2010) mostly because they act as transcriptional regulators of lac genes (Piscitelli et al., 2011). Thus, we evaluated the effect of culture supplementation with six aromatic compounds (veratryl alcohol, veratraldehyde, catechol, vanillin, p-anisidine, and ferulic acid) and two metal ions (copper and manganese), in the production of lac by the LGMF1504 strain. In general, supplementation with aromatics or metal ions did not affect the biomass yield, except for p-anisidine and ferulic acid ($p < 0.05$) (Fig. 6A). However, a slight improvement in the mycelium-associated destaining activity was observed when cultures were performed in the presence of veratryl alcohol, veratraldehyde, and ferulic acid and this was accompanied by similar behavior in the expression of laccase (Fig. 6B).

Metal ions and aromatics modulate lac gene expression through the interaction with specific response elements (Piscitelli et al., 2011). Although a great deal of literature describes their variable effect on wood-rotting fungi, few studies regarding the production of lac by *Pestalotiopsis* species are available. Feng et al. (2013) using *Pestalotiopsis sp.* J63, a marine-related fungus, reported a higher lac yield using phenol supplementation. Moreover, the addition of other aromatics such as guaiacol and veratryl alcohol led to the expression of other lac isoenzymes. The addition of different metal ions resulted in a significant boost of the laccase secretion for different strains of this genus; however for other *Pestalotiopsis* isolates, supplementation with guaiacol did not enhance lac production (Hao et al., 2007; Yadav et al., 2019).

3.6. Fungal destaining activity remained high in different pH values and salt

The effect of pH and salt concentration on the lac mediated dye destaining of the LGMF1504 strain was evaluated. The enzyme effectively destained RB220 dye over a broad range of pH values (2.0–9.0), with the best performance being observed in the pH range 2.0–6.0 (retaining respectively 100 and 60% of the maximum activity). Enzyme activity on pH values among 7 to 9 was 50% of the maximum (Fig. 7A).

The pH was set to 5.0 for the subsequent experiments and the effect of different salt concentrations on dye destaining was determined. The LGMF1504 mycelial destaining activity remained high and basically unaltered in all assessed conditions (0.05 M to 0.6 M) (Fig. 7B). Textile dye effluents possess extremely variable pH values and salt content, thus, an enzyme with a potential dye-biodegrading application must be able to adapt to a wide range of working conditions. Most of the reported fungal laccases act in the 3.0–6.0 pH range (Bollag and Leonowicz, 1984; Madhavi and Lele, 2009), with a drastic decrease of activity in alkaline environments. Nevertheless, there have been reported some exceptions as laccases derived from *Pycnoporus* (Wang et al., 2010) and *Trametes* species (Si et al., 2013), acting in pH values among 7.0 to 10. In *Pestalotiopsis,* laccases described in the literature displayed similar behavior to our LGMF1504 strain. Thus, Chen et al. (2011) reported a laccase in the marine J63 *Pestalotiopsis* isolate with the highest production in pH 4.5–5.5 and culture media containing 30 g L^{-1} of salt. Yanto et al. (2014a), using the *Pestalotiopsis* sp. NG007 isolate was able to decolorize different textile effluents in a wide range of pHs (3–12) and salt content (0–10%), with an optimum activity of the responsible lac at pH 3.0. In contrast, Arfi et al. (2013) when characterizing the proteome of a mangrove *Pestalotiopsis* isolate, described a 67% reduction of the enzyme activity when incubated in a 3% salt medium.

In summary, the LGMF1504 mycelium associated lac showed a remarkable activity in wide pH conditions including acid environments, even with very low or high salt content. The relative resistance of the enzyme activity to different pH and salt concentrations is probably the result of an association with the mycelium, which may act as a physical protective barrier in the cell membrane (Svobodová et al., 2008).

