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Purification, characterization and decolourization ability of *Fomes fomentarius* laccase produced in solid medium

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ABSTRACT

Laccase produced by *Fomes fomentarius* grown on wheat bran in solid cultures was purified to electrophoretic homogeneity by ammonium sulfate precipitation, size-exclusion chromatography and anion-exchange chromatography. A single laccase was found having apparent molecular mass of 51 kDa. The N-terminal amino acid sequence was IGPKTDLTIATGDVSPDG and the highest similarity value was found to the laccase from *Trametes* sp. 420 (94%). The enzyme exhibits a temperature optimum of 60 °C and has a half-life of 66 min at 60 °C. It manifested maximal activity at pH 4 and showed K_m, k_{cat} and k_{cat}/K_m values of 26 μM, 106 s^{-1} and 4×10^6 s^{-1} M^{-1}, respectively, with 2,6-dimethoxyphenol as substrate. The purified laccase was resistant to several metal ions such as Cd^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} and Cu^{2+}. In addition, the enzyme had ability to decolourize the anthraquinone dye Remazol Brilliant Blue R without mediators.

1. Introduction

Laccases (benezenediol:oxygen oxidoreductase, EC 1.10.3.2) are multinuclear copper-containing enzymes that catalyze the oxidation of a variety of phenolic and inorganic compounds, with the concomitant reduction of oxygen to water [1]. Due to their broad substrate specificity, laccases have great potential in various applications including pulp delignification, xenobiotics degradation and textile dye bleaching [2,3]. Moreover, due to their high stability, selectivity for phenolic substructures, and mild reaction conditions, laccases are attractive for fine chemical synthesis and numerous synthetic processes have now been reported [4,5].

Laccases are widely distributed in the higher plants, some insects, a few bacteria, and fungi. Most of the known laccases are of fungal origin, in particular from the white-rot fungi [6]. Fungal laccases can be produced using solid-state or submerged cultivation systems, the former is advantageous in obtaining concentrated metabolites and subsequent purification procedures are economical [7].

A review of literature shows a number of reports on purification and characterization of fungal laccases produced in submerged cultures. The purified laccases differ widely from each other in their biochemistry and mode of action, e.g. in molecular mass (43–383 kDa), p*I* (2.6–6.9), optimum temperature (25–80 °C), optimum pH (2–8), K_m (3–30,000 μM), k_{cat} (90–360,000 s^{-1}), etc. [8]. De Souza and Peralta [9] have reported effective purification of laccase produced by white-rot fungus *Pleurots pulmonarius* on wheat bran solid medium.

The white-rot fungus *Fomes fomentarius* has been recently described as a good producer of laccase on wheat bran solid-state medium [10,11]. In this paper, the laccase produced by *F. fomentarius* under this condition was purified to apparent electrophoretic homogeneity and some properties of the enzyme were studied.

2. Experimental

2.1. Chemicals

Unless otherwise stated all chemicals were purchased from Sigma–Aldrich or Fluka and were of certified reagent grade.

2.2. Microorganism

F. fomentarius (MUCL 35117) used in this study was obtained from the Belgian Coordinated Collections of Microorganisms/Mycothèque de l'Université Catholique de Louvain (BCCM/MUCL) and maintained at 4 °C on 2% malt extract agar (MEA).

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2.3. Solid-state fermentation (SSF) and enzyme production

The laccase was produced under solid-state fermentation (SSF) conditions by *F. fomentarius* as described by Neifar et al. [11]. Cylindro-conical flasks (125 ml) containing 2.5 g of wheat bran (WB) and 15 ml of sodium acetate buffer 20 mM, pH 5.0 were inoculated with five agar plugs (diameter, 6 mm), coming from an actively growing fungus on MEA medium. The flasks were capped with cotton stoppers, which permitted passive aeration, and were incubated at 30 °C under dark. Extracellular enzymes from SSF were extracted with the same buffer (10 ml buffer/g substrate), by shaking for 1 h at 160 rpm at room temperature.

