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Laccase purification and characterization from *Trametes trogii* isolated in Tunisia: decolorization of textile dyes by the purified enzyme

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Abstract

A white-rot basidiomycete, isolated from decayed acacia wood (from Northwest of Tunisia) and identified as *Trametes trogii*, was selected in a broad plate screening because of its ability to degrade commercial dyes. In liquid cultures using a glucose–peptone medium, the sole ligninolytic activity detected was laccase. The highest laccase levels were obtained in presence of $CuSO_4$ as inducer (around 20000 U/l). Two isoenzymes, were purified using anion-exchange and size-exclusion chromatographies. Both isoenzymes are monomeric proteins, with M_w around 62 kDa and isoelectric points of 4.3 and 4.5, showing similar stability at pH and temperature, optimum pH and substrate specificity. The highest oxidation rate was obtained at pH 2 and 2.5 for ABTS and DMP, respectively. They were stable up to 50 °C for 24 h and the stability was higher at alkaline pH. Activity increased by the addition of 10 mM Ni, Mo or Mn but it was not affected by Cd, Al, Li and Ca. Identical N-terminal sequences were determined in both laccases. The crude enzyme, as well as the purified laccase, was able to decolorize dyes from the textile industry.

Keywords: Fungi; Basidiomycete; Enzymes; Industrial dyes; *Trametes trogii*

1. Introduction

White rot fungi are believed to be the most effective lignin-degrading microbes in nature. They produce different kinds of extracellular oxidoreductases, including laccases [26], peroxidases [10] and oxidases producing H_2O_2 [18]. These enzymes are involved in the degradation of lignin [20], but also of other aromatic recalcitrant compounds causing environmental problems. Both laccases and peroxidases can catalyze the one-electron oxidation of aromatic lignin units, resulting in various non-enzymatic reactions. The peroxidases have the highest redox potential, being able to catalyze directly the oxidation of non-phenolic compounds. However, the use of oxygen (a non-limiting electron acceptor) by the laccases makes these enzymes more adequate for industrial and environmental applications.

Laccase is an enzyme secreted by the most of the lignin degrading basidiomycetes [23] and it has been reported as an essential enzyme for lignin degradation in fungi without peroxidases [15]. This enzyme catalyzes the oxidation of a wide number of phenolic compounds and aromatic amines but its substrate range have been extended to non-phenolic compounds in the presence of low molecular mass compounds acting as mediators [5,14]. Most of the studies have been carried out with laccases from eukaryotes, principally with enzymes secreted by basidiomycetes being their distribution in prokaryotes more recently reported [7].

The textile industry, by far the most avid user of synthetic dyes, is in need of ecologically efficient solutions for its colored effluents. Wastewaters from textile industries are a complex mixture of many polluting substances such as organochlorine-based pesticides, heavy metals, pigments and dyes. Dye effluents are poorly decolorized by conventional biological wastewater treatments and may be toxic for the microorganisms present in the treatment plants due to their complex aromatic structures. Furthermore, following anaerobic digestion, nitrogen-containing dyes are transformed into aromatic amines that are more toxic and mutagenic than the parent molecules. To overcome these

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difficulties, and taking into account the broad substrate specificity of white-rot fungi (WRF) to degrade aromatic compounds, these fungi and their ligninolytic enzymes are being investigated for their potential application in textile effluent treatments. Among them, the most widely studied is the white-rot fungi *Phanerochaete chrysosporium*, producing two kind of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP) in ligninolytic conditions and a multicopper oxidase only when cellulose is present as carbon source. This fungus requires the addition of oxygen to the cultures to carry out the degradative process, but other fungi being able to degrade pollutants under environmental conditions could be more promising. This is the case of *Trametes versicolor*, which produces a high level of laccase, and is being studied for industrial applications [2].

Trametes trogii, a worldwide distributed white-rot basidiomycete, is also another example. It has been demonstrated to be a good producer of laccases and other ligninolytic enzymes including LiP and MnP [12,23,27,30]. This fungus was also shown to be efficient tool for the degradation of several organic pollutants including nitrobenzene and anthracene [29], PCB mixture (Aroclor 1150) and an industrial PAH mixture (10% V/V of PAHs, principal components hexaethylbenzene, naphthalene, 1-methyl naphthalene, acenaphthylene, anthracene, fluorene and phenanthrene) [19].

