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Purification and characterization of a fungal laccase from the ascomycete *Thielavia* sp. and its role in the decolorization of a recalcitrant dye

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ABSTRACT

A laccase-producing ascomycete was isolated from arid soil in Tunisia. This fungus was identified as *Thielavia* sp. using the phylogenetic analysis of rDNA internal transcribed spacers. The extracellular laccase produced by the fungus was purified to electrophoretic homogeneity, showing a molecular mass around 70 kDa. The enzyme had an optimum pH of 5.0 and 6.0 for ABTS and 2,6-DMP, respectively and it showed remarkable high thermal stability, showing its optimal temperature at 70 °C (against 2,6-DMP). It presented slight inhibiting effect by EDTA, SDS and L-cyst although this effect was more marked by sodium azide (0.1 mM). On the other hand, it showed tolerance to up to 300 mM NaCl, retaining around 50% of its activity at 900 mM. Among the metal ions tested on TaLac1, Mn^{2+} showed an activating effect. Their kinetic parameters K_m and k_{cat} were 23.7 μM and 4.14 s^{-1} for ABTS, and 24.3 μM and 3.46 s^{-1} towards 2,6-DMP. The purified enzyme displayed greater efficiency in Remazol Brilliant Blue R decolorization (90%) in absence of redox mediator, an important property for biotechnological applications.

1. Introduction

Fungi are widespread in nature and they are essential organisms for carbon cycling in nature when inhabit in the soil. In this habitat there are diverse groups, belonging mainly to the Dikarya subkingdom, with a strong dominance of ascomycetous fungi [1].

During last years a wide number of studies have focused on ligninolytic fungi because they have a versatile enzyme system (including laccases, peroxidases and oxidases producing H_2O_2) which participate in the degradation of lignin but also of other recalcitrant aromatic compounds causing environmental problems [2]. Among these groups of extracellular enzymes, laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) have received great attention because they possess huge applicability in different biotechnological and industrial applications [3–5], such as textile dye decolorization, pulp bleaching, detoxification and bioremediation of environmental pollutants and delignification or second generation ethanol production [2,4–6]. These enzymes catalyze the monoelectronic oxidation of a wide number of phenolic compounds and aromatic amines using oxygen as a nonlimited electron acceptor and their substrate range is expanded to nonphenolic aromatic compounds in the presence of low-molecular-mass compounds, which acts as redox mediators [4,7,8]. Fungal laccases are glycoproteins with monomeric, dimeric or tetrameric structure, containing four redox-active copper atoms that are distributed in three distinct types (T1, T2 and T3) based on their spectroscopic properties [3].

In the last decades, while the production of laccases from basidiomycetes fungi has been intensively studied, very little is known about ascomycete laccases [9], being most of them about laccases from Xylariaceae family [10]. Albeit the important laccase-like genes reported in ascomycete fungi, little is known about the presence, function and potential use of asco-laccases as biocatalysts. In this study, a fungus isolated from Tunisian arid soil, was identified as *Thielavia* sp., an ascomycete from Chaetomiaceae family. An extracellular laccase from this ascomycetous was purified and characterized and studied its ability to decolorize, without mediator, Remazol Brilliant Blue R, a recalcitrant dye producing environmental problems.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); 2,6-DMP, 2,6-dimethoxyphenol; MALDI-TOF, Matrix Assisted Laser Desorption and Ionization-Time Of Flight; TaLac1, *Thielavia* sp. laccase; EDTA, ethylenediaminetetraacetate-dinatrium salt; SDS, sodium dodecyl sulfate; NaN_3, sodium azide; L-cyst, L-cysteine.

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2. Materials and methods

2.1. Chemicals

2,6-Dimethoxyphenol (2,6-DMP), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) and the synthetic dye Remazol Brilliant Blue R (RBBR) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Isolation, screening and identification of the laccase producing fungal strain

The fungal strains isolated from arid soil regions in southern Tunisia were maintained on 2% (w/v) malt extract agar (MEA) plates and incubated at 40 °C for a week. After inoculation into 2,6-DMP (5 mM) amended MEA plates, the laccase producing strain HJ22, showing intense red-brown halos, was selected for further identification studies. For the molecular identification, genomic DNA was extracted from fungal mycelium using DNeasy Plant Mini Kit (QIAGEN) according to the supplier's instruction. The partial sequence of the 18S ribosomal RNA gene, the internal transcribed spacer 1, the 5.8S ribosomal RNA gene, the internal transcribed spacer 2, and the 28S ribosomal RNA gene partial sequence, were amplified using universal primers ITS1/ITS4 [11]. The PCR reaction was made in a total volume of 50 μL, containing 1.25 μL of each primer (10 μM stock), 50 ng of genomic DNA, 1 U of *Taq* DNA polymerase, 1.25 μL of the mixture dNTPs (10 mM stock), 1.5 μL of $MgCl_2$ (25 mM stock) and 5 μL of *Taq* DNA buffer. The reagents were combined in 200 μL microtubes and placed in a thermocycler programmed as follows: pre-PCR denaturation at 95 °C for 5 min followed by 30 cycles of heat denaturation at 95 °C for 45 s, primer annealing at 52 °C for 45 s, and extension at 72 °C for 1.5 min. Final extension of the amplified DNA fragments were done at 72 °C for 10 min. Amplified DNA was separated on 0.7% (w/v) agarose gel in TAE buffer. The amplified amplicon of around 600 bp was purified from the gel using the QIAquick® Gel Extraction Kit, sequenced using the BigDye Terminator v3.1 cycle sequencing kit and the automated ABI Prism 3730 DNA sequencer, in Secugen (Madrid, Spain), and compared with the sequences in GenBank database using the Basic Local Alignment Search Tool (BLAST) software [11]. Downloaded homologous sequences were aligned with MUSCLE as implemented in MEGA7 [12] with bootstrap values based on 1000 replicates [13]. The evolutionary distances were computed by the Maximum Likelihood approach [14]. All positions containing gaps and missing data were eliminated. Neighbor-Joining method was applied to the distance matrix to infer the phylogenetic tree [15].