3.7. Analysis of the redox mediators in the lac-mediated dye destaining

In order to expand the number of potential dye substrates of the LGMF1504 mycelial laccase, destaining assays using nine additional dyes (Table S2) were performed in the presence of laccase natural redox mediators: vanillin, p-coumaric acid, acetosyringone, and syringaldehyde. In the absence of redox mediators, the mycelium-associated lac reached low decolorization (0–2 DAU) of 7 dyes; moderate decolorization (2–4 DAU) of RY107 and high decolorization (4–6 DAU) of MG (Fig. 10); however it is not supported by statistical analysis. The addition of redox mediators resulted in an improvement in the dye destaining scores of all commercial dyes tested. Thus, the number of dyes presenting moderate decolorization switched from 1 to 5 dyes using different compounds, while those presenting high decolorization changed from 1 to 2. Although there was observed some improvement, for AB194, PR3B, RB198 and AB193 dyes, decolorization values remained low even in the presence of redox mediators. Syringaldehyde was the most effective redox mediator, followed by vanillin (Fig. 8). In the presence of syringaldehyde, it was observed a 7-fold increase in the decolorization of MB dye and 3.7-fold improvement in the decolorization of RB198 over 24 h. Vanillin also showed superior performance enhancing RR120 and RY84 dye destaining, with a 3.4-fold increase in DAU for both dyes. The decolorization taxes obtained by LGMF1504 lac reached 44% for AB193 and MB and 87% for AB194 and MG dyes. The effectiveness of each mediator depended on the type of dye as observed in other studies (Camarero et al., 2005; Grassi et al., 2011). Syringaldehyde is considered one of the fastest and most efficient natural lac mediators (Camarero et al., 2005) and it has improved lac activity in several studies (Valle et al., 2015). Also, vanillin effect is well documented and has been a requirement as a mediator for some fungal laccases (Camarero et al., 2005), improving the dye-destaining dynamics in some fungi (Camarero et al., 2005; Grassi et al., 2011; Piscitelli et al., 2011; Valle et al., 2015). The presence of these mediators resulted in higher levels of laccase also in *Pestalotiopsis* strains although it is not clear if the compound acted as an inducer of lac expression or as a redox mediator since they were used directly in fungal cultures (Feng et al., 2013; Yadav et al., 2019).

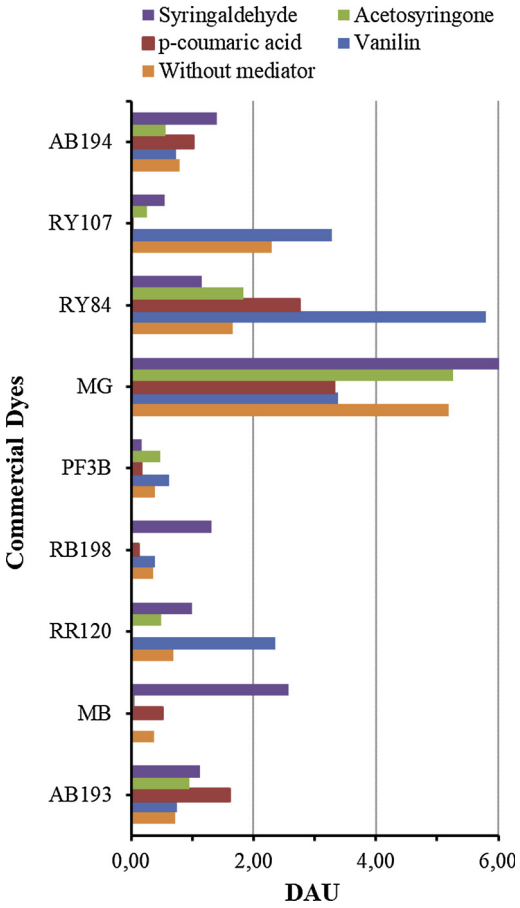

Fig. 8. Effect of the redox mediators vanillin, p-coumaric acid, acetosyringone, and syringaldehyde in the decolorization of nine different commercial dyes by the mycelium-associated laccase of LGMF1504. No statistical significance was observed (Kruskal-Wallis test, $p > 0.05$).