2.4. Enzyme assays

The laccase activity was measured by monitoring the oxidation of 5 mM 2,6-dimethoxyphenol (DMP) buffered with 0.1 M tartrate buffer (pH 4.0) at 469 nm for 1 min [12]. To calculate enzyme activity an absorption coefficient of 27,500 M^{-1} cm^{-1} was used. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of DMP per minute.

2.5. Protein determination

Protein concentration was measured as described by Bradford [13] using bovine serum albumin (BSA) as the reference.

2.6. Enzyme purification

Laccase from *F. fomentarius* was purified from 14-day-old solid culture. The culture supernatant was separated from mycelia by filtration on Whatman paper. Proteins were precipitated by the addition of $(NH_4)_2SO_4$ up to 80% saturation at 4 °C and centrifuged at 10,000 × *g* for 30 min. The precipitate was resuspended in 20 mM citrate-phosphate buffer, pH 6. The concentrated enzyme preparation were applied to a size-exclusion chromatography (Biogel P100, 2.5 × 90 cm) (Bio-Rad) equilibrated with the same buffer at a flow rate of 20 ml h^{-1}. Fractions with laccase activity were pooled and loaded onto a Mono-Q anion-exchange column (Q-Sepharose Cl 6B, 1.6 × 11 cm) (Pharmacia LKB Biotechnology) equilibrated with 20 mM citrate-phosphate buffer, pH 6.0 at a flow rate of 20 ml h^{-1}. The retained proteins were eluted using the NaCl gradient from 0.05 to 1 M. The laccase peak fractions were pooled and stored at 4 °C or at −20 °C in glycerol.

2.7. Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor the development of the purification process, to determine the homogeneity and apparent molecular mass of the purified laccase. SDS-PAGE was carried out on a 12% resolving gel and a 5% stacking gel according to the method of Laemmli [14]. Proteins were visualized by staining with Coomassie Brilliant Blue G-250.

2.8. Zymograms

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 [15]. The gel was also stained with RBBR (150 mg l^{-1}) for decolourization as described by Murugesan et al. [16].

2.9. Characterization of the purified laccase

The UV–vis spectrum of the purified laccase was recorded in 10 mM sodium phosphate buffer, pH 6.5.

N-terminal sequence of laccase was determined by automated Edman degradation of 5 pg of protein using an Applied Biosystems protein sequencer (Procise 492 cLC; Perkin-Elmer, New York, USA). N-terminal sequence homology was analyzed using the BLAST database.

The optimum pH of the purified enzyme was studied over a pH range of 2.0–9.0. To determine the pH stability, enzyme was kept at room temperature (22–24 °C) for 1 h and 24 h in different buffers (0.1 M) and the residual laccase activity was determined under standard assay conditions. The buffer systems used were sodium citrate buffer for pH 2.0–3.0, tartrate buffer for pH 4.0–5.0, citrate-phosphate buffer for pH 6.0–7.0 and citrate-phosphate-borate buffer for pH 8.0–9.0. To analyze the effect of temperature, the activity was tested at different temperatures (30–80 °C) by standard enzyme assay (0.1 M tartrate buffer, pH 4.0). To determine the enzyme stability with change in temperature, purified enzyme was incubated at different temperatures (25, 50, 60 and 70 °C) for 4 h and then relative activities were assayed at standard assay conditions.

Kinetic constants were calculated for DMP using the Lineweaver–Burk transformation of Michaelis–Menten equation. The DMP concentrations were ranged from 0 to 1.5 mM in 0.1 M tartrate buffer, pH 4.0.

Some phenolic compounds were tested as *F. fomentarius* laccase substrates by studying the qualitative changes in the absorbance spectra of reaction mixtures containing 150 μM potential substrate, 130 U l^{-1} purified laccase, and 0.1 M tartrate buffer, pH 4.0. These substrates are pyrocatechol, pyrogallol, 2,6-dimethoxyphenol, caffeate, vanillin, vanillate, ferulate, 4-methyl catechol, 3,4-dihydroxybenzoate, and 3,4-dihydroxybenzaldehyde.