2.3. Fungal culture conditions for laccase production

For the inoculum preparation, three mycelial plugs of isolated fungus (identified as *Thielavia* sp.) were transferred into 50 mL malt extract medium in a 250 mL Erlenmeyer flask. After 3 days of growing on a rotary shaker (160 rpm) at 40 °C, the mycelial pellets were harvested and 5 mL were used as inoculum for further experiments. The growth medium was prepared according to Mtibaà et al. [16]. Submerged cultures were incubated at 40 °C with shaking at 160 rpm and samples were taken periodically. After 8 days of incubation, when maximal laccase activity was obtained, the culture broth was filtered and applied to the enzyme purification process.

2.4. Enzymatic assays and protein concentration

Laccase activity was measured spectrophotometrically by the oxidation of 2,6-DMP and ABTS. Enzyme activity was estimated by monitoring the absorbance change at 436 nm for 5 mM ABTS oxidation (ε_{ABTS} = 29,300 M^{-1} cm^{-1}) [17] and at 469 nm for 5 mM 2,6-DMP oxidation (ε_{DMP} = 27,500 M^{-1} cm^{-1}) [16] in 0.1 M Britton-Robinson buffer, pH 5.0 at room temperature. One unit was defined as the amount of enzyme transforming 1 μmol of substrate per min. The enzyme activities were expressed as unit (U mL^{-1} or U mg^{-1}). Protein concentrations were determined using the Pierce BCA protein assay kit (ThermoFisher) and bovine serum albumin (BSA) as the standard according to the supplier's instruction [18].

2.5. Purification of laccase

Mycelia were removed from eight-day-old cultures liquid by filtration using three tangential flow filters of 0.8, 0.45 and 0.22 μm pore size sequentially. Then, the filtrated supernatant was concentrated by ultrafiltration, using a Pellicon system with a 10-kDa-cutoff membrane, at 4 °C. The concentrated sample was dialysed against buffer A (10 mM sodium citrate buffer, pH 5.5). The extract was then applied to a HiTrap QFF 5 mL strong anionic-exchange column (GE Healthcare) pre-equilibrated with buffer A. Proteins were eluted with a 0–25% NaCl 1 M linear gradient, at 1 mL min^{-1} flow rate. Two peaks were found with activity on ABTS, suggesting the presence of two different laccases. The first peak, with the major activity (named as TaLac1) was dialysed against 10 mM sodium citrate buffer pH 4.0, concentrated and subjected to cationic-exchange chromatography using a HiTrap SPFF 5 mL column (GE Healthcare) pre-equilibrated with the same buffer. The retained proteins were eluted using 0–25% NaCl 1 M linear gradient, at a flow rate of 1 mL min^{-1}. Laccase-rich fractions were pooled, dialysed to remove excess of NaCl and concentrated by ultrafiltration. The active blue fraction of TaLac1 was used for further studies.

2.6. Laccase characterization

2.6.1. Molecular mass determination and absorbance spectrum

The determination of the molecular mass of the purified protein, as well as its homogeneity, was performed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), according to Laemmli [19], using high-molecular-mass protein marker (Bio-Rad). Protein bands were stained with Coomassie Brilliant Blue R-250 after SDS-PAGE.

As part of the physicochemical characterization of the purified laccase, its UV–visible spectrum (200 to 800 nm) at resting state in 10 mM sodium acetate buffer, pH 5.0, was recorded (UV 160 Spectrophotometer, Shimadzu) [20].

2.6.2. MALDI-TOF analysis and molecular amplification of TaLac1

For peptide mass fingerprinting, the purified protein was analyzed by SDS-PAGE in a 12% polyacrylamide gel, stained with SYPRO Ruby (Bio-Rad) and subjected to tryptic in-gel digestion in a DigestPro MS digestor (Intavis AG) in accordance with the protocol reported by Shevchenko et al. [21]. MS analyses of the tryptic peptides were performed in an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) controlled by the flexControl 3.0 software (Bruker Daltonics), and mass values from trypsin, keratin, matrix or sodium adducts were removed. Data were performed against the NCBInr database (National Center for Biotechnology Information non-redundant) with the 2.3 MASCOT search engine (Matrix Science). Relevant search parameters were set as follows: trypsin as enzyme, carbamidomethylation of cysteines as fixed modification, methionine oxidation as variable modification, 1 missed cleavage allowed, peptide tolerance of 50 ppm, and MS/MS tolerance of 0.5 Da. Protein scores >75 were considered significant.