4. Conclusions

We explored the dye destaining potential of six fungal strains isolated from the medicinal plant *M. ilicifolia*. The study showed the ability of *Neopestalotiopis* sp. LGMF1504 to produce an active and stable mycelium-associated laccase capable to destain 12 synthetic dyes, at different rates, and in the presence of redox mediators. Also, the enzyme was not affected by variations in pH or salt concentrations. Characteristics such as the association of the enzyme to the mycelium acting as a physical immobilization support, the wide range of dye substrates on which it can act, and its ability to remain active in a broad spectrum of pH values and salt concentrations make strain LGMF1504 an excellent candidate to technological applications as dye biodegradation. Additionally, it is demonstrated the importance of *M. ilicifolia* as a repository of fungal species with biotechnological applications, highlighting the significance of plant conservation programs in Brazil.

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Declaration of Competing Interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126299.

References

Abadulla, E., Tzanov, T., Costa, S., Robra, K.H., Cavaco-Paulo, A., Gubitz, G.M., 2000. Decolorization and detoxification of textile dyes with a laccase from Trametes hirsute. Appl Environ Microb 66, 3357–3362. https://doi.org/10.1128/AEM.66.8.3357-3362.2000.

Afzal, M., Shabir, G., Tahseen, R., Islam, E.U., Iqbal, S., Khan, Q.M., Khalid, Z.M., 2014. Endophytic *Burkholderia* sp. strain PsJN improves plant growth and phytoremediation of soil irrigated with textile effluent. Clean–Soil Air Water 42, 1304–1310. https://doi.org/10.1002/clen.201300006.

Almeida, E.J.R., Corso, C.R., 2014. Comparative study of toxicity of azo dye procion red MX-5B following biosorption and biodegradation treatments with the fungi *Aspergillus niger* and Aspergillus terreus. Chemosphere 112, 317–322. https://doi.org/10.1016/j.chemosphere.2014.04.060.

Arfi, Y., Chevret, D., Henrissat, B., Berrin, J.-G., Levasseur, A., Record, E., 2013. Characterization of salt-adapted secreted lignocellulolytic enzymes from the mangrove fungus *Pestalotiopsis* sp. Nat. Commun. 4 (1810), 1–9. https://doi.org/10.1038/ncomms2850.

Asgher, M., Kausar, S., Bhatti, H.N., Hassan Shah, S.A., Ali, M., 2008. Optimization of medium for decolorization of solar golden yellow R direct textile dye by *Schizophyllum commune* IBL-06. Int Biodeter Biodegr 61, 189–193. https://doi.org/10.1016/j.ibiod.2007.07.009.

Baldrian, P., 2006. Fungal laccases-occurrence and properties. FEMS Microbiol. Rev. 30, 215–242. https://doi.org/10.1111/j.1574-4976.2005.00010.x.

Bischoff, K.M., Wicklow, D.T., Jordan, D.B., De Rezende, S.T., Liu, S., Hughes, S.R., Rich, J.O., 2009. Extracellular hemicellulolytic enzymes from the maize endophyte Acremonium zeae. Curr. Microbiol. 58, 499–503. https://doi.org/10.1007/s00284-008-9353-z.

Bollag, J.M., Leonowicz, A., 1984. Comparative studies of extracellular fungal laccases. Appl Environ Microb 48, 849–854.

Bulla, L.M.C., Polonio, J.C., Portela-Castro, A.L., de, B., Kava, V., Azevedo, J.L., Pamphile, J.A., 2017. Activity of the endophytic fungi *Phlebia* sp. and *Paecilomyces formosus* in decolourisation and the reduction of reactive dyes' cytotoxicity in fish erythrocytes. Environ. Monit. Assess. 189, 88. https://doi.org/10.1007/s10661-017-5790-0.

Camarero, S., Ibarra, D., Martínez, M.J., Martínez, A., 2005. Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Appl Environ Microb 71, 1775–1784.

Carbone, I., Kohn, L.M., 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91, 553–556. Stable URL. http://www.jstor.org/stable/3761358.

Champagne, P.P., Nesheim, M.E., Ramsay, J.A., 2013. A mechanism for NaCl inhibition of reactive Blue 19 decolorization and ABTS oxidation by laccase. Appl. Microbiol. Biotechnol. 97, 6263–6269. https://doi.org/10.1007/s00253-012-4525-y.