Since the enzymatic system secreted by basidiomycetes depends of the kind of fungus, the strain and the culture conditions, more white-rot fungi have to be screened for their ability to degrade recalcitrant aromatic compounds, including dyes present in industrial effluents causing environmental problems. In this paper, we isolated a promising fungal strain from the north of Tunisia, identified as a *Trametes* (=*Funalia*) *trogii* strain, able to oxidize ABTS and to decolorize the commercial dye Poly R478. We characterized the laccase produced by this strain, the single ligninolytic enzyme secreted by this fungus in the studied conditions, and studied its role in the decolorization of some textile dyes.

2. Material and methods

2.1. Chemicals

2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) and 2,6-dimethoxyphenol (DMP) were from Sigma–Aldrich, H_2O_2 (Perhydrol, 30%) was obtained from Boehringer, aromatic compounds were from Sigma–Aldrich or Fluka. All other chemicals used were of analytical grade. The azo dyes Neolane blue, Neolane pink Neolane yellow and Maxilon blue and the indigoid dyes Basacryl yellow and Bezaktiv S-BF turquoise, were provided by the institute of textile at Ksar Helal, Tunisia.

2.2. Media and culture conditions

The solid medium used for isolation of the fungal strains contained per liter: 10 g of malt extract, 4 g of yeast extract, 4 g of glucose and 20 g of agar. The pH was adjusted to 5.5 with 2N NaOH. For the detection of ligninolytic activity the fungi were grown on malt–agar plates supplemented with 0.5 mM ABTS or Poly R478.

For laccase production and induction studies, 3.0 ml of homogenized mycelium were used for inoculation of 1000-ml Erlenmeyer flask containing 300 ml of culture medium. This basal medium contained (per liter): glucose, 10 g; peptone, 5 g; yeast extract, 1 g; ammonium tartrate, 2 g; KH_2PO_4, 1 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; KCl, 0.5 g; trace elements solution, 1 ml. The trace elements solution composition per liter was as follow: $B_4O_7Na_2{\cdot}10H_2O$, 0.1 g; $CuSO_4{\cdot}5H_2O$, 0.01 g; $FeSO_4{\cdot}7H_2O$, 0.05 g; $MnSO_4{\cdot}7H_2O$; 0.01; $ZnSO_4{\cdot}7H_2O$, 0.07 g; $(NH4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.01 g. The pH of the solution was adjusted to 5.5. Cultures were incubated at 30 °C on a rotary shaker (160 rpm). Basal medium was supplemented with $CuSO_4{\cdot}5H_2O$ (0–600 μM) and ethanol (3% V/V) as inducer of laccases and $MnSO_4{\cdot}4H_2O$ (150 μM) was added as inducers of MnP.

2.3. Isolation of the fungal strain

The fungus used in this study was isolated in 2003 from decaying acacia wood in the vicinity of Bousalem, Northwest of Tunisia. The selected strain, denominated B6J, was identified by the Spanish culture collection of microorganisms as *T. trogii* and selected by their capability to oxidize ABTS in solid medium and decolorize Poly R478. The culture was maintained on 2% malt extract agar plates grown at 30 °C and stored at 4 °C.

2.4. Enzyme assays and analysis of protein

Laccase activity was assayed using 10 mM DMP in 100 mM sodium tartrate buffer, pH 5 ($\varepsilon_{469} = 27{,}500\ M^{-1}\ cm^{-1}$, referred to DMP concentration) [40]. Mn-oxidizing peroxidase activity was estimated by the formation of Mn^{3+}-tartrate complex (ε_{238}: $6500\ M^{-1}\ cm^{-1}$) during the oxidation of 0.1 mM Mn^{2+} ($MnSO_4$) in 100 mM sodium tartrate buffer (pH 5) in the presence of 0.1 mM H_2O_2. LiP activity was determined by the H_2O_2-dependent veratraldehyde (3,4-dimethoxybenzaldehyde) formation ($\varepsilon_{310} = 9300\ M^{-1}\ cm^{-1}$) from 2 mM veratryl alcohol (3,4-dimethoxybenzyl alcohol) in 100 mM sodium tartrate buffer (pH 3) in the presence of 0.4 mM H_2O_2. Aryl-alcohol oxidase activity was also estimated by veratraldehyde formation from 5 mM veratryl alcohol in 100 mM phosphate buffer, pH 6 [18].

The enzymatic reactions were carried out at room temperature (22–25 °C) and one unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of substrate min^{-1}. Extracellular protein was determined by the Bradford method, using Bio-Rad protein assay and bovine serum albumin as standard.