Degenerate primers to amplify the TaLac1 gene were designed based on the conserved regions of laccase sequences from related ascomycete fungi. The designed primers were TaLac Fw 5′-GGCACSASCTGGTACCA CTC-3′ and TaLac Rv 5′-TCGTGGCCGTGRAGRTGCAT-3′. Amplification of laccase gene from *Thielavia* sp. was done by adding 50 ng of genomic DNA in 50 μL final volume reaction containing the following quantities: 10× PCR buffer, 5 μL; equimolar mixture of 10 mM dNTPs, 1.5 μL; Taq

polymerase enzyme 5 U, 1 μL; 60 mM $MgCl_2$, 1.75 μL; 10 μM degenerated primers, 1.5 μL of each one; and sterile distilled water (35 μL). The PCR conditions were set as follows: a pre-PCR denaturation of 5 min at 95 °C followed by 35 cycles of denaturation for 45 s at 95 °C, primer annealing of 55 °C for 45 s, extension for 1.5 min at 72 °C and a final PCR extension of 5 min at 72 °C. The PCR amplicons were analyzed on a 0.7% (w/v) agarose containing 0.5 μg mL^{-1} ethidium bromide, and the bands 1000–1800 bp were excised and purified with QIAquick Gel Extraction Kit. The amplified sequences were ligated into pGEM-T easy vector at 4 °C overnight and the resulting plasmids were transformed into *Escherichia coli* DH5α for plasmid amplification [22]. Cells containing plasmids were selected by plating in LB supplemented with 10% Ampicillin. Plasmid purification was carried out using plasmid DNA purification Kit (Roche), and the inserted fragments were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems). The nucleotide sequences were translated and the introns were eliminated. The deduced laccase sequence was submitted to the National Center for Biotechnology Information (NCBI) database and deposited in the GenBank nucleotide sequence database under accession number of KX618207.

A three-dimensional model of the partial sequence of the laccase was generated using the programs implemented by the automated protein homology-modeling server SWISS-MODEL (http://swissmodel.expasy.org/). The model was comprehensively analyzed using PyMol 1.1 (http://pymol.org/).

2.6.3. Effect of pH and temperature on laccase activity and stability

The optimum pH of TaLac1 was studied over a pH range from 3.0 to 7.0, in 10 mM Britton-Robinson buffer, using ABTS and 2,6-DMP as substrates. The relative activity was calculated considering the maximum activity as 100%. The pH stability was assessed by incubating the purified enzyme at different pH in the same buffer (10 mM) during 24 h determining the residual activity by using 2,6-DMP as described above. The initial activity of the enzyme was taken as 100%. The optimum temperature was investigated at temperatures between 30 °C and 90 °C. Thermal stability was tested by incubating the enzymes at 30 °C, 40 °C, 50 °C and 60 °C in Britton-Robinson buffer (10 mM, pH 5.0), and the remaining activity was determined after 3 h. The initial activity of the enzyme was taken as 100%.

2.6.4. Effect of metal ions and inhibitors on laccase activity

Various metal ions (Ca^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+} and Hg^{2+}) were tested at concentrations of 5, 10, 100 and 200 mM on TaLac1. The purified enzyme was also tested in the presence of typical laccase inhibitors (SDS, NaN_3, EDTA and L-cysteine) at different concentrations, depending on the effect of each compound on the enzyme. Remaining laccase activity was determined using ABTS as substrate in 10 mM Britton and Robinson buffer at pH 5.0 and at room temperature. The laccase activity in absence of these compounds was considered as 100%.

The effects of various concentrations of sodium chloride (0, 1, 2 and 3 M) on laccase activity was investigated at room temperature, using 2,6-DMP as substrate at pH 5.0 and in 10 mM Britton and Robinson buffer. Control without salt was regarded as 100%.

2.6.5. Kinetic properties of purified laccase

Kinetic constants (K_m and k_{cat}) of the purified laccase were determined using the Lineweaver-Burk plot by measuring the enzyme activities in the presence of different concentrations (0.625–6.25 mM) of ABTS and 2,6-DMP at pH 5.0 and 6.0, respectively.

2.7. Dye decolorization and biodegradation analysis

2.7.1. RBBR decolorization and its UV–vis spectrum

The decolorization of Remazol Brilliant Blue R was carried out in triplicate by measuring spectrophotometrically the decrease of absorbance at 592 nm. The reaction mixture (5 mL) contained 50 mg L^{-1} dye dissolved in 100 mM Britton and Robinson buffer, pH 5.0, and 3 U mL^{-1} of TaLac1 (against 2,6-DMP). The reactions were incubated at 30 °C for 24 h under mild shaking conditions. Control samples, without the enzyme, were processed in parallel. UV–visible spectrum of RBBR was recorded using a JENWAY 7315 spectrophotometer at different wavelengths ranging from 200 to 800 nm.

2.7.2. High Performance Liquid Chromatography (HPLC) analysis

The untreated and treated RBBR dye samples were analyzed by HPLC (DIONEX Softron GmbH, Germany), equipped with quaternary gradient pump system, a UV–vis detector and a DIONEX UltiMate 3000 (Thermo Scientific), using a C-18 column at room temperature. The mobile phase consisted of water: methanol (30:70) with a flow rate of 1 mL min^{-1}. 20 μL of the filtered samples were injected into the column and eluted with a gradient elution program starting with 100% water for 5 min, followed by 30–70% water-methanol for 15 min and ending by a linear increase of methanol to 100% during 5 min. The separated compounds were monitored at 280, 560, 592 and 600 nm using a UV–vis detector in the range of 200–800 nm.

2.7.3. Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR analysis was performed on a Cory 630 FTIR spectrophotometer (Agilent), to investigate the changes in the chemical structure of the synthetic dye RBBR, before and after decolorization. The metabolites obtained after RBBR decolorization were freeze-dried and their transmittance spectra were recorded in the range of 4000 to 600 cm^{-1}.

3. Results and discussion

3.1. Isolation, screening and identification of the laccase producing fungal strain

Sixteen fungal strains isolated from arid soils were identified as laccase producers because they oxidize 2,6-DMP in malt extract agar plates producing red-brown halos. Among these strains, the isolate HJ22 was selected for further study based on its ability to grow in a wide temperature range, between 28 and 45 °C. The identification of HJ22 isolate was carried out using the sequences of the ribosomal DNA as a suitable target for analysis of fungal phylogeny. A fragment around 600 bp was amplified by PCR and the sequence deposited in GenBank (accession number KX618198). It showed 99% identity with the ITS region of *Thielavia arenaria* (HQ647318) and 99% with other *Thielavia* species (*T. subthermophila* AM909688). This information, as well as its morphological characteristics, contributes to the identification of the fungus as a new strain of *Thielavia* sp.