Chen, H.Y., Xue, D.S., Feng, X.Y., Yao, S.-J., 2011. Screening and production of ligninolytic enzyme by a marine-derived fungal *Pestalotiopsis* sp. J63. Appl Biochem Biotech 165, 1754. https://doi.org/10.1007/s12010-011-9392-y.

Doss, R.P., 1999. Composition and enzymatic activity of the extracellular matrix secreted by germlings of botrytis cinerea. Appl Environ Microb 65 (2), 404–408 PMCID: PMC91039; PMID: 9925560.

El-Rahim, W.M.A., Moawad, H., Abdel, A.Z., Sadowsky, M.J., 2017. Optimization of conditions for decolorization of azo-based textile dyes by multiple fungal species. J. Biotechnol. 260, 11–17. https://doi.org/10.1016/j.jbiotec.2017.08.022.

Feng, X., Chen, H., Xue, D., Yao, S., 2013. Enhancement of laccase activity by Marine-derived *Deuteromycete Pestalotiopsis* sp. J63 with agricultural residues and inducers. Chinese J Chem Eng 21, 1182–1189. https://doi.org/10.1016/S1004-9541(13)60567-4.

Fillat, Ú., Martín-Sampedro, R., Macaya-Sanz, D., Martín, J.A., Ibarra, D., Martínez, M.J., Eugenio, M.E., 2016. Screening of eucalyptus wood endophytes for laccase activity. Process Biochem. 51, 589–598. https://doi.org/10.1016/j.procbio.2016.02.006.

Givaudan, A., Effosse, A., Faure, D., Potier, P., Bouillant, M.L., Bally, R., 2004. Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: evidence for a laccase in non-motile strains of *Azospirillum lipoferum*. FEMS Microbiol. Lett. 108, 205–210. https://doi.org/10.1111/j.1574-6968.1993.tb06100.x.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microb 61, 1323–1330.

Gomes-Figueiredo, J., Pimentel, I.C., Vicente, V.A., Pie, M.R., Kava-Cordeiro, V., Galli-Terasawa, L., Pereira, J.O., de Souza, A.Q.L., Glienke, C., 2007. Bioprospecting highly diverse endophytic *Pestalotiopsis* spp. with antibacterial properties from *Maytenus ilicifolia*, a medicinal plant from Brazil. Can. J. Microbiol. 53, 1123–1132.

Grassi, E., Scodeller, P., Filiel, N., Carballo, R., Levin, L., 2011. Potential of *Trametes trogii* culture fluids and its purified laccase for the decolorization of different types of recalcitrant dyes without the addition of redox mediators. Int Biodeter Biodegr 65, 635–643. https://doi.org/10.1016/j.ibiod.2011.03.007.

Hall, T., 2013. BioEdit v7.1.11., Ibis Biosciences. 760-476-3375. An Abbott company, 2251 Faraday Avenue, Carlsbad, CA, pp. 92008. http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

Hao, J., Song, F., Huang, F., Yang, C., Zhang, Z., Zheng, Y., Tian, X., 2007. Production of laccase by a newly isolated deuteromycete fungus *Pestalotiopsis* sp. and its decolorization of azo dye. J Ind Microbiol Biot 34, 233–240. https://doi.org/10.1007/s10295-006-0191-3.

Harms, H., Schlosser, D., Wick, L.Y., 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat. Rev. Microbiol. 9, 177–192. https://doi.org/10.1038/nrmicro2519.

Hassan, S.H.A., Koutb, M., Nafady, N.A., Hassan, E.A., 2018. Potentiality of *Neopestalotiopsis clavispora* ASU1 in biosorption of cadmium and zinc. Chemosphere 202, 750–756. https://doi.org/10.1016/j.chemosphere.2018.03.114.

Hoang, D.T., Chernomor, O., Von Haeseler, A., Minh, B.Q., Vinh, L.S., 2018. UFBoot2: improving the ultrafast bootstrap approximation. Mol. Biol. Evol. 35, 518–522. https://doi.org/10.1093/molbev/msx281.

Hoopes, J.T., Dean, J.F., 2004. Ferroxidase activity in a laccase-like multicopper oxidase from Liriodendron tulipifera. Plant Physiol. Biochem. 42, 27–33. https://doi.org/10.1016/j.plaphy.2003.10.011.