The effect of various inhibitors was investigated using DMP as substrate. Ethylene diamine tetra-acetic acid (EDTA) was investigated at concentrations up to 20 mM. Sodium azide was investigated up to a concentration of 0.05 mM, and L-cysteine up to a concentration of 5 mM. For K_i determination of sodium azide, DMP concentration was varied from 0.0005 to 0.005 mM, at pH 4.0 with 0.1 M tartrate buffer.

The activity of laccase was tested in the presence of several metal salts including $Co(NO_3)_2$, $Ni(NO_3)_2$, $CdCl_2$, $HgCl_2$, $FeSO_4$, $ZnSO_4$, $MgSO_4$, $MnSO_4$ and $CuSO_4$. Two concentrations were used: 10 and 100 mM. The residual activities were determined using the standard laccase assay.

2.10. Enzymatic dye decolourization

Dye decolourization capability of the purified enzyme, obtained from *F. fomentarius* SSF culture, was accessed using RBBR dye. Reaction mixture contained, 150 mg l^{-1} dye concentration, 0.2 U ml^{-1} purified enzyme in 0.1 M tartrate buffer (pH 4.0) in a total volume of 1 ml in an Eppendorf tube. The reaction mixture was incubated at 30 °C in dark and the dye decolourization was measured by monitoring the decrease in absorbance maximum of dye (RBBR 592 nm) in a Shimadzu UV-VIS Scanning spectrophotometer (UV-2101-PC) and expressed in terms of percentage.

2.11. Statistical analysis

The data presented are the average of the results of two replicates with a standard error of less than 5%.

Table 1
Purification of *F. fomentarius* laccase from the WB solid culture.

Purification step	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Yield (%)	Purification factor (X-fold)
Culture supernatant	1430	127	11	100	1.0
Ammonium sulfate (80%)	1260	59	21	88	1.9
Biogel P100	706	10	71	49	6.4
Mono-Q	459	0.4	1150	32	104

3. Results

3.1. Purification of laccase

The laccase was purified to homogeneity from the SSF medium. Table 1 summarizes the results obtained from the different steps of laccase purification. During the first chromatographic step (gel filtration on Biogel P100), the laccase activity was separated from some contaminant proteins (Fig. 1A). After this step, a purification factor of 6.4 was noticed for the laccase. During the second anion-exchange chromatography, laccase activity was detected as a single peak eluting in 50 mM NaCl. This step permitted to separate the laccase enzyme from other proteins and removed all dark coloured pigments (which showed strong absorbance at 280 nm) (Fig. 1B). At the end of the process, laccase was purified 104-fold. The overall yield of the purification was about 32% with respect to the culture supernatant. The specific activity of the purified enzyme was 1150 U/mg.

3.2. Molecular mass and activity staining

The purified *F. fomentarius* laccase showed a single band on SDS-PAGE with a mobility corresponding to the molecular mass of 51 kDa as visualized by Coomassie Brilliant Blue staining (Fig. 2A). Activity staining of the laccase, with DMP as substrate, revealed a single protein band corresponding with the position of the laccase activity (Fig. 2B).