The phylogenetic tree showed in Fig. S1 was built with the partial sequence obtained from *Thielavia* sp. HJ22 and related species. It is clear also from the dendrogram that the isolate HJ22 has the highest similarity with different *Thielavia* species (99%) but also with *Chaetomium* sp. strains (90% identity), which has been classified into the same family.

3.2. Purification of laccase and electrophoresis analysis

The extracellular *Thielavia* sp. laccase, denominated TaLac1, was purified from 8-day-old cultures after three chromatographic steps: ultrafiltration (10-kDa cut-off membrane), HiTrap QFF and HiTrap SPFF anion exchange chromatography columns. The concentrated proteins were loaded first onto HiTrap QFF. The elution profile showed two active fractions. The large eluted fraction represented the not bound laccase TaLac1, which was further fractionated in HiTrap SPFF against 10 mM sodium citrate buffer pH 4.0, to yield an electrophoretically homogeneous protein. A summary of the purification steps is presented in Table 1. Respect to the purification process, TaLac1 was been purified 90.57-fold with a yield of 11.95%.

The purified laccase TaLac1 appeared as a single band in SDS-PAGE with apparent molecular mass around 70 kDa (Fig. 1). A similar

Table 1
Summary of the purification process of laccase from *Thielavia* sp. HJ22.

Purification step	Total laccase activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification factor
Culture filtered	7400	0.26	100	1
Concentration	6460	4.64	87.30	17.84
Ultrafiltration	5705	5.42	77.00	20.84
HiTrap QFF	4652	3.31	62.90	12.73
HiTrap SPFF	884.30	23.55	11.95	90.57

molecular mass was previously reported for ascomycete fungal laccases, such as laccases from *Melanocarpus albomyces* [23] and *Cladosporium cladosporioides* [24].

3.3. Absorbance spectrum of TaLac1

The absorbance spectrum of TaLac1 showed a shoulder at 280 nm and an absorption peak at 600 nm. This spectrum (Fig. S2) is typical of blue laccases. The peak around 600 nm is due to the presence of type 1 copper site (T1), while the shoulder around 300 nm represents additional copper atoms present in these enzymes in the binuclear cluster (T2 and T3) [3,25,26].

3.4. MALDI-TOF analysis and molecular study of TaLac1

The tryptic digestion of the purified laccase TaLac1 exhibited several fragments. The resulted peptides were analyzed by MALDI-TOF and their sequences were compared with those available in NCBI protein database. The deduced peptides had 96–98% amino acid identity with the laccase reported previously in *T. arenaria* (PDB: 3PPS).

A sequence of 825 nucleotides was obtained (GenBank accession number KX618207) by amplification using the degenerate primers designed in conserved regions of the laccase gene. This sequence was translated and compared to the sequences of closely related laccases.

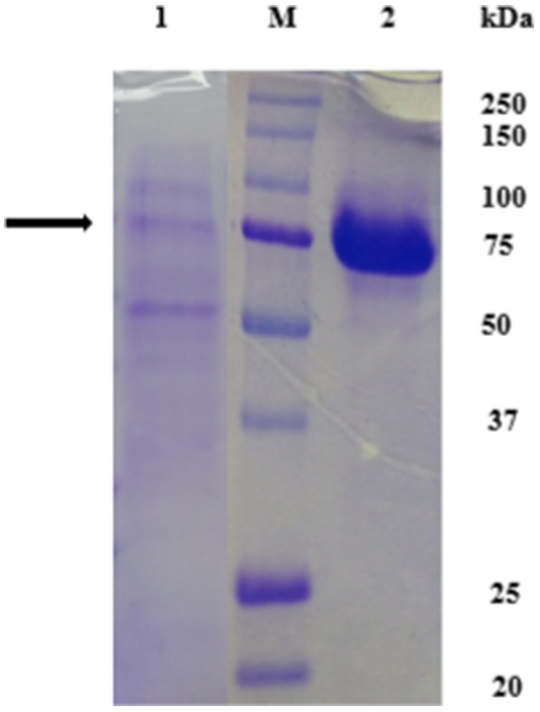

Fig. 1. SDS-PAGE analysis of *Thielavia* sp. HJ22 purified laccase (TaLac1). The gel was stained with Coomassie Brilliant Blue. Lane M: molecular weight markers; Lane 1: crude laccase from culture supernatant; Lane 2: purified laccase.

The partial protein sequence (275 aa) aligned with laccase from *T. arenaria* strain (PDB: 3PPS) from residues 179 to 453 sharing 99% of identity. TaLac1 also shared 80% and 77% of identity with laccases from *Chaetomium globosum* (GenBank accession number XP 0012288061) and *M. albomyces* (PDB: 2Q9OA), respectively.

These results suggest that TaLac1 could be a new asco-laccase with high homology in the sequenced region to other ascomycete laccases. A three-dimensional model of the partial sequence of the laccase was generated using 3PPS structure as template with a Qmean of 0.99 (Fig. S3). The model includes T2 site and shows similarity with *Thielavia arenaria* laccase sequence although it does not include the total sequence of TaLac1, and thus the biochemical differences found between these enzymes may be justified in structural differences in the rest of the molecule.