Husain, Q., 2006. Potential applications of the oxidoreductive enzymes in the decolorization and detoxification of textile and other synthetic dyes from polluted water: a review. Crit. Rev. Biotechnol. 26, 201–221. https://doi.org/10.1080/07388550600969936.

Kern, M.E., Blevins, K.S., 1999. Micologia médica, second ed. Premier, São Paulo.

Kenkebashvili, N.V., Elisashvili, V., Hadar, Y., 2009. Effect of nutrient medium composition on laccase and manganese peroxidase activity in medicinal mushrooms. Int J Med Mushrooms 11, 191–198. https://doi.org/10.1615/IntJMedMushr.v11.i2.80.

Khandare, R.V., Govindwar, S.P., 2015. Phytoremediation of textile dyes and effluents: current scenario and future prospects. Biotechnol. Adv. 33, 1697–1714. https://doi.org/10.1016/j.biotechadv.2015.09.003.

Kirk, T.K., Farrell, R.L., 1987. Enzymatic "Combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. 41, 465–505. https://doi.org/10.1146/annurev.mi.41.100187.002341.

Kristanti, R.A., Hadibarata, T., 2015. Biodegradation and identification of transformation products of fluorene by Ascomycete fungi. Water Air Soil Pollut. 226, 406. https://doi.org/10.1007/s11270-015-2674-1.

Kumar, S., Raut, S., Bandyopadhyay, P., 2016a. Fungal decolouration and degradation of azo dyes: a review. Fungal Biol Rev 30, 112–133. https://doi.org/10.1016/j.fbr.2016.06.003.

Lii, S.B.W., Wong, C., Al-Obaidi, J.R., Rahmad, N., Mujahid, A., Müller, M., 2017. Ability of endophytic fungi isolated from *Nepenthes ampullaria* to degrade polyurethane. Malays J Microbiol 13, 172–179.

Lin, J.P., Lian, W., Xia, L.M., Cen, P.L., 2003. Production of laccase by *Coriolus versicolor* and its application in decolorization of dyestuffs: (II). Decolorization of dyes by laccase containing fermentation broth with or without self-immobilized mycelia. J Environ Sci-China 15, 5–8 PMID: 12602596.

Lopez, C., Moreira, M.T., Feijoo, G., Lema, J.M., 2004. Dye decolorization by manganese peroxidase in an enzymatic membrane bioreactor. Biotechnol. Prog. 20, 74–81. https://doi.org/10.1021/bp030025c.

Madhavi, V., Lele, S.S., 2009. Laccase: properties and applications. Bioresources 4, 1694–1717.

Maharachchikumbura, S.S.N., Hyde, K.D., Groenewald, J.Z., Xu, J., Crous, P.W., 2014. *Pestalotiopsis* revisited. Stud Mycol 79, 121–186. https://doi.org/10.1016/j.simyco.2014.09.005.

Majeau, J.A., Brar, S.K., Tyagi, R.D., 2010. Laccases for removal of recalcitrant and emerging pollutants. Bioresour. Technol. 101, 2331–2350. https://doi.org/10.1016/j.biortech.2009.10.087.

Naranjo-Briceño, L., Pernía, B., Guerra, M., Demey, J.R., De Sisto, Á., Inojosa, Y., González, M., Fusella, E., Freites, M., Yegres, F., 2013. Potential role of oxidative exoenzymes of the extremophilic fungus *Pestalotiopsis palmarum* BM-04 in biotransformation of extra-heavy crude oil. Microb. Biotechnol. 6, 720–730. https://doi.org/10.1111/1751-7915.12067.

Nguyen, L.-T., Schmidt, H.A., Von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. Mol. Biol. Evol. 32, 268–274. https://doi.org/10.1093/molbev/msu300.

Niebisch, C.H., Foltran, C., Domingues, R.C.S., Paba, J., 2014. Assessment of secretion extracts for decolorization of textile dyes. Int Biodeter Biodegr 88, 20–28. https://doi.org/10.1016/j.ibiod.2013.11.013.