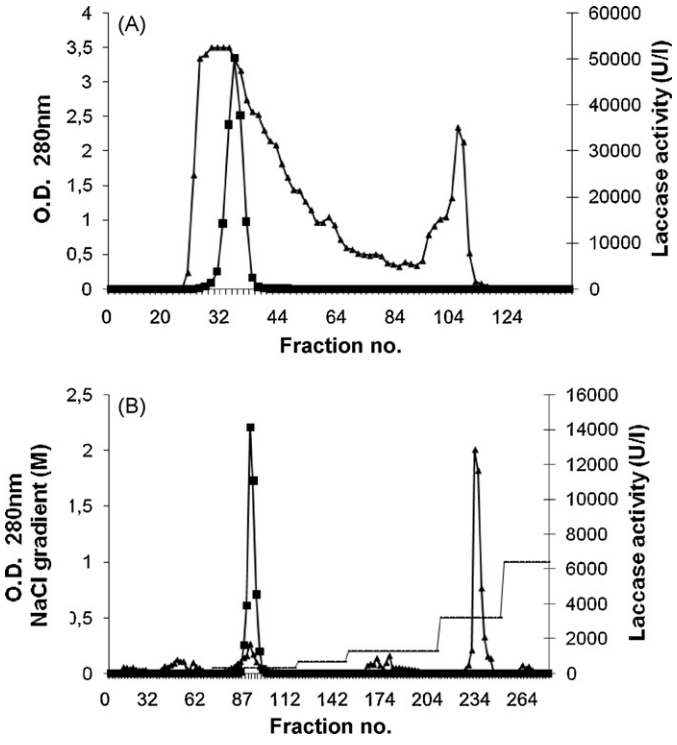

Fig. 1. Purification of *F. fomentarius* laccase. Subsequent chromatography steps on Biogel P100 (A) and Mono-Q (B) columns. Profiles corresponding to optical density at 280 nm (–▲–), NaCl gradient (dashed line) and laccase activity (–■–).

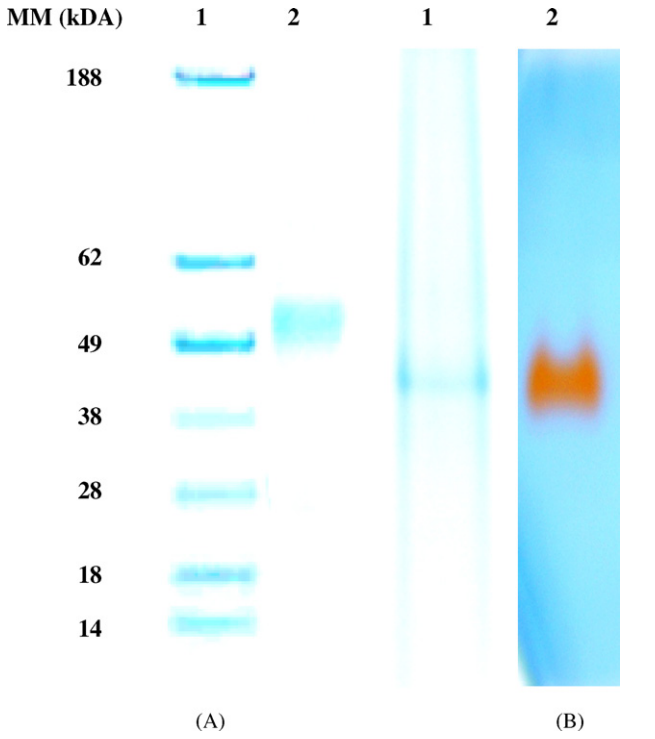

Fig. 2. Electrophoretic analysis of the purified *F. fomentarius* laccase. (A) Determination of molecular weight on SDS-PAGE. Lane 1: marker protein. Lane 2: purified laccase. (B) Electrophoretic patterns of the purified laccase under non-denaturating conditions. Lane 1: stained gel by Coomassie Brilliant Blue G 250. Lane 2: The laccase activity band was detected by zymogram with DMP as substrate.

3.3. Absorption spectrum of *F. fomentarius* laccase

The UV–vis spectrum of the purified *F. fomentarius* laccase has typical characteristics of multicopper oxidases including a shoulder at 325 nm (type III binuclear copper) and a peak at 614 nm (type I blue copper atom) (Fig. 3).

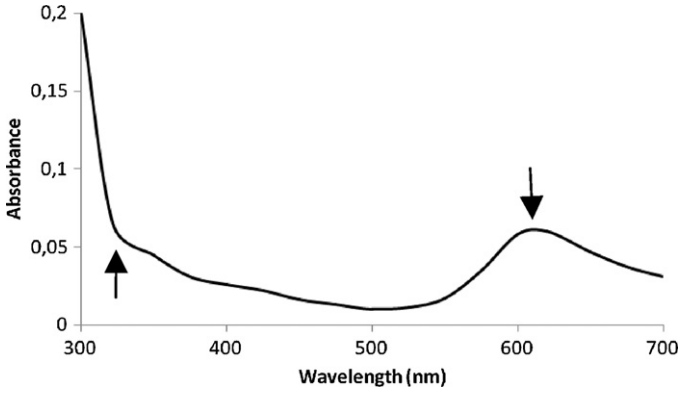

Fig. 3. Absorption spectrum of *F. fomentarius* laccase. Arrows indicate the characteristic absorbance peaks corresponding to type I (ca. 614 nm) and III (ca. 325 nm) Cu(II).