2.5. Laccase purification

Laccase from *T. trogii* was purified from basal medium with 150 μM $CuSO_4$. The culture liquid from 10 days was separated from mycelia by filtration on Whatman paper, concentrated and dialyzed against 10 mM tartrate buffer (pH 5.5) by ultrafiltration (Filtron, 3-kDa cutoff membrane). Samples of 50 ml of this crude enzyme preparation were applied to a Hitrap Q FF cartridge (Amersham Biosciences) equilibrated with the same buffer at a flow rate of 1.5 ml min^{-1}. The retained proteins were eluted over 100 min using the following NaCl gradient: 0 to 400 mM, 60 min; 400 to 1000 mM, 30 min and 1000 mM 10 min.

Fractions with laccase activity were pooled, concentrated (Filtron Microsep, 3-kDa cutoff), and samples of 0.2 ml were applied to a Superdex 75 (Pharmacia HR 10/30) column equilibrated with 10 mM sodium tartrate buffer, pH 5.5 containing 150 mM NaCl, at a flow rate of 0.4 ml min^{-1}. The laccase peak was pooled, concentrated (Filtron Microsep, 3-kDa cutoff), dialyzed against 10 mM sodium tartrate buffer, pH 5.5, and 1 ml samples applied to a Mono-Q anion-exchange column (Pharmacia HR 5/5) equilibrated with the same buffer. Retained proteins were eluted with a linear NaCl gradient from 0 to 120 mM over 30 min, at a flow rate of 0.8 ml min^{-1}.

2.6. Properties of purified laccases

The molecular mass of the laccase was determined by SDS/PAGE and gel filtration. SDS/PAGE was performed with 12% polyacrylamide gels, using high-molecular-mass standards (Bio-Rad) ovalbumin (45 kDa), bovine serum albumin (66.2 kDa) and phosphorylase b (97.4 kDa), β-galactosidase (116.25 kDa), and Myosin (200 kDa). Isoelectricfocusing was performed on 5% polyacrylamide gels with a thickness of 1 mm and a pH gradient from 2.5 to 5.5 [38] (determined using a contact electrode). Zymograms were obtained using 10 mM DMP in 100 mM sodium tartrate buffer, pH 5, after washing the gels for 10 min with the same buffer. Protein bands were stained with Coomassie brilliant blue R-250. Gel filtration was carried out on Superdex 75, calibrated with aldolase (158 kDa),

albumin (67 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa), to determine the molecular mass of native protein. N-terminal sequence of laccase was determined by automated Edman degradation of 5 μg of protein in an Applied Biosystem protein sequencer (Perkin-Elmer, Procise 494). The UV-visible spectrum of the purified laccase was recorded in 10 mM sodium phosphate buffer pH 6.5. The effect of temperature on laccase stability was investigated in sodium tartrate buffer pH 4, whereas pH stability and optimum pH were determined in citrate–borate-phosphate buffer (pH range between 2.0–11).

2.7. Substrate specificity

Substrate specificity was qualitatively studied by the changes in the absorption spectra of reaction mixtures with phenolic and non-phenolic aromatic compounds, which contained 0.5 mM substrate, 130 mU ml^{-1} purified laccase, and 100 mM sodium tartrate buffer, pH 4. These compounds are listed in Table 2.

Kinetic constants for ABTS were calculated in 100 mM sodium tartrate buffer, pH 4. The molar extinction coefficient for ABTS was $\varepsilon_{436} = 29{,}300\ M^{-1}\ cm^{-1}$.

2.8. Decolorization of textile dyes

To test the ability of the fungal culture to decolorize industrial dyes, six different dyes were solubilized in water, membrane-filtered through a 0.45 μm cellulose nitrate filter and mixed with the malt extract agar medium, previously autoclaved (final concentration of 50 mg l^{-1}). After three weeks of incubation, dye decolorization was determined qualitatively for each dye by comparing color in the inoculated plates with that of plates containing the medium and the dyes, without the fungus.

Decolorization of textile dyes was investigated also by the crude and purified laccase (Lac I). The reaction mixture (5 ml) contained 100 mM sodium tartrate buffer pH 5, dye (50 mg l^{-1}) and laccase (0.5 U ml^{-1}). The reaction was initiated with enzyme and incubated at 30 °C. Samples were withdrawn at 4 h intervals and subsequently analyzed. Spectra were recorded between 200 and 800 nm using a Shimadzu UV–vis spectrophotometer. The dyes partially or non-decolorized by laccase were tested in the presence of 0.5 mM HBT (1-hydroxybenzotriazole), a common laccase mediator, to increase the oxidative effect of the enzyme.