3.5. Effect of pH on laccase activity and stability

Two substrates, ABTS and 2,6-DMP, were used to determine the effect of pH on *Thielavia* sp. laccase. The obtained optimum pH were in the acidic range, which is representative of typical laccases (4.0 to 6.0) [7,26,27]. The purified laccase exhibited high activity at pH 5.0 and 6.0 for ABTS and 2,6-DMP, respectively (Fig. 2a). The pH-activity profile determined for TaLac1 using 2,6-DMP as substrate, is in agreement to that reported for other fungal laccases [17,28]. Using ABTS, similar results were reported by the purified laccases from the ascomycete *Scytalidium thermophilum* [28], *Agaricus bisporus* AU13 [7] and *Leptosphaerulina* sp. [29]. The difference in pH optima for substrates is typical for laccases

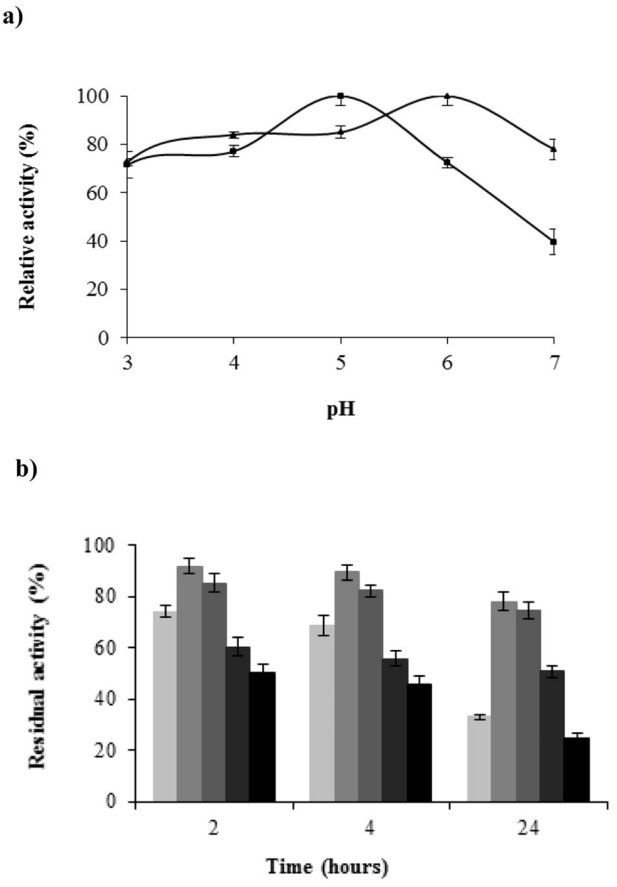

Fig. 2. Effect of pH on activity and stability of the purified laccase TaLac1: (a) optimum pH using ABTS (■) and 2,6-DMP (▲) as substrates at room temperature; (b) pH stability on pH 3.0 (□), pH 4.0 (■), pH 5.0 (■), pH 6.0 (■) and pH 7.0 (■). Enzyme stability was checked by measuring the residual activity after 24 h using 2,6-DMP as substrate at pH 5.0. Data points represent the mean of three experiments ± SD.

and it is related to the oxidation mechanisms depending on the substrate [17].

From the pH stability data obtained (Fig. 2b), we can conclude that TaLac1 is highly stable during 24 h under slight acidic conditions (4.0–5.0). The purified enzyme retained around 80% of its initial activity after 4 h of incubation at pH 5.0 while *Myceliophthora thermophila* laccase retained only 62% of its activity at pH 5.0 after 3 h incubation [30]. TaLac1 showed great stability compared to *A. bisporus* CU13 Lacc2 which retained only 51% of its original activity after 4 h of incubation at pH 5.0 [7]. At neutral pH values (pH 7.0), the enzyme retained over 50% of its initial activity after 2 h. The same result was reported for purified laccase from *Trametes* sp. LS-10C and *M. albomyces* [23,31]. Moreover, Kittl et al. [32] showed similar pH stability range of BaLac which was highest at pH 5.5, followed by pH 4.5 and worse at pH 6.5.

3.6. Effect of temperature on laccase activity and stability

The effect of temperature on enzyme activity was studied in the range from 30 to 90 °C using ABTS and 2,6-DMP as substrates. As shown in Fig. 3, the purified laccase showed maximum activity at 70 °C using 2,6-DMP and at 60 °C with ABTS. This result was in the range of typical fungal laccases which were characterized by optimum temperature activity between 50 and 80 °C [25,33,34]. Similar optimum temperatures were obtained with 2,6-DMP by some ascomycetes laccases from *M. albomyces* (60–70 °C), *Chaetomium* sp., and the white rot fungus *Abortiporus biennis* (60 °C) [16,23,35].

Thermal stability studies (Fig. 3) showed that TaLac1 possessed a modest thermostability, retaining all the activity between 30 and 50 °C after 6 h of incubation, similarly to those reported for *M. albomyces* [23] and *Sporothrix carnis* [26] laccases. As illustrated in Fig. 3, thermal stability decreased with increasing temperature above 50 °C. After 6 h at 60 °C, TaLac1 retained 50% of its initial activity. Similar results were reported for *M. albomyces* laccase with a half-life of over 5 h at 60 °C [23]. Laccases from *Trametes trogii* 463 and *A. bisporus* CU13, however, showed less stability, with a half-life of 2 h at 60 °C [7,36]. The activity of this enzyme is comparable to the thermostable laccases described in basidiomycetous fungi of the genera *Ganoderma* [37] and *Trametes pubescens* [38]. The thermostability of TaLac1 between 30 and 50 °C suggests its potential for broad range of industrial applications. Interestingly, TaLac1 showed apparent thermal activation at 30 °C within 360 min and at 40 °C after 90 min of incubation. This activation was up to 130% of the original activity, which is in agreement to that reported by Hildén et al. [39]. According to Hildén et al. [39], the thermal activation might be due to the flexibility of the enzyme conformation at temperature below 50 °C.