Table 2
Comparison of the N-terminal sequence of *F. fomentarius* laccase with those of other fungal laccases.

Micro-organism	N-terminal amino acid sequence	Identity (%)	Reference
Fomes fomentarius	IGPKTDLTIATGDVSPDG	100	This work
Trametes sp. 420	IGPKTDLTIADGDVSPDG	94	[17]
Trametes villosa	IGPVTDLTISNGDVSPDG	83	[18]
Trametes versicolor	IGPVTDLTISNADVSPDG	77	[19]
Trametes pubescens	IGPVTDLTISNADVTPDG	72	[20]
Trametes versicolor	IGPVTDLTISNADVTPDG	72	[21]
Polyporus brumalis	IGPVADLTISNADISPDG	66	[22]
Pycnoporus coccineus	IGPMADLTLTNAAVSPDG	61	[23]
Trametes hirsuta	VGPVADLTITDAAVSPDG	61	[24]
Pleurotus eryngii	IGPIADMYIVNEDVSPDG	61	[25]

The different aminoacids are depicted in italics.

3.4. N-terminal sequence of F. fomentarius laccase

The N-terminal amino acid sequence of laccase was IGPKTDLTIATGDVSPDG, which showed the highest similarity to *Trametes* sp. 420 laccase (94%) (Table 2).

3.5. Effect of pH and temperature on enzyme activity

The optimum pH for the maximum laccase activity was observed at 4.0 when DMP was used as substrate (Fig. 4A). The pH stability experiments indicated that the purified enzyme was more stable at alkaline values of pH (8.0) than at acidic values of pH (Fig. 4B).

When laccase activity was studied as a function of temperature, the enzyme was found to be active in a temperature range of 30–80 °C, with the maximum activity at 60° C (Fig. 4C). Half-life of the laccase was up to 240 min at 50 °C and 66 min at 60 °C (Fig. 4D).

3.6. Determination of kinetic parameters

The kinetic constants of the purified enzyme were determined on DMP, a substrate frequently used in laccase specificity studies. The Michaelis–Menten constant (K_m), the turnover number (k_{cat}) and the specificity constant (k_{cat}/K_m) values for *F. fomentarius* laccase were found to be 26 μM, 106 s^{-1} and 4×10^6 s^{-1} M^{-1} respectively.

3.7. Phenolic compounds oxidation by F. fomentarius laccase

The ability of laccase from *F. fomentarius* to oxidize variously substituted phenols was qualitatively studied by monitoring changes in the absorption spectra of reaction mixtures. Oxidation was detected by formation of products with absorbance peaks at the wavelength shown in Table 3. The purified enzyme had a wide substrate specificity oxidizing all the phenolic compounds assayed.

3.8. Effect of inhibitors on laccase activity

Effect of a range of potent laccase inhibitors on the enzyme activity was tested with DMP as substrate and the results were presented in Table 4. The most potent of the inhibitors assessed was sodium azide (NaN_3). Indeed, total inhibition was observed with 0.02 μM sodium azide versus 5 and 20 mM for L-Cys and EDTA respectively.