3. Results

3.1. Isolation of fungal strain and production of ligninolytic activity in liquid medium

Screening of local fungi for ligninolytic activities was performed using samples of decayed wood collected from a forest in the north west of Tunisia. A high number of fungal strains were isolated on malt extract–agar medium, and screened for enzyme production using the same medium supplemented with ABTS or Poly R478. The strain B6J, identified as *T. trogii,* was chosen because it exhibited a fast and large oxidation of ABTS on agar plates, as demonstrated by the dark green color appeared in the plates.

The production of extracellular laccase and peroxidase activities, proteins and reducing sugars was studied in this *T. trogii* strain, in the presence of different enzyme inducers. Low laccase activity was detected in the absence of Mn^{2+} or Cu^{2+} in the cultures. The presence of Mn^{2+} in the basal medium increased slightly the laccase activity levels, but the higher induction was obtained in presence of Cu^{2+} in the basal medium. The presence of ethanol in these cultures did not increase laccase activity. MnP, LiP, and aryl-alcohol oxidase were not detected in any of the conditions assayed.

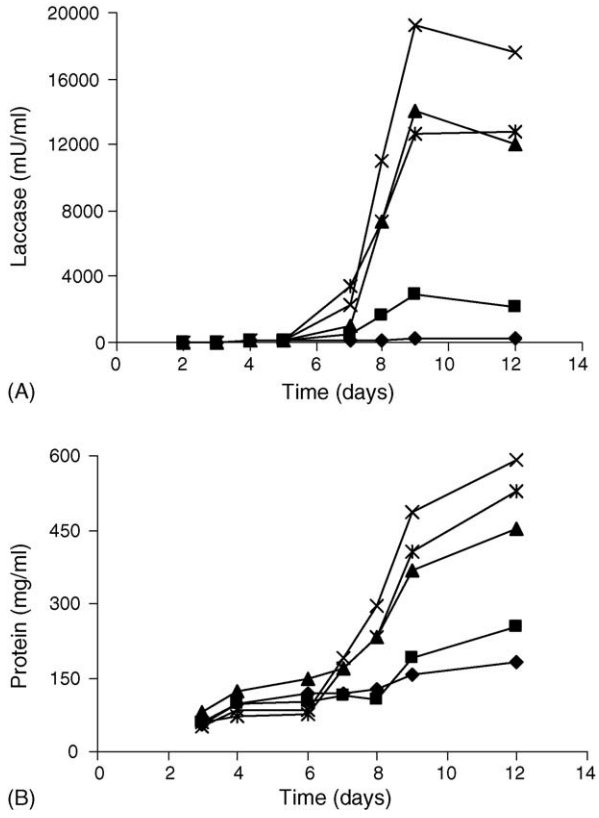

Fig. 1. Time course of extracellular laccase activity (A) and proteins (B) in *T. trogii*. Control (♦), 75 μM Cu(■), 150 μM Cu (▲), 300 μM Cu (×), 600 μM Cu (✱).

The effect of different Cu^{2+} concentration on *T. trogii* laccase production is shown in Fig. 1. Maximal laccase activity and proteins were obtained with 300 μM $CuSO_4$, decreasing this activity when the Cu^{2+} concentration was higher. Zymograms after isoelectrofocusing of crude enzyme preparations obtained from cultures carried out in the absence of Cu^{2+} resulted in two laccase activity bands, a major band and a minor band. The zymograms from Cu^{2+}-induced cultures showed that the induced protein corresponded to the major protein band after staining the gel with Coomassie blue R-250 (data not shown).

Partial characterization of the laccase in the crude preparation, to optimize the purification process, showed an optimal pH around 4. This activity was stable in the crude at pH 7 at room temperature for 24 h, but retained more than 50% of its activity at pH 5. The laccase in the crude extract was also stable for 24 h at 50 °C however, it lost more than 90% of its activity at 60 °C.

3.2. Purification of the laccases

Two proteins with laccase activity were purified to homogeneity from the basal medium supplemented with 150 Cu^{2+}. Table 1 summarizes the results obtained from 10-days old culture. During the first chromatographic step (Q-Cartridge) the laccase activity was separated from most impurities, which include a brown pigment absorbing strongly at 280 nm (Fig. 2A). During the filtration chromatography (Superdex 75), laccase activity was detected as a symmetrical peak separated of other

Table 1
Scheme of purification of *T. trogii* strain B6J laccase

	Total activity (U)	Protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification factor (fold)
UF	5395	665	8.11	100.0	1.0
Hittrap	4420	425	10.40	81.9	1.3
Superdex	3216	64	50.25	59.6	6.2
MonoQ Lac1	1983	28	70.66	36.8	8.7
MonoQ Lac2	729	13	53.56	13.5	6.6
Total Lac	2712	41		50.3	