Niladevi, K.N., Prema, P., 2008. Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolourization. Bioresour. Technol. 99, 4583–4589. https://doi.org/10.1016/j.biortech.2007.06.056.

O'Donnell, K., Cigelnik, E., 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus fusarium are nonorthologous. Mol. Phylogenet. Evol. 7, 103. https://doi.org/10.1006/mpev.1996.0376.

Oses, R., Valenzuela, S., Freer, J., Baeza, J., Rodríguez, J., 2006. Evaluation of fungal endophytes for lignocellulolytic enzyme production and wood biodegradation. Int

Biodeter Biodegr 57, 129–135. https://doi.org/10.1016/j.ibiod.2006.01.002.
Piscitelli, A., Giardina, P., Lettera, V., Pezzella, C., Sannia, G., Faraco, V., 2011. Induction and transcriptional regulation of laccases in fungi. Curr Genomics. 12, 104–112. https://doi.org/10.2174/138920211795564331.
Pakhadnia, Y.G., Malinouski, N.I., Lapko, A.G., 2009. Purification and characteristics of an enzyme with both bilirubin oxidase and laccase activities from mycelium of the basidiomycete Pleurotus ostreatus. Biochemistry 74, 1027–1034. https://doi.org/10.1134/S0006297909090119.
Paradis, E., 2010. Pegas: an R package for population genetics with an integrated-modular approach. Bioinformatics 26, 419–420. https://doi.org/10.1093/bioinformatics/btp696.
Patil, S.M., Chandanshive, V.V., Rane, N.R., Khandare, R.V., Watharkar, A.D., Govindwar, S.P., 2016. Bioreactor with *Ipomoea hederifolia* adventitious roots and its endophyte *Cladosporium cladosporioides* for textile dye degradation. Environ. Res. 146, 340–349. https://doi.org/10.1016/j.envres.2016.01.019.
R Core Team, 2017. R: A Language and Environment for Statistical Computing. URL. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org.
Ramos, J.A.T., Barends, S., Verhaert, R.M.D., De Graaff, L.H., 2011. The *Aspergillus niger* multicopper oxidase family: analysis and overexpression of laccase-like encoding genes. Microb. Cell Fact. 10, 78. https://doi.org/10.1186/1475-2859-10-78.
Rauf, M.A., Salman, S.R., 2012. Survey of recent trends in biochemically assisted degradation of dyes. Chem. Eng. J. 209, 520–530. https://doi.org/10.1016/j.cej.2012.08.015.
Robinson, T., McMullan, G., Marchant, R., Nigam, P., 2001. Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. Bioresour. Technol. 77, 247–255. https://doi.org/10.1016/S0960-8524(00)00080-8.
Rodríguez, C.S., Rodríguez, A., Paterson, R.R.M., Lima, N., Teixeira, J.A., 2006. Laccase activity from the fungus *Trametes hirsuta* using an air-lift bioreactor. Lett. Appl. Microbiol. 42, 612–616. https://doi.org/10.1111/j.1472-765X.2006.01879.x.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574. https://doi.org/10.1093/bioinformatics/btg180.
Ruhl, M., Majcherczyk, A., Kues, U., 2013. Lcc1 and Lcc5 are the main laccases secreted in liquid cultures of *Coprinopsis cinerea* strains. Antonie van Leeuwenhoek 103, 1029–1039. https://doi.org/10.1007/s10482-013-9883-7.
Saparrat, M.C.N., Hammer, E., 2006. Decolorization of synthetic dyes by the deuteromycete *Pestalotiopsis guepinii* CLPS no. 786 strain. J Basic Microb 46, 28–33. https://doi.org/10.1002/jobm.200410543.
Saratale, R.G., Saratale, G.D., Chang, J.S., Govindwar, S.P., 2011. Bacterial decolorization and degradation of azo dyes: a review. J Taiwan Inst Chem E 42, 138–157. https://doi.org/10.1016/j.jtice.2010.06.006.