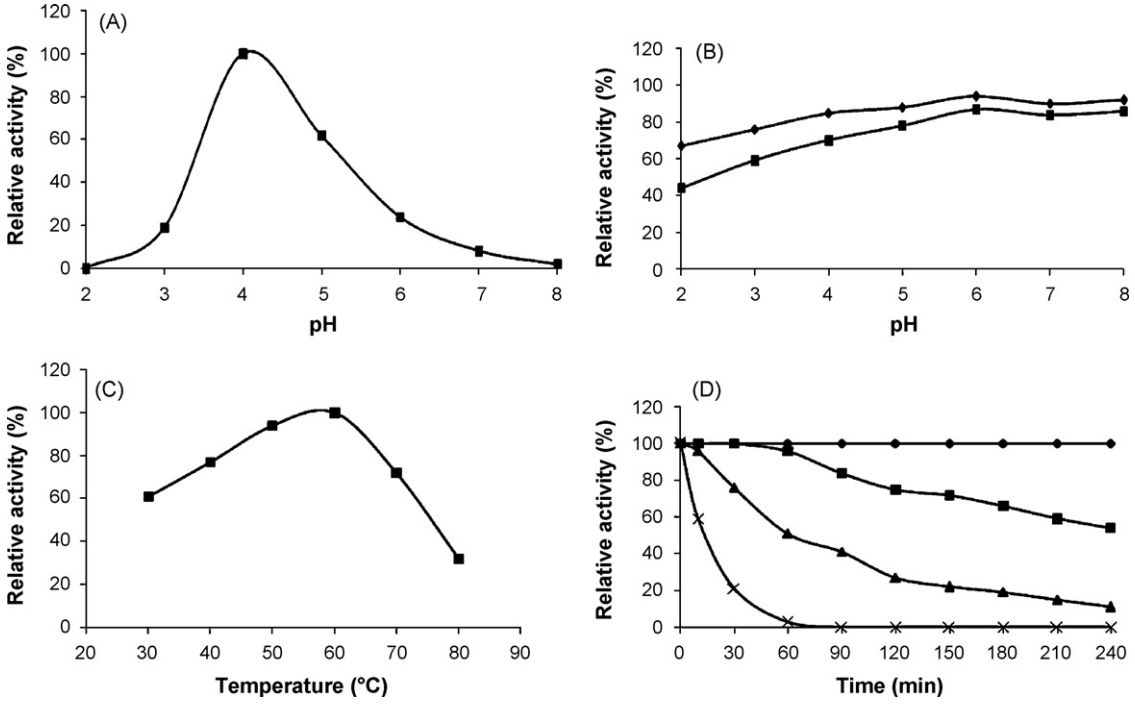

Fig. 4. Effect of pH and temperature on activity and stability of purified laccase from *F. fomentarius*: (A) optimum pH using DMP as substrate. (B) pH stability after 1 h (–♦–) and 24 h (–■–) incubation at room temperature. (C) Optimum temperature using DMP as substrate. (D) Thermal stability at 25 °C (–♦–), 50 °C (–■–), 60 °C (–▲–) and 70 °C (–×–).

Table 3
Wavelengths of the oxidation products formed by the action of *F. fomentarius* laccase on different phenolic compounds.

Compound	Wavelength (nm)[a]
2,6-Dimethoxyphenol	469
Pyrocatechol	389
Caffeate	413
Vanillin	355
Vanillate	316
Ferulate	287
4-Methyl catechol	244
3,4-Dihydroxybenzoate	291
Pyrogallol	450
3,4-Sihydroxybenzaldehyde	291

[a] New absorption peaks observed after incubation with the *F. fomentarius* laccase.

Table 4
Effect of various inhibitors on oxidation of DMP by purified *F. fomentarius* laccase.

Compound	Concentration (mM)	Inhibition (%)
NaN_3	0.001	59
	0.005	90
	0.01	96
	0.02	100
L-cys	0.1	20
	0.5	66
	1	87
	5	100
EDTA	1	9
	5	25
	10	71
	20	100

Sodium azide demonstrated pure non-competitive inhibition with a calculated K_i of 1 μM (data not shown).

3.9. Effect of metal ions on laccase activity

The stability of laccase activity against several metal compounds was tested (Table 5). Metal ions such as Co, Cd, Hg, Fe, Zn, Mg, Mn, Ni and Cu were assessed. It was found that for a concentration of 10 mM, Co and Ni inhibited laccase activity by 53 and 59%, respectively; whereas the laccase activity was highly sensitive to 10 mM Hg showing 94% inhibition. It should be also noted that the other ions are without effect on laccase activity. When the metal ions concentration was increased to 100 mM, laccase activity remained stable in the presence of Cd, Fe, Zn, Mg and Mn. It was completely inhibited by Co, Ni and Hg and it was activated by 18% in the presence of 100 mM Cu.

Table 5
Effect of metal ions on laccase activity.