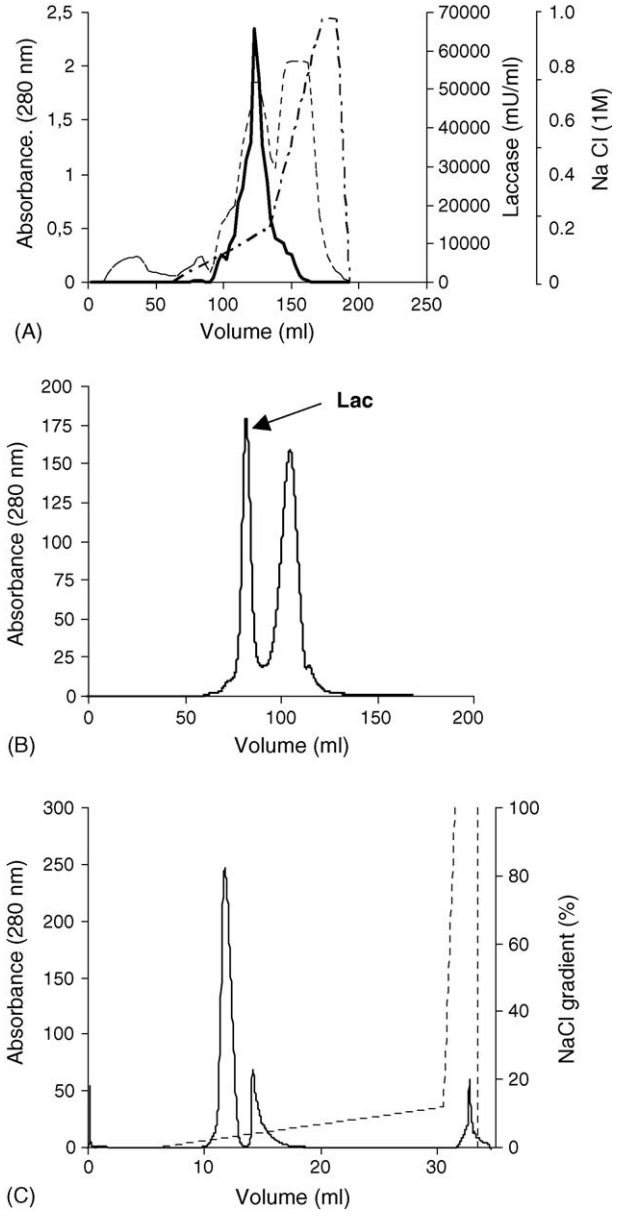

Fig. 2. Purification of *T. trogii* laccases from 150 μM Cu^{2+}-induced cultures: chromatography on Hitrap Q-cartridge (A). Superdex 75 (B). Mono-Q columns (C). Profiles corresponding to optical density at 280 nm (solid line), NaCl gradient (dashed line) and laccase activity (solid bold line).

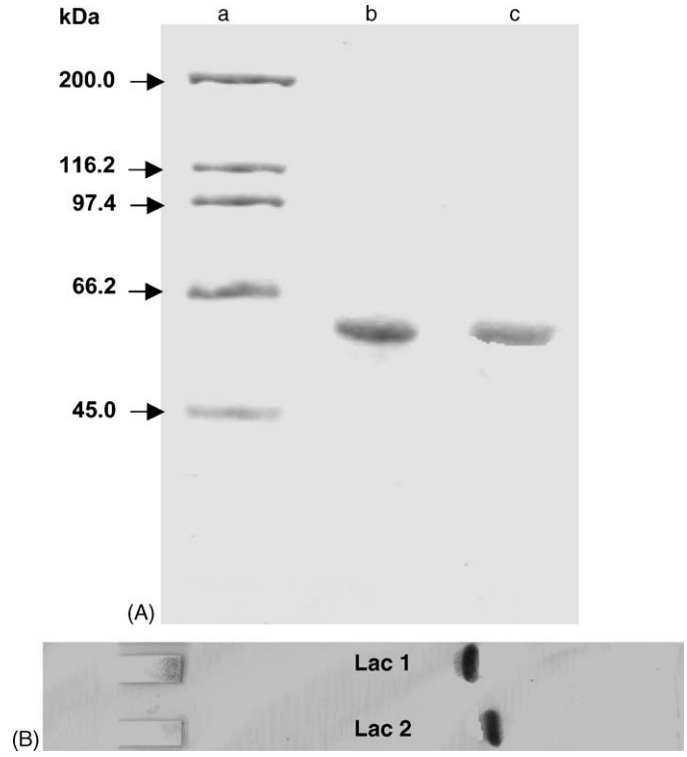

Fig. 3. Estimation of the molecular mass and the isolectric point of the *T. trogii* laccases. (A) SDS/PAGE (12% polyacrylamide gels) of purified laccases. High-molecular-mass standards (lane a), Lac I (lane b) and Lac II (lane c). (B) Isoelectricfocusing of LacI and LacII on 5% polyacrylamide gel.