Sarayu, K., Sandhya, S., 2012. Current technologies for biological treatment of textile wastewaters- a review. Appl Biochem Biotech 167 (3), 645–661. https://doi.org/10.1007/s12010-012-9716-6.
Saunders, M., Glenn, A.E., Kohn, L.M., 2010. Exploring the evolutionary ecology of fungal endophytes in agricultural systems: using functional traits to reveal mechanisms in community processes. Evol. Appl. 3 (5-6), 525–537. https://doi.org/10.1111/j.1752-4571.2010.00141.x.
Shanmugam, S., Ulaganathan, P., Swaminathan, K., Sadhasivam, S., Wu, Y.R., 2017. Enhanced biodegradation and detoxification of malachite green by *Trichoderma asperellum* laccase: degradation pathway and product analysis. Int Biodeter Biodegr 125, 258–268. https://doi.org/10.1016/j.ibiod.2017.08.001.
Singh, R.L., Singh, P.K., Singh, R.P., 2015. Enzymatic decolorization and degradation of azo dyes - a review. Int Biodeter Biodegr 104, 21–31. https://doi.org/10.1016/j.ibiod.2015.04.027.
Si, J., Peng, F., Cui, B., 2013. Purification, biochemical characterization and dye decolorization capacity of an alkali-resistant and metal-tolerant laccase from Trametes pubescens. Bioresour. Technol. 128, 49–57. https://doi.org/10.1016/j.biortech.2012.10.085.
Sinha, A., Osborne, W.J., 2016. Biodegradation of reactive green dye (RGD) by indigenous fungal strain VITAF-1. Int Biodeter Biodegr 114, 176–183. https://doi.org/10.1016/j.ibiod.2016.06.016.
Soden, D.M., Dobson, A.D.W., 2003. The use of amplified flanking region-PCR in the isolation of laccase promoter sequences from the edible fungus Pleurotus sajor-caju. J. Appl. Microbiol. 95, 553–562. https://doi.org/10.1046/j.1365-2672.2003.02012.x.
Stone, J.K., Bacon, C.W., White, J.F., 2000. An overview of endophytic microbes: endophytism defined. In: Bacon, C.W., White, J.F. (Eds.), Microbial Endophytes. Marcel Dekker Inc, New York, pp. 3–29.
Sunitha, V., Nirmala Devi, D., Srinivas, C., 2013. Extracellular enzymatic activity of endophytic fungal strains isolated from medicinal plants. World J Agric Res 9, 1–9. https://doi.org/10.5829/idosi.wjas.2013.9.1.72148.
Svobodová, K., Majcherczyk, A., Novotný, Č., Kües, U., 2008. Implication of mycelium-associated laccase from *Irpexlacteus* in the decolorization of synthetic dyes. Bioresour. Technol. 99, 463–471. https://doi.org/10.1016/j.biortech.2007.01.019.
Kumar, S., Stecher, G., Tamura, K., 2016b. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054.
Tapia-Tussell, R., Pérez-Brito, D., Rojas-Herrera, R., Cortes-Velazquez, A., Rivera-Muñoz, G., Solis-Pereira, S., 2011. New laccase-producing fungi isolates with biotechnological potential in dye decolorization. Afr J Biotechnol 10, 10134–10142. https://doi.org/10.5897/AJB11.331.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Valle, J.S., Vandenberghe, L.P.S., Oliveira, A.C.C., Tavares, M.F., Linde, G.A., Colauto, N.B., Soccol, C.R., 2015. Effect of different compounds on the induction of laccase production by Agaricus blazei. Genet Mol Res 14, 15882–15891. https://doi.org/10.4238/2015.December.1.40.
Verma, P., Madamwar, D., 2002. Decolorization of synthetic textile dyes by lignin peroxidase of Phanerochaete chrysosporium. Folia Microbiolog 47, 283–286. https://doi.org/10.1007/BF02817653.
Verma, A.K., Raghukumar, C., Verma, P., Shouche, Y.S., Naik, C.G., 2010. Four marine-derived fungi for bioremediation of raw textile mill effluents. Biodegradation 21, 217–233. https://doi.org/10.1007/s10532-009-9295-6.