Metal ions	Concentration (mM)	Relative activity (%)
Control	–	100
Co^{2+}	10	47
	100	0
Hg^{2+}	10	6
	100	0
Fe^{2+}	10	99
	100	98
Ni^{2+}	10	41
	100	0
Zn^{2+}	10	98
	100	97
Mg^{2+}	10	100
	100	99
Mn^{2+}	10	100
	100	99
Cd^{2+}	10	99
	100	98
Cu^{2+}	10	97
	100	118

3.10. Enzymatic dye decolourization

As illustrated in Fig. 5A, RBBR showed a degree of decolourization of about 80% after 1 h of laccase treatment. This result was also confirmed by gel electrophoresis (Fig. 5B). After the gel was stained with 150 mg l^{-1} RBBR and incubated at 30 °C for 1 h, colourless zone was observed. Afterwards, the gels were treated with DMP. This colourless zone turned into orange colour after incubation for 5 min at 30 °C.

4. Discussion

The laccase produced by *F. fomentarius*, under SSF using wheat bran as solid substrate was purified and characterized. The enzyme was a typical blue laccase as demonstrated by its UV–visible spectrum that shows an intensive peak around 614 nm, corresponding to the type 1 copper site, which is responsible for the deep blue colour of the enzyme. It has a shoulder peak at 325 nm,

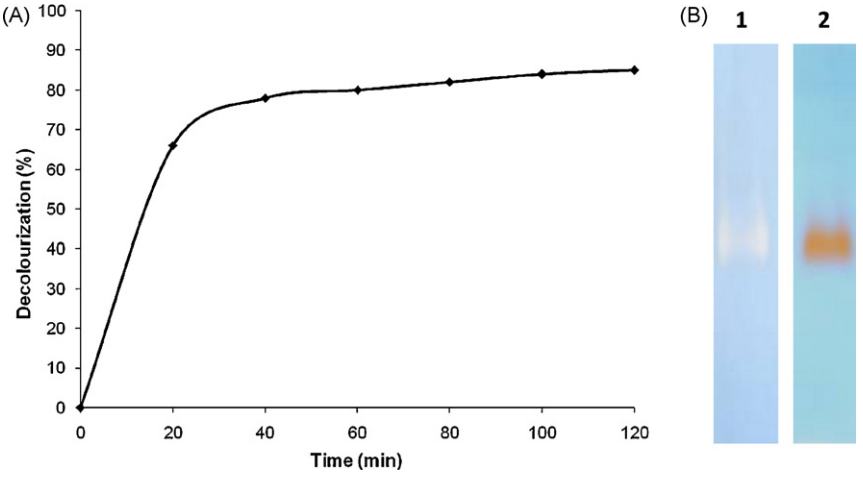

Fig. 5. (A) Decolorization of RBBR (150 mg l^{-1}) by purified laccase from *F. fomentarius*. (B) Lane 1: Gel incubated with dye showing single band of decolorized zone. Lane 2: Gel incubated with DMP showing the laccase activity at decolorized zone.

corresponding to the presence of the type 3 copper site of a typical blue laccase [26]. The N-terminal sequence of *F. fomentarius* laccase was not identical to any known laccase N-terminal sequence. However, it showed similarity to those laccases from *Trametes* sp. 420 (94% identity), *Trametes villosa* (83%), *Trametes versicolor* (77%), and *Trametes pubescens* (72%) (Table 2).

Laccase from *F. fomentarius* has some properties similar to or in the range of most fungal laccases. The molecular mass of the purified laccase (51 kDa) was consistent with typical fungal laccases (50–80 kDa) [27]. *F. fomentarius* laccase had an optimum pH of 4.0 for DMP. At pH values larger than 4.0, the enzyme activity decreased gradually. This phenomenon can be explained by the difference in redox potential between a reducing substrate and the type 1 copper in the active site of the enzyme and the inhibition of type 3 copper by hydroxide ion at higher pH [28]. When laccase activity was studied as a function of temperature, the enzyme was found to be active in a temperature range of 30–80 °C, with the maximum activity at 60 °C. The typical optimum temperature range for laccases is 50–60 °C [12,29]. *F. fomentarius* laccase was more stable at alkaline pH than at acidic pH and at lower temperature. Similar temperature and pH stabilities were reported for other fungal laccases [30,31].