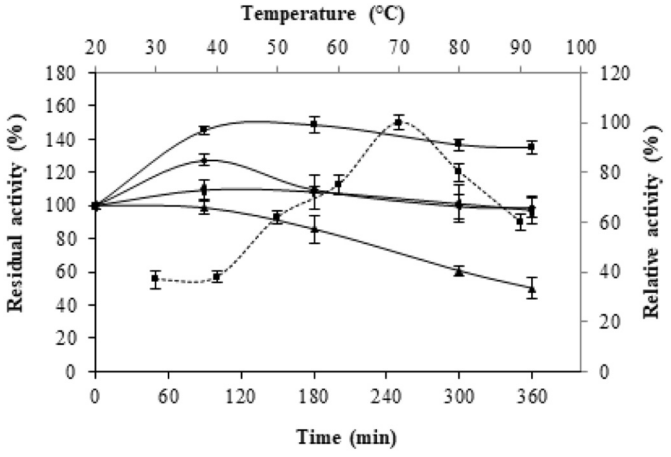

Fig. 3. Effect of temperature on the activity of purified laccase from *Thielavia* sp. HJ22 with 2,6-DMP as substrates at pH 5.0 (dashed line, secondary axes) and thermostability at pH 5.0 of purified laccase from *Thielavia* sp. HJ22 at 30 °C (■), 40 °C (♦), 50 °C (●), 60 °C (▲) (full lines, primary axes). Laccase activity was determined using 2,6-DMP as the substrate at pH 5.0. Data points represent the mean of three replicates ± SD.

3.7. Effect of metal ions on laccase activity

The effect of different metal ions was studied for the purified laccase (Table 2). The enzyme activity is not affected by the presence of most of the tested ions except, Hg^{2+} and Fe^{2+}, which inhibited it drastically at high concentrations. Similar inhibition had been reported for laccases from the basidiomycete *Sporothrix carnis* CPF-05 [26] and the ascomycete *Xylaria* sp. [10]. A further increase in Mg^{2+}, Mn^{2+} and Zn^{2+} concentrations seems to have no pronounced inhibition on TaLac1 activity. This finding is similar to that obtained for the laccase from *Xylaria* sp. [10], which retained around 90% of its activity at 10 mM of Mg^{2+}, Mn^{2+} and Zn^{2+}. TaLac1 showed better activity compared to that of laccase from *S. carnis* CPF-05 which was inhibited in the presence of 1–10 mM Mg^{2+} [26]. The presence of Mn^{2+} at concentrations of 5 and 10 mM promoted the enzymatic activity. Similar activation of fungal laccases by Mn^{2+} has been reported for laccases from the ascomycete *Cladosporium cladosporioides* and the basidiomycete *S. carnis* CPF-05 [24,26], while an important inhibitory effect was reported in presence of Mn^{2+} at 1–10 mM in other studies [7,40]. In presence of 10 mM Zn^{2+} and Mn^{2+}, TaLac1 showed higher activity compared to that obtained by *Cerrena unicolor* GSM-01 laccase [2]. Moreover, there was inactivation of TaLac1 by >10 mM Cu^{2+}, which is consistent with many reports showing the inhibitory effect associated with the increase of Cu^{2+} in the reactions [26,41]. These results also suggest that TaLac1 could be suitable for catalyzing reactions of industrial effluents treatment containing high amount of diverse metal ions, such as textile dye wastewater.

3.8. Effect of inhibitors on laccase activity

The effects of four classical inhibitors (L-cyst, EDTA, SDS and NaN_3) on the activity of the purified laccase were carried out at different concentrations (Fig. 4). No significant loss of laccase activity was observed using the chelating agent EDTA at concentrations below 10 mM. In the presence of 5 mM EDTA, only 10% of the initial activity was lost. Similar result had been reported for *M. albomyces* laccase [23]. However, EDTA had an inhibitory effect on most of characterized fungal laccases [42,43]. The resistance against this chelator agent was explained by its low accessibility to the structural copper atoms present in the active site, required for catalytic enzyme activity [3].

On the other hand, the surfactant SDS affected slightly the laccase activity at 10 mM concentration (the enzyme maintained 80% of activity). It has been previously reported for other laccases that the presence of SDS (up to 1 mM) did not significantly affect laccase activity [3,26]. Pawlik et al. [33] explained this effect by a partial unfolding of a compact protein structure, leading then to the improvement of the hydrophobic interactions between the substrate and the active site. Other studies described opposite effects of SDS on laccase activity [26,44].

Table 2
Effect of various metal ions on the activity of the purified *Thielavia* laccase (TaLac1).

Metal ions	Relative laccase activity (% of control)			
	5 mM	10 mM	100 mM	200 mM
Cu^{2+}	96.33 ± 7.70	90.37 ± 1.10	78.38 ± 1.20	65.15 ± 2.10
Ca^{2+}	89.07 ± 2.00	76.98 ± 5.80	61.74 ± 5.50	66.72 ± 3.50
Mn^{2+}	126.75 ± 2.70	117.21 ± 4.00	98.94 ± 6.80	98.61 ± 1.30
Zn^{2+}	99.02 ± 2.10	99.02 ± 2.70	87.52 ± 2.10	85.80 ± 2.40
Mg^{2+}	105.63 ± 1.50	93.55 ± 7.50	90.70 ± 2.00	81.32 ± 1.50
Fe^{2+}	96.41 ± 2.70	87.92 ± 6.30	30.42 ± 1.30	0.00 ± 0.00
Hg^{2+}	49.96 ± 4.00	17.62 ± 1.20	5.80 ± 0.40	0.00 ± 0.00

The values represent the mean ± standard deviation (SD) of triplicate measurements (n = 3). Laccase activity in the absence of any metal ions was regarded as 100%.