Verma, S.K., Kumar, A., Lal, M., Debnath, M., 2015. Biodegradation of synthetic dye by endophytic fungal isolate in *Calotropisprocera* root. Int J Appl Sci Biotechnol 3, 373–380. https://doi.org/10.3126/ijasbt.v3i3.13136.
Wang, J.W., Wu, J.H., Huang, W.Y., Tan, R.X., 2006. Laccase production by *Monotospora* sp., an endophytic fungus in *Cynodondactylon*. Bioresour. Technol. 97, 786–789. https://doi.org/10.1016/j.biortech.2005.03.025.
Wang, Z.X., Cai, Y.J., Liao, X.R., Tao, G.J., Li, Y.Y., Zhang, F., Zhang, D.B., 2010. Purification and characterization of two thermostable laccases with high cold adapted characteristics from *Pycnoporus* sp. SYBC-L1. Process Biochem. 45, 1720–1729. https://doi.org/10.1016/j.procbio.2010.07.011.
Wesenberg, D., Kyriakides, I., Agathos, S.N., 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol. Adv. 22, 161–187. https://doi.org/10.1016/j.biotechadv.2003.08.011.
White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a Guide to Methods and Applications. Academic, New York, pp. 315–322.
Wilson, D., 1995. Endophyte: the evolution of a term, and clarification of its use and definition. Oikos 73, 274–276. https://doi.org/10.2307/3545919.
Yadav, M., Bista, G., Maharjan, R., Poudyal, P., Mainali, M., Sreerama, L., Joshi, J., 2019. Secretory laccase from *Pestalotiopsis* species CDBT-F-G1 fungal strain isolated from high altitude: optimization of its production and characterization. Appl Sci 9, 340. https://doi.org/10.3390/app902034.
Yang, Q., Yang, M., Pritsch, K., Yediler, A., Hagn, A., Schloter, M., Kettrup, A., 2003. Decolorization of synthetic dyes and production of manganese-dependent peroxidase by new fungal isolates. Biotechnol. Lett 25, 709–713. https://doi.org/10.1023/A:1023454513952.
Yang, J., Wang, G., Ng, T.B., Lin, J., Ye, X., 2016. Laccase production and differential transcription of laccase genes in *Cerrena* sp. in response to metal ions, aromatic compounds, and nutrients. Front. Microbiol. 6, 1558. https://doi.org/10.3389/fmicb.2015.01558.
Yanto, D.H.Y., Tachibana, S., 2013. Biodegradation of petroleum hydrocarbons by a newly isolated *Pestalotiopsis* sp. NG007. Int Biodeter Biodegr 85, 438–450. https://doi.org/10.1016/j.ibiod.2013.09.008.
Yanto, D.H.Y., Tachibana, S., 2014. Enhanced biodegradation of asphalt in the presence of Tween surfactants, Mn^{2+} and H2O2 by *Pestalotiopsis* sp. in liquid medium and soil. Chemosphere 103, 105–113. https://doi.org/10.1016/j.chemosphere.2013.11.044.
Yanto, D.H.Y., Tachibana, S., Itoh, K., 2014a. Biodecolorization and biodegradation of textile dyes by the newly isolated saline-pH tolerant fungus *Pestalotiopsis* sp. J of Envir Sci Tech 7, 44–55.
Yanto, D.H.Y., Tachibana, S., Itoh, K., 2014b. Biodecolorization of textile dyes by immobilized enzymes in a vertical bioreactor system. Procedia Environ. Sci. 20, 235–244. https://doi.org/10.1016/j.proenv.2014.03.030.
Zilly, A., da Silva Coelho-Moreira, J., Bracht, A., Marques de Souza, C.G., Carvajal, A.E., Koehnlein, E.A., Peralta, R.M., 2011. Influence of NaCl and Na_2SO_4 on the kinetics and dye decolorization ability of crude laccase from *Ganoderma lucidum*. Int Biodeter Biodegr 65, 340–344. https://doi.org/10.1016/j.ibiod.2010.12.007.
Zhu, X., Gibbons, J., Garcia-Rivera, J., Williamson, P.R., Casadevall, A., 2001. Laccase of *Cryptococcus neoformans* is a cell wall-associated Virulence factor. Infect. Immun. 69, 5589–5596. https://doi.org/10.1128/IAI.69.9.5589.