The reaction kinetics of the *F. fomentarius* laccase were determined using DMP. The catalytic efficiency (k_{cat}/K_m) value of the enzyme ($41 \times 10^5\ s^{-1}\ M^{-1}$) was found to be higher than those reported for other fungal laccases such as *Pycnoporus coccineus* laccase ($34 \times 10^5\ s^{-1}\ M^{-1}$) [12], *Pycnoporus sanguineus* laccase ($4 \times 10^5\ s^{-1}\ M^{-1}$) [29] and *Xylaria polymorpha* laccase ($25 \times 10^5\ s^{-1}\ M^{-1}$) [32].

The enzyme secreted by *F. fomentarius was* able to oxidize a variety of phenolic compounds, including hydroxy- and methoxy-substituted phenols as pyrocatechol, pyrogallol, caffeate, vanillate and ferulate. This result should open widely the possibility to use this laccase in chemical synthesis. In fact, the oxidation of ferulic acid by laccase was recently used to synthesize stable phenolic colourants that could be used in cosmetics and foodstuffs [33].

Effect of a range of potent laccase inhibitors on the enzyme activity was tested with DMP. These inhibitors included: sodium azide that complexes to the coppers in the active site, cysteine that is a sulfhydryl organic compound with a reducing effect on the copper-containing active site of laccase and EDTA that exhibits metal chelating properties. Sodium azide was the most efficient inhibitor. It demonstrated pure non-competitive inhibition with a calculated K_i of 1 μM. This result is in agreement with the findings of Jordaan et al. [34] who noted non-competitive inhibition with sodium azide for laccase from an unidentified basidiomycete with a calculated K_i of 10 μM. The *F. fomentarius* laccase exhibited a higher stability towards the chelator EDTA. Most laccases purified from other white-rot fungi are inhibited by 1.0 mM EDTA [35,26]. Nevertheless, exceptions have already been described, such as the laccases of *Pleurotus ostreatus* [36] and *Phellinus ribis* [37], where only high concentrations of EDTA inhibited the enzyme.

The interaction of metals with extracellular laccase was particularly important for the better understanding of the biotechnological processes of xenobiotic degradation [38]. *F. fomentarius* laccase was resistant to several metal cations such as Cd^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} and Cu^{2+}. In fact, Cu^{2+} slightly stimulated the enzyme. This activation may be due to the filling of type-2 copper binding sites with copper ions [39]. The laccase activity was highly sensitive to Hg^{2+}, indicating the presence of thiol groups, essential for its activity [40]. These observations indicated that the effect of metal ions on laccase activity was highly dependent on the type of metals used, which had a great influence on the catalytic activity of the enzyme.

Remazol Brilliant Blue R is a typical anthraquinone dye used in the textile industry and represents an important class of toxic and recalcitrant organopollutants and is therefore used as a model dye for decolourization investigation by many researchers [41,42]. In this study, *F. fomentarius* laccase decolourized efficiently the dye down to 80% of 150 mg l^{-1} initial concentration of RBBR when only 0.2 U ml^{-1} of laccase was used in the reaction mixture. Laccases from other white-rot fungi are also able to decolourize RBBR without redox mediator [43,44] but these results differ from those found by Soares et al. [41], who reported that the addition of a redox mediator was necessary for RBBR decolourization by a laccase from a genetically modified *Aspergillus* sp. The fact that *F. fomentarius* laccase was able to decolourize the dye RBBR without mediator addition is very interesting, since this dye is frequently used as a starting material in the production of polymeric dyes [45]. In addition to dye degradation, laccase was also reported to catalyze oxidative coupling reactions leading to the formation of dye compounds [46,47].

5. Conclusion

In summary, the biochemical characteristics of the purified *F. fomentarius* laccase and its highly ability to oxidase phenolic compounds and anthraquinonic dyes as well as its relatively easy production and purification, make it very attractive for application in different biotechnological areas, particularly in bioremediation and in chemical synthesis.

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