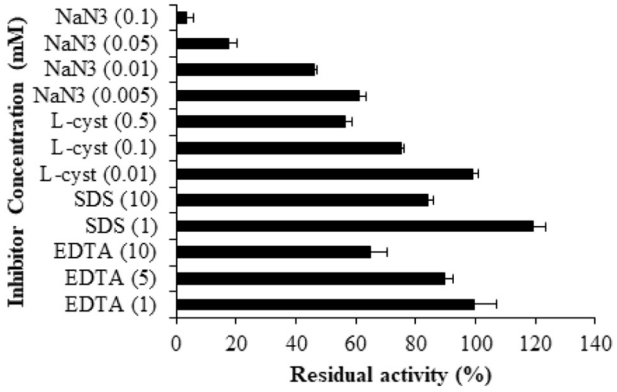

Fig. 4. Effect of different typical inhibitor compounds on laccase activity of the purified *Thielavia* sp. TaLac1. The results are expressed as mean of three replicate samples.

It is quite surprising that L-cyst, which had been previously reported as an efficient laccase inhibitor, has no significant effect on TaLac1 activity at concentration below 0.1 mM. At a concentration of 0.5 mM, TaLac1 lost almost 40% of its activity. This thiol has no strong inhibitory effect on TaLac1 compared to other laccases [3,17]. The purified enzyme retained around 80% of its initial activity in presence of 0.1 mM L-cyst while enzyme from *Trametes orientalis* retained only 31% of its activity under the same condition [45]. A pronounced decrease of laccase activity was observed in presence of 0.1 mM of NaN_3 which agrees with the effect observed in other fungal laccases [25,33,44]. In fact, the binding of N_3^- to the trinuclear copper center blocks the internal electron transfer and then abolishes the oxidation reaction catalyzed by laccase [3,26,33]. These results are in agreement with the general properties of laccase from a diverse range of fungal sources [3,24].

3.9. Effect of sodium chloride on laccase activity

Fig. S4 shows that TaLac1 activity was not significantly affected by 300 mM NaCl, retaining around 90% of its initial activity, although at higher concentrations (1 M), the activity decreased around 40%. Most fungal laccases are strongly inhibited by sodium chloride at high concentrations [3,33,46], as laccases produced from *Chaetomium* sp. (IC_{50} for 100 mM), *Xylogone sphaerospora* (IC_{50} for 200 mM) and *Sinorhizobium meliloti* (70% inhibition for 1 mM) [16,33]. Safary et al. [3] suggest that this inhibition is due to the binding of chloride ions to the putative channel between T1 and T2/T3 copper present in the laccase active site, which disrupt the internal electron transfer. According to previous data, TaLac1 enzyme is more chloride tolerant than those of some basidiomycetes, such as *Pycnoporus sanguineus* CS43 [42] and *Cerrena* sp. HYB07 [47]. Thus, this tolerance also pointed out that this fungal laccase could be a good option for industrial applications, such as coir and textile effluents [3,48,49].

3.10. Kinetic properties of purified laccase

The kinetic parameters of the purified *Thielavia* sp. laccase were investigated using nonphenolic (ABTS) and phenolic (2,6-DMP) typical laccase substrates (Table 3). Compared to other fungal laccases, TaLac1 demonstrated a considerably high affinity towards ABTS and 2,6-DMP (K_m values of 23.7 and 24.0 μM, respectively). Apparent K_m values against these substrates are in the range of 4–770 μM for 2,6-DMP and 8–14,720 μM for ABTS for different fungal and bacterial laccases [27]. The strong affinity of TaLac1 against ABTS and 2,6-DMP, was similar to those reported for the basidiomycete fungus *Pleurotus ostreatus* (K_m = 20 μM) and the bacterium *Bacillus subtilis* (K_m = 26 μM) [33]. Fungal laccase are known to have a very broad range of substrate affinities [28]. TaLac1 showed no differences in its catalytic efficiencies (k_{cat}/K_m) when substrates were varied. These parameter values were 0.17 $\mu M^{-1}\ s^{-1}$ and 0.14 $\mu M^{-1}\ s^{-1}$ in the presence of ABTS and 2,6-DMP, respectively. The catalytic efficiency of TaLac1 towards 2,6-DMP was similar to those earlier reported for laccases from the basidiomycetes *Moniliophthora roreri* (Mrl2) (0.2 $\mu M^{-1}\ s^{-1}$) [50] and *Trametes trogii* BAFC 463 (LCC3) (0.16 $\mu M^{-1}\ s^{-1}$) [51]. For ABTS, close catalytic efficiencies of 0.267 $\mu M^{-1}\ s^{-1}$ and 0.177 $\mu M^{-1}\ s^{-1}$ have been reported for laccases from *Physisporinus rivulosus* strain T241i (Lac-3.5) [39] and *Pleurotus ostreatus* HAUCC 162 (rLACC6) [52], respectively. K_m value for ABTS of TaLac1 was lower than that reported for laccases from *Cerrena unicolor* GSM-01 (302.7 μM) [2] and *S. carnis* CPF-05 (31.6 μM) [26]. TaLac1 possessed higher affinity for ABTS than some previously reported fungal laccases [2,26,53].

When comparing catalytic properties of laccase TaLac1 with Lcc1 enzyme from *T. arenaria* some differences were found. K_m value of TaLac1 against 2,6-DMP was significantly lower than that of Lcc1 enzyme [54], although they kept the same optimum pH for that substrate [55]. Since both proteins shared 99% homology in the sequenced region (275 aa) these results indicate that the non-sequenced portions of protein at the N- and C-terminal could contain key residues that could affect enzyme activity and characteristics and, thus, TaLac1 should be considered as a new enzyme. Since the sequenced region of TaLac1 comprised mainly the T2 copper binding region, the structural differences could be involved in the other copper binding sites, or in substrate access channels.