contaminant proteins (Fig. 2B). Last step on a high efficiency exchange anion column (Mono-Q) resolved two laccase activity peaks: a major LacI and LacII (Fig. 3c). At the end of the process, LacI and LacII had been purified 31 and 11-fold, respectively.

The molecular masses of both proteins LacI and LacII were 58 kDa as estimated by gel filtration chromatography and 62 kDa as determined by SDS-PAGE (Fig. 3a). These results suggest that both enzymes are monomeric proteins.

Analytical isoelectric focusing showed a pI of 4.3 for LacII and 4.5 for LacI (Fig. 3b). The N-terminal amino acid sequences of LacI and LacII were identical: SIGPVADLTISNGAVSPDGF. Both purified laccases showed the optimum activity, estimated in 100 mM tartrate buffer, at pH 2.5 and 3 for the oxidations of ABTS and DMP, respectively.

3.3. Substrate specificity of T. trogii laccase

The substrate specificity of both laccases obtained from the *T. trogii* cultures supplemented with 300 μM Cu^{2+}, was qualitatively studied on different phenolic and non-phenolic aromatic compounds (Table 2). Both laccases present similar activity on the studied substrates. They are able to oxidize substituted phenols, catechol, phenolic aldehydes and acids. No activity was observed on, phenol, chloro-substituted phenols, and the non-phenolic aromatic compounds (Table 2). The kinetic studies were carried out using ABTS as substrate (Table 3). The results showed significant differences in the K_m and k_{cat} of Lac I and Lac II but similar efficiency to oxidize this substrate.

Table 2
Substrate specificity of *T. trogii* laccases

Substrate	Wavelength[a] (nm)
DMP	469
ABTS	436
2-Methoxyphenol	464
3,5-Dimethoxy-4-hydroxybenzoate (syringate)	296
3-Methoxy-4-hydroxybenzoate (vanillate)	248
3-Hydroxybenzoate	NC
3,4-Dihydroxyphenylacetate	308
1,2,3-Trihydroxybenzene (pyrogallol)	450
Phenol	NC
3-Chlorobenzoate	NC
3,4,5-Trimethoxybenzoate	NC
3,4-Dihydroxycinnamate (caffeate)	308
3-Methoxy-4-hydroxybenzaldehyde (vanillin)	230
2-Methylphenol (*o*-cresol)	NC
3-Methylphenol (*m*-cresol)	NC
4-Methylphenol (*p*-cresol)	NC
4-Hydroxyphenylethanol (tyrosol)	NC
3,4-Dihydroxybenzoate (protocatechuate)	308
Benzoate	NC
3,4-Dimethoxybenzoate (veratrate)	NC
2-Methoxybenzoate (*o*-anisate)	NC
3,4,5-Trihydroxybenzoate (gallate)	450
3-Methoxy-4-hydroxycinnamate (ferulate)	287
4-Methoxy-3-hydroxybenzoate (isovanillate)	248
4-Hydroxycinnamate (*p*-coumarate)	NC
2-Hydroxycinnamate (*o*-coumarate)	NC
3,5-Dinitrobenzoate	NC
4-Chlorophenol	NC
1,2 Benzenediol (catechol)	396
4-Hydroxybenzoate	NC
Phenylacetate	NC
4-Hydroxyphenylacetate	NC

NC: no changes in the reaction.
[a] New absorption maxima observed after incubation of the substrate with the laccases.

Table 3
Kinetic constants against ABTS of laccases from *T. trogii*

	K_m (mM)	k_{cat} (s^{-1})	Efficiency (s^{-1} mM^{-1})
Lac I	0.050	344	6888
Lac II	0.033	207	6263

3.4. Decolorization of textile dyes

The ability of *T. trogii* strain B6J to decolorize textile dyes was tested first on solid media. The decolorization was possible in the presence and in the absence of Cu^{2+}, although it was faster when Cu^{2+} was present in the medium. The fungus grew well in the plates and decolorized the dyes Neolane blue, Neolane yellow, Neolane pink and Bezaktiv S-BF turquoise but a slow growth was obtained on the plates containing Basacryl yellow and Maxilon blue and no decolorization was obtained for these dyes, either in the presence of Cu.