Table 3
Kinetic parameters of purified *Thielavia* sp. laccase.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
ABTS	23.70	4.14	0.17
DMP	24.30	3.46	0.14

3.11. Dye decolorization and biodegradation analysis

3.11.1. RBBR decolorization and its UV–vis spectrum

The ability of the purified laccase TaLac1 for dye decolorization was performed using the recalcitrant dye RBBR (Fig. S5). An efficient decolorization was obtained within 24 h with an extent of 90%. This is a very interesting result since numerous studies have reported that RBBR is hardly decolorized in absence of redox mediators, compounds usually required to efficiently degrade recalcitrant structures [2,56]. In this sense, *Arthrospira maxima* laccase decolorized only 49% of RBBR within 4 days [56], in absence of mediators. Other laccases, like that of *Myceliophthora thermophila*, were not able of decolorizing this recalcitrant dye, even in the presence of mediators [57].

The efficient degradation of RBBR found in our work was reported also by other previous studies. Murugesan et al. [58] showed that RBBR (50 mg L^{-1}) was decolorized by 90% within 20 h by 20 U mL^{-1} of crude laccase produced from the white rot fungus *Ganoderma lucidum* KMK2 without redox mediator. Grassi et al. [36] investigated the decolorization capacity of the purified laccase (6.5 U mL^{-1}) from *T. trogii* BAFC 463 on RBBR and revealed the complete removal of this dye (>85%) without mediator after 24 h of incubation. In both cases, laccase doses exceeded the used in our work. Wang et al. [2] reported that 2 U mL^{-1} of *C. unicolor* purified laccase decolorized only 10.8% of RBBR (100 mg L^{-1}) within 24 h without the help of redox mediators, while Yang et al. [47] reported the decolorization of 100 mg L^{-1} of RBBR with extent of >90% in 40 min when 2 U mL^{-1} of purified *Cerrena* sp. HYB07 laccase was used in the absence of any mediators.

The UV–visible spectrum in the wavelength range 200–800 nm of treated and untreated RBBR is shown in Fig. S5a. At 592 nm, a significant decrease in absorbance values was observed. This change in absorbance was associated with an oxidation of the RBBR native molecule. Previous study showed that the shift in color intensity is due to the presence of benzene ring with substitution group or conjugated carbonyl group in

the dye molecule [59]. The results demonstrated that the laccase from *Thielavia* sp. TaLac1 showed good activity in anthraquinonic dye color removal. Therefore, this enzyme has great potential to be employed in textile effluents treatment.

3.11.2. High Performance Liquid Chromatography (HPLC) analysis

Samples withdrawn before and after treatment, were analyzed by HPLC (Fig. S5b). The untreated sample showed a major peak at retention time of 3.5 min (80 mAU). After RBBR treatment, the predominant peak was reduced considerably (40 mAU) and led to the appearance of three new peaks in the retention times of 0.2, 3 and 4 min. These new peaks could be intermediates generated by dye degradation. It was established by the FTIR data, which revealed the presence of aromatic rings (1500 cm^{-1}) and amines (3000–3400 cm^{-1}).

3.11.3. Fourier Transform Infrared Spectroscopy (FTIR) analysis

Fig. S5c displays the FTIR analysis of RBBR dye before and after decolorization. It is clear that both chromophore and functional group of RBBR were degraded. The absorption peaks corresponding to the control dye were observed at around 3400–3100 cm^{-1} (N—H (—NH_2)); 1541–1401 cm^{-1} (C═C); 1699 cm^{-1} (C═O anthraquinone ring); 717 cm^{-1} (C—H out-plane bending vibration, anthraquinone ring); 1636 cm^{-1} for C—N stretching and at 1339 cm^{-1} and 1049 cm^{-1} for SOO symmetric and asymmetric sulphonic acid groups, respectively [60]. The SOO peaks values confirm the presence of —SO_3Na group in the RBBR dye structure.

On comparing the spectra with laccase-treated sample, there was no significant charge in the band position but an important shift was observed at around 700–900 cm^{-1}, suggesting that it can be responsible for substituted structure out-of-plane C—H bending vibrations of benzene ring [61]. The cleavage of chromophore having tricyclic anthraquinone structures was also confirmed by a shift in peak intensity of absorptions in the range of 1541–1401 cm^{-1}, which denote aromatic C═C conjugated with C═O stretching vibration [62]. Furthermore, the disappearance of the peak at 1194 cm^{-1} can be assigned to the complete destruction of C—N bonds connecting between monobenzene ring and anthraquinone ring, which was confirmed by the presence of a large new peak at 1177 cm^{-1} due to the formed byproducts [63]. A significant shift was observed in N—H groups indicating the degradation of the chromophore structure of RBBR dye. Obtained results agree with previous report on treated RBBR dye using laccase from the white-rot fungus *Pleurotus florida* [64].

4. Conclusion

An effective purification protocol was successfully applied to purify the major laccase secreted from *Thielavia* sp., a fungus isolated from arid soil in Tunisia. Activity of purified laccase was not significantly affected in presence of typical laccase inhibitors, at the assayed concentrations, and the presence of Mn^{2+} enhanced its activity. The purified enzyme was able to decolorize efficiently the recalcitrant dye RBBR, without redox mediator. All the results suggest that it could be an interesting tool for different biotechnological applications. Further molecular studies will be needed in order to complete the complete structural characterization of the protein.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.09.175.

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