Similar results were obtained with the crude enzyme and the purified laccase (Table 4). Neolane blue and Neolane pink were completely decolorized after 24 h of treatments. The dyes Neolane yellow and Bezaktiv S-BF turquoise were only partially decolorized after 4 days of treatments. No decolorization was detected after 4 days of treatments for Basacryl yellow and Maxilon blue.

Table 4
Decolorization of some textile dyes by the laccase of *Trametes trogii* strain B6J

Dyes	Incubation time (days)	Decolorization Without HBT		Decolorization With HBT
		Crude enzyme	Pure enzyme	Crude enzyme
Neolane yellow[a]	1	18	26.6	100
	4	39.6	40.5	100
Maxillon blue[a]	1	0	0	15.1
	4	4.1	0	35.3
Neolane pink	1	63.6	63.6	nd
	4	86.4	86.4	nd
Basacryl yellow[a]	1	0	0	0
	4	0	0	0
Neolane blue	1	91.5	91.5	nd
	4	91.2	91.2	nd
Bezaktiv yellow	1	54	53	nd
	4	71	68.5	nd

nd: not determined.
[a] Dyes which were slightly (less than 50% decolorization) or not decolorized were treated with crude enzyme in the presence of 0.5 mM HBT.

The dyes not decolorized by the laccase, and the Neolane yellow, partially decolorized by this enzyme, were tested in the presence of HBT as laccase mediator (Table 4). The results show that the addition of mediator increased the decolorization of Neolane yellow, being complete after 24 h of incubation, Maxilon blue was partially decolorized in this conditions (4 days of incubation) and no effect was detected on Basacryl yellow after laccase-mediator treatment.

4. Discussion

The *T. trogii* strain isolated from the north of Tunisia, which was able to oxidize ABTS and decolorize the commercial dye Poly R478, secreted only laccase in the conditions studied, even though peroxidase inducers were added to the culture medium. The presence of Mn^{2+} increase slightly the laccase activity levels in the cultures, but no Mn^{2+}-oxizidizing activity was detected. The addition of Cu^{2+} produced the strongest laccase induction. These results are in agreement with that reported for other *T. trogii* strains [29].

Although it has been reported that the presence of ethanol in the cultures can increase laccase production in some basidiomycetes [22,32,33], the addition of ethanol to the copper supplemented cultures in *T. trogii* did not produce a significant effect. Copper induction has been reported in other fungal laccases, including the laccases secreted by different *Trametes* species [16,17,35] and in the case of *T. versicolor*, it has been described the regulation by copper of laccase gene at the transcription level [9]. In the *T. trogii* strain isolated in Tunisia, the addition of $CuSO_4$ to basal medium enhanced more than 80-fold the laccase activity at 150 μM copper. The best results were obtained in presence of 300 μM copper (100-fold more than basal medium) but higher concentration produced a decrease

Table 5
Properties of laccases from different *Trametes trogii*

Strain	*K*m (mM)	Mr kDa	pI	Glycosilation %	pH opt	pH stability	Stable temp.	N-terminal acid sequence
T. trogii Strain B6J								
LacI	0.050[a]	62	4.3	nd	2.5[a]-3[d]	7	50 °C (24h)	S **I G P V A D L** T **I S N G A V** S **P D G F**
LacII	0.033[a]	62	4.5	nd	2.5[a]-3[d]	7	50 °C (24 h)	S **I G P V A D L** T **I S N G A V** S P D G F
T. trogii Strain 201 [17]	0.03[a]	70	3.6	12	3–3.5[a,d]	nd	nd	A **I G P V A D L** V **I S N G A V** T **P D G F**
Basidiomycete C30 [11]	0.0071[s]	63	3.6	nd	4.5[g]	nd	60 °C (1h)	S **I G P V A D L** T **I S N G A V** S **P D G F**
Basidiomycete PM1 [8]	0.5[g]	64	3.6	6.5	4.5[g]	3–9	60 °C (1 h)	S **I G P V A D L** T **I S N G A V** S **P**
T. trogii BAFC 463								
Lac1[28]	nd	60	nd	nd	3.4[a]	>4.4	30 °C (5d)	nd
Lac 2[28]	nd	38	nd	nd				

nd: not determined.
[a] ABTS.
[d] 2,6-dimethoxyphenol.
[g] Guiacol.
[s] Syringaldazine.

of fungal biomass and laccase activity, probably due to a toxic effect on the fungal culture.

Although it has been described laccases with two subunits from *Trametes villosa* [41], laccases from basidiomycetes, including *Trametes* species, are generally monomeric protein with a molecular mass between 50 and 80 kDa [28,40]. The two laccases purified from *T. trogii* are monomeric proteins with the same molecular mass (62 kDa), but a small difference in the pI values have permitted their separation in Mono-Q column. Compared to the laccases of other *T. trogii* strains, the laccases from our strain has a molecular mass close to most laccases except that of strain 201 [17], and one isoenzyme from strain BAFC 463 [28] which were 70 kDA and 38 kDa, however the pI of the two isoenzymes from our strain were less acid than all the other laccases from *T. trogii* strains (Table 5).

The N-terminal sequence of new *T. trogii* laccases was the same for both purified proteins. The 20 residues analyzed showed 100% identity with laccases from Basidiomycete PM1 [8] and Basidiomycete C30 (formerly *Marasmius quercophilus* reclassified as a *Trametes* species [11,25], but only 85% identity with *T. trogii* strain 201 [17] (Table 5).

The comparison of the N-terminal sequence of the laccases from *T trogii* strain B6J with those of laccases from other white-rot fungi showed 85% identity with laccases from *T. versicolor* [4], 80% with laccases from *Pycnoporus cinnabarinus* and *P. coccineus* [13,21], and 75% with *Phlebia radiata* and *Coriolopsis rigida* laccases [37,38].

As a typical laccase, the enzyme secreted by *T. trogii* showed no activity on tyrosine and had a wide substrate specificity oxidizing hydroxy- and methoxy-substituted phenols. The kinetic study on ABTS showed a high affinity of *T. trogii* laccase on this substrate, in the same order that *P. coccineus* and *C. rigida* laccases [21,38] and higher than laccases from *Pleurotus eryngii*. Differences in affinity of this substrate could be related with the redox potential of these enzymes and its role in the degradation of recalcitrant compounds present contaminated soils or residual wastewater [22].

The ability of white-rot fungi to decolorize synthetic textile dyes has been widely studied, particularly with *P. chrysosporium* and *T. versicolor* [3]. The role of laccases and peroxidases secreted by these fungi is controversial although, in the case of *P. chysosporium* only peroxidases seem to be involved in the process [33]. In *Trametes* species laccases are the major enzymes but peroxidases also are secreted during dye decolorization [24,31].

The ability of *T. trogii* to decolorize industrial dyes was demonstrated in several studies as reported by Apohan and Yesilada [1], Cing and Yesilada [6], Levin et al. [30], Ozsoy et al [35], Ünyayar et al. [39] and Yesilada et al. [42,43].

Textile dyes Drimarene blue X3LR, Remazol brilliant blue R, Astrazon blue and red, orange II, Ponceau 2R (a xylidine derivative), malachite green, anthraquinone blue, and Reactive Black 5 (RB5) were used to study the ability of *T. trogii* to decolorize textile dyes. In most of these studies the culture or the fungal pellet of *T trogii* were used however no studies using purified laccase were reported. Our study is the first to report the use of purified laccase from *Trametes trogii.*

The *T. trogii* strain isolated in Tunisia was able to decolorize Poly R 478 in the agar-plates and Cu^{2+} addition stimulated decolorization, suggesting that laccase could be involved in the process. The results on dye decolorization with the crude enzyme, without peroxidase activity, also indicated that laccase is the enzyme involved in the process. The results obtained with the purified enzyme were similar to those obtained with the crude enzyme, confirming that *T. trogii* laccase decolorizes industrial dyes.

Dye decolorization with the fungal culture and the crude and purified laccase show the different nature of the industrial dyes. Probably an inhibitory effect of the dyes Maxillon blue and Basacryl yellow, at the assayed concentration, are the responsible of the low growth obtained in the agar-plates in the presence of these industrial dyes. The similar results obtained with crude and purified laccase confirmed the role of the enzyme in dye decolorization. The presence of laccase mediator in the process increased the range and rate of decolorization. This effect has

been also reported using laccase from *Coriolopsis gallica* [36] and *Trametes modesta* [34].

The ability of *Trametes* species to decolorize different dyes is evident, but the advantage of laccase treatment is a shorter treatment period. The *T. trogii* isolated from Tunisia is a promising fungal strain since it produces a high laccase levels in the studied conditions. Currently, the optimization of laccase production from this fungal strain is being studied and industrial dyes effluents from Tunisia textile industry are being treated with the enzyme to check its potential use in decolorization and detoxification of the effluents